An Ahemolytic Pneumolysin of *Streptococcus Pneumoniae* Manipulates Human Innate and CD4+ T-Cell Responses and Reduces Resistance to Colonization in Mice in a Serotype-Independent Manner

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**Background.** Some *Streptococcus pneumoniae* serotypes express an ahemolytic pneumolysin (PLYa). Serotypes that commonly express PLYa, including serotype 8 (ST8) and ST1, are often associated with a low prevalence during colonization but a higher propensity to cause invasive disease. We sought to study the host response to ST8 PLYa in a homologous and heterologous capsular background.

**Methods.** We genetically exchanged the PLYa of ST8 strain 6308 with the hemolytic PLY (PLYh) of ST3 A66.1 and vice versa and determined the impact of the exchange on nasopharyngeal colonization in mice. Then, to compare the response of human cells to PLYa-expressing and PLYh-expressing strains, we infected human peripheral blood mononuclear cells (PBMCs) with PLY-switched strains and assessed dendritic cell and CD4+ T-cell responses by intracellular cytokine staining.

**Result.** Mice colonized with PLYa-expressing strains had significantly higher colonization densities than those colonized with PLYh-expressing strains, irrespective of capsular background. Compared with infection of PBMCs with PLYh-expressing strains, infection with PLYa-expressing strains induced diminished innate (dendritic cell cytokines, costimulatory receptor, and apoptotic) and adaptive (CD4+ T-cell proliferative and memory interleukin 17A) responses.

**Conclusion.** Our findings demonstrate that PLYa has the potential to manipulate host immunity irrespective of capsule type. PLY exchange between STs expressing PLYa and PLYh could lead to unexpected colonization or invasion phenotypes.

**Keywords.** *Streptococcus pneumoniae*; pneumolysin; serotype; colonization; invasion; mice; apoptosis; dendritic cell; T cell.

Pneumolysin (PLY), a 52-kDa pneumococcal toxin, has been considered as a potential vaccine candidate. In addition to its hemolytic and complement-fixing properties and its effects on host cellular processes, PLY can induce innate immune responses to pneumococcus [1–5]. At noncytotoxic concentrations it activates the NLRP3 inflammasome, promotes Toll-like receptor 4 (TLR-4)–mediated release of proinflammatory cytokines, and induces apoptosis [4, 6], but at higher concentrations it functions as a toxin and causes pores in cholesterol-containing membranes [7]. Some PLY-deficient mutants are avirulent in mice [2, 8, 9]. However, engineering the PLY gene (*ply*) to lessen but not eliminate PLY production reduced serotype 3 (ST3)
virulence by enhancing immune stimulation and bacterial clearance, whereas ply deletion increased virulence [10].

There are multiple alleles of PLY, classified by relatedness and hemolytic activity, with some being ahemolytic [11]. ST1, ST7, and ST8 strains expressing ply alleles with reduced or no activity (ie, ahemolytic strains) have been identified [12]. PLY hemolytic activity in the ST2 background did not contribute to virulence in one mouse model [13], but an ahemolytic ST1 PLY conferred an early growth advantage in another [14]. In patients, ST1, ST7F, and ST8 are considered to have low colonization but high invasive potential [15]. ST1 and/or ST8 have been identified in epidemics [12,16] and invasive disease in human immunodeficiency virus–infected and homeless individuals [17,18]. In the post–7-valent pneumococcal conjugate vaccine (PCV7) era, ST1 and ST3 emerged as major causes of necrotizing pneumonia and empyema [19–22]. ST1, ST3, and ST7F are included in PCV13 but were not included in PCV7. ST8 is a non-PCV ST. Given that PCV and antibiotic use have led to the emergence of non-PCV STs and to drug-resistant and other genetic variants [23,24], ply could also be exchanged between STs. This study was designed to determine the effects of hemolytic (PLYh) and ahemolytic PLY (PLYa) on ST3 and ST8 virulence. We constructed PLY-switched strains in different capsular backgrounds and determined their ability to colonize the mouse nasopharynx and activate human dendritic and CD4+ T cells in vitro.

**MATERIAL AND METHODS**

**Animals**

Male C57BL/6 mice were obtained from the National Cancer Institute (Fredrick, MD) and used with the approval of and in accordance with the guidelines of the Einstein Institutional Animal Care and Use Committee.

**Pneumococcal Strains and Reagents**

Bacterial strains and plasmids used in this study are shown in Table 1. ST3 strain A66.1 was originally obtained from David Briles (University of Alabama, Birmingham), and ST8 strain 6308 was obtained from American Tissue Culture Collection (Rockville MD). All bacterial cultures were grown from a single colony as described elsewhere [10]. Recombinant PLY variants were expressed in *Escherichia coli* and purified using Ni-NTA chromatography as previously described [25]. Recombinant PLYs were tested for endotoxin contamination, using the Pierce endotoxin kit, and results were negative.

**Construction and Characterization of PLY-Switched ST3 and ST8 Strains**

The ply sequences of A66.1 (ST3) and 6308 (ST8) were determined using established primers [10]. ST8 ply was redesigned to eliminate certain restriction sites without changing its codon sequence, as described elsewhere [10]. ST3 and ST8 ply were cloned in the carrier plasmid pUCminus with flanking Pst1 and EcoR1 restriction sites. The fragments were excised and cloned into plasmids pPLY-ST3 and pPLY-ST8 containing a 6308-expressing ST3 pneumolysin and Kmr cassette. We also performed an independent transformation and verified that colonies exhibited the same activity as that of the original A66.1-PLYa strain. Hemolytic activity of PLY-switched ST3 and ST8 strains was determined as described elsewhere [7,10,26]. PLY protein levels were determined by Western blot, using anti-PLY antibody with equal counts of log-phase bacterial cultures as described [10].

**Mouse Studies**

**Pneumococcal Colonization**

Mice (5 per group) were infected with 10 μL of phosphate-buffered saline (PBS) containing either 1 × 10^7 colony-forming units (CFU) of wild type (A66.1) or A66.1-PLYa or 5 × 10^4 CFU of wild type (6308), 6308-PLYh, or 6308-PLYa (reconstituted). Inocula of each ST were established in preliminary experiments as colonization doses that did not disseminate to lungs or blood.

<table>
<thead>
<tr>
<th>Name</th>
<th>Designation</th>
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<tbody>
<tr>
<td>A66.1 PLY (recombinant)</td>
<td>PLYh</td>
</tr>
<tr>
<td>6308 PLY (recombinant)</td>
<td>PLYa</td>
</tr>
<tr>
<td>Wild-type A66.1</td>
<td>A66.1</td>
</tr>
<tr>
<td>A66.1-expressing ST8 PLY (PLYa)</td>
<td>A66.1-PLYa</td>
</tr>
<tr>
<td>Wild-type 6308</td>
<td>6308</td>
</tr>
<tr>
<td>6308-expressing ST3 PLY (PLYh)</td>
<td>6308-PLYh</td>
</tr>
<tr>
<td>6308 PLYh reconstituted with PLYa</td>
<td>6308-PLYa</td>
</tr>
<tr>
<td>pUCminus</td>
<td>Synthetic carrier plasmid, Blue Heron Biotechnology</td>
</tr>
<tr>
<td>pPLY-ST3</td>
<td>Derivative of pUCminus that contains the wild-type ST3 pneumolysin and Kmr cassette</td>
</tr>
<tr>
<td>pPLY-ST8</td>
<td>Derivative of pPLY-ST3 that encodes the nonhemolytic pneumolysin from strain 6308</td>
</tr>
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On day 7 after inoculation, mice were euthanized, an upper respiratory tract wash was done, and 200 µL of wash was collected from the nostrils [27]. Nasal wash specimens were serially diluted and plated on blood agar containing gentamicin (BD Biosciences). CFU were enumerated the next day. In another experiment, a higher colonization dose (5 × 10^6) of all strains was administered, and mice were observed for morbidity and mortality.

**Phenotyping of Mouse Nasopharyngeal Lavage**

Nasal wash specimens from infected mice were pooled, pelleted, and washed twice with PBS. Samples were stained to detect CD4+ T cells (CD3+, CD44), neutrophils (Ly6G+, CD11b-), and macrophages (F4/80+, CD11b+) as previously described [28]. BD LSR II was used to acquire 10 000 events, and data were analyzed using FlowJo (Tree Star).

**Studies With Human Peripheral Blood Mononuclear Cells (PBMCs)**

**Cell Stimulations**

PBMCs were stimulated or infected to detect (1) intracellular cytokines in myeloid dendritic cells (mDCs; HLA-DR+, CD11c+) and CD4+ T-cell subsets, (2) CD4+ T-cell proliferation, and (3) bacterial uptake, maturation, and apoptosis of in vitro monocyte (CD14+)-derived DCs. Briefly, PBMCs were thawed, rested overnight, and plated in 96-well tissue culture plates (5 × 10^4 cells/mL). One million cells were used for stimulations with recombinant PLYh or PLYa (500 ng/mL) or for infection with live wild-type or PLY-switched ST3 and ST8 strains in a multiplicity of infection of 1:1 (CD4+ T-cell proliferation assay after infection for 10 days, apoptosis after infection for 6 or 18 hours) or 1:10 (Intracellular cytokines in mDCs and CD4+ T-cells after 7 hours of infection, bacterial uptake by DCs after infection for 2 hours, maturation and apoptosis of DCs after infection for 18 and 24 hours). Medium and lipopolysaccharide (LPS; 1 µg/mL, for mDCs and DCs) or phorbol myristate acetate/ionomycin (for T cells) were used as unstimulated controls, respectively. After 2 hours of infection, gentamicin (100 µg/mL) was added to kill extracellular bacteria. For intracellular cytokine staining (ICS) experiments, gentamicin was supplemented with brefeldin A (10 µg/mL; Sigma).

**Cell Surface Staining and CD4+ T-cell and mDC Cytokine Profiling by ICS**

In separate panels, surface staining was done for mDCs and CD4+ T cells. For mDC surface staining, anti-CD3, CD66b (Pacific blue), MHCII (Alexa700), CD11c (perdinin chlorophyll protein [PerCP]/Cy5.5; Biolegend), and CD14 (Qdot 605; Invitrogen) were used. T cells were surface stained with anti-CD3 (Pacific blue), anti-CD4 (fluorescein isothiocyanate [FITC]), and anti-CD45RA (allophycocyanin [APC]). After staining, cells were washed, treated with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer’s instructions, and stained intracellularly to detect cytokines in DCs (interleukin 6 [IL-6; APC], interleukin 1β [IL-1β; FITC], tumor necrosis factor α [TNF-α; phycoerythrin [PE]/Cy7], and interleukin 10 [IL-10; PE]) or CD4+ T cells (interleukin 17A [IL-17A–PE]; Biolegend). Surface and ICS reactions were done as described previously [29]. A BD LSR II was used to acquire 100 000 gated events, and data were analyzed using FlowJo (Tree Star).

**Blocking of TLR-2, TLR-4, and Cytokine Responses in mDCs**

PBMCs (1 million/well) in 96-well plates were incubated with 100 µL (5 µg/mL) of either TLR-2 or TLR-4 neutralizing antibodies (mAb-Htlr2 and mAb-Htlr4, respectively; Invitrogen) for 15 minutes at 37°C. Cells were then stimulated with recombinant PLYh, PLYa, or LPS as described above. Both surface and ICS reactions were performed as described above to detect IL-6, IL-1β, and TNF-α in mDCs.

**DC Maturation and Pneumococcal Uptake**

Monocytes were magnetic sorted from human PBMCs by using CD14 microbeads (Miltenyi Biotec). DCs were derived in vitro [30] and infected as described above. DC phenotype was assessed by examination of CD11c (PerCP/Cy5.5; Biolegend) and CD1a (FITC; Biolegend) expression by flow cytometry. To examine DC maturation, expression of surface maturation markers CD80 (PE), CD86 (APC), and HLA-DR (Alexa 700; Biolegend) was examined. For uptake, DCs (1 × 10^5 cells/well) were infected as described above, washed, and lysed with 0.1% saponin, and the number of viable internalized bacteria was enumerated on blood agar plates.

**Apoposis of PBMCs and DCs**

The effect of PLY on cell viability was determined using a FITC annexin V apoptosis detection kit with PI (Biolegend). Cells were either stimulated with PLYh or PLYa (100 ng/mL-10 µg/mL) or infected with wild type or PLY switched strains as above. The numbers of apoptotic and necrotic cells were determined by staining with annexin V (FITC) and propidium iodide, according to the manufacturer instructions. BD LSR II was used to acquire 50 000 events and data were analyzed using FlowJo (Tree Star).

**CD4+ T-Cell Proliferation**

PBMCs were labeled with CFSE (e-biosciences) according to the manufacturer’s instructions, followed by stimulation with 500 ng/mL of PLYa or PLYh, or were infected with wild-type or PLY-switched strains and incubated at 37°C in a humidified incubator with 5% CO2 for 10 days as described earlier [31]. After incubation, cells were stained with anti-human CD4 (APC) antibody (Biolegend) for 20 minutes at room temperature. BD LSR II was used to acquire 100 000 gated events, and data were analyzed using FlowJo (Tree Star). CD4+ T-cell...
proliferative responses in infected cells were compared with those in unstimulated controls.

Statistical Analysis
Data were analyzed using GraphPad Prism software. Statistical significance among groups was examined by means of analysis of variance, followed by the Dunn post hoc test for comparisons between 2 groups. Survival data were analyzed by the Mantel-Cox log-rank test. A P value of < .05 was considered statistically significant.

RESULTS

Construction and Characterization of PLY-Switched ST3 and ST8 Strains
Plasmid *ply*-ST8 containing *plya* was used to replace the hemolytic *plyh* of ST3 A66.1, resulting in an A66.1 strain expressing PLYa, A66.1-PLYa. Similarly, plasmid *ply*-ST3 containing *plyh* from ST3 was used to replace the *plya* of ST8 6308, resulting in a 6308 strain expressing PLYh, 6308-PLYh (Figure 1A). A hemolytic assay confirmed that 6308, A66.1-PLYa, and 6308-PLYh reconstituted with PLYa were ahemolytic (Figure 1B). Expression of the PLY upstream and downstream genes SPD-1725 and SPD-1727 (Supplementary Figure 1) was equivalent in A66.1-PLYa and wild-type A66.1, making a polar effect unlikely. Expression of pneumococcal surface proteins (PspA, PhtD, PLY, PcpA, and LytB) was also evaluated by qPCR. There was no difference in expression between wild-type (ST3 or ST8) and PLY-switched mutants (data not shown). Gene sequence analysis of 6308 *ply* revealed it to be closest to alleles 3 or 5 of ST8 *ply* (GenBank accession number EF368014.1). The amounts of PLY (Figure 1C) and capsule expression (Figure 1D) of wild-type and PLY-switched strains were comparable. ST3 strains produced more capsule than ST8 strains, but wild-type and PLY-switched strains expressing the same capsule produced similar amounts of capsule (Figure 1D).

Effect of PLYa and PLYh on Colonization
At day 7, mice colonized with A66.1-PLYa had significantly more nasal CFU than mice colonized with A66.1, and mice

Figure 1. Construction and characterization of pneumolysin (PLY)–switched serotype 3 (ST3) and ST8 strains. A, Design of gene construct used for the construction of PLY-switched strains. B, Confirmation of PLY-switched ST3 and ST8 strains by hemolytic assay. Data represent mean (+SD) of 1 experiment in duplicate. C, Western blot of in vitro–grown PLY-switched strains. Lane 1, Hemolytic PLY (PLYh); lane 2, A66.1; lane 3, A66.1–ahemolytic PLY (PLYa); lane 4, 6308; lane 5, 6308-PLYh; lane 6, 6308-PLYa; lane 7, PLY knockout. D, Quantitation of ST3 and ST8 polysaccharides in in vitro–grown culture supernatants and pellets by enzyme-linked immunosorbent assay. Data is representative of 1 experiment in triplicate and expressed as mean (+SD). Abbreviation: NS, not significant.
colonized with 6308 had more CFU than mice colonized with 6308-PLYh ($P < .001$; Figure 2A). Thus, PLYa led to a higher bacterial burden, irrespective of capsule type. The colonization density of 6308-PLYh complemented with PLYa (6308-PLYa) was higher than that of 6308-PLYh and A66.1, but this difference was not statistically significant (Supplementary Figure 2A). Mice colonized with PLYh-expressing strains (A66.1, 6308-PLYh) had significantly more F480$^+$/CD11b$^-$ nasal cells (Figure 2B), and mice colonized with 6308-PLYa had significantly fewer such cells (Supplementary Figure 2B) than mice colonized with 6308-PLYh. PLYh-expressing strains had more nasal Ly6G$^+$/CD11b$^+$ cells, but this difference was not significant. There was no difference in CD4$^+$ T cells. Thus, PLYh-expressing strains recruit more macrophages than PLYa-expressing strains, irrespective of capsule type.

To assess the impact of PLYh and PLYa expression on invasion, we increased the colonization dose. All mice receiving PLYh-expressing strains (A66.1, 6308-PLYh) survived irrespective of capsule type, but 57% of mice colonized with A66.1-PLYa died by 4 days after infection ($P = .02$; Figure 3C). Thus, PLYa enhanced the invasiveness of ST3. One hundred percent of 6308-PLYh colonized mice also survived; this finding did not differ from that for mice colonized with 6308 ($P = .13$; Figure 2C). Mice colonized with 6308-PLYa had a level of survival similar to that of 6308-colonized mice (Supplementary Figure 2C).

**Effect of PLYa and PLYh on Human DC Response**

To extend colonization results to the response of human cells to PLY-switched strains, we stimulated human PBMCs with recombinant PLYs and wild-type and PLY-switched strains and assessed cytokine production in mDCs. PLYh and PLYh-expressing strains induced higher frequency of IL-1$\beta$-producing (CD11c$^{hi}$ IL-1$\beta$) and IL-6-producing (CD11c$^{lo}$ IL-6$^+$) mDCs than PLYa or PLYa-expressing strains ($P < .05$; Figure 3B). Recombinant PLYh induced more TNF-$\alpha$-producing mDCs than
PLYa \( (P < .05) \), but there were no differences in the frequency of TNF-\(\alpha\)-producing mDCs between PLYa- and PLYh-expressing strains (data not shown). Recombinant PLYa induced more IL-10–producing mDCs than PLYh, and 6308 induced more IL-10–producing mDCs than 6308-PLYh \( (P < .05) \), but there was no difference between A66.1 and A66.1-PLYa. Thus, the ST3 capsule could have an independent effect on IL-10 production.

Previous work established that PLYh and a detoxified PLY variant (PdT) bind TLR-4 \([3, 6]\). We assessed the TLR-4 dependence of PLYa-mediated human inflammatory responses. In contrast to TLR-2 blockade, which had no effect, TLR-4 blockade reduced the frequency of PLYa- and PLYh-induced IL-6– and TNF-\(\alpha\)-producing mDCs similarly, but PLYa induced a less robust response (Figure 3C). Neither TLR-2 nor TLR-4 blockade had an effect on IL-1\(\beta\) expression.

We also examined the effect of recombinant PLYs and PLYa- and PLYh-expressing strains on DC maturation and bacterial uptake. PLYa-expressing strains exhibited significantly less...
bacterial uptake than PLYh-expressing strains (Figure 4A). There was significantly higher upregulation of CD86 and HLA-DR on DCs infected with recombinant PLYh or PLYh-expressing strains (Figure 4B). These effects were irrespective of capsule type.

**Effect of PLYa and PLYh on Apoptosis**

Because PLYh of ST1, ST3, and acapsular ST4 induced apoptosis [6, 30], we determined the effect of PLYa and PLYh in the ST3 and ST8 background on the viability of PBMCs and DCs. We did not detect apoptosis at 6 hours after infection (data not shown). However, 18 hours after infection, consistent with findings shown in Figure 4A, cells infected with PLYh-expressing strains had more annexin V–positive cells than those infected with PLYa-expressing strains (Figure 4C and 4D). Cells infected with PLYh-expressing strains also had more evidence of cell shrinkage, loss of forward-scatter height (Figure 4E), and more propidium iodide–positive cells (data not shown).

**Effect of PLYa and PLYh on CD4+ T-Cell Proliferative Responses**

The effect of PLYh and PLYa on CD4+ T-cell proliferative responses was examined in PBMCs. PBMCs infected with
PLYh-expressing strains had greater CD4+ T-cell proliferation than PLYa-expressing strains, irrespective of capsule type \((P < .05; \text{Figure 5A and 5B})\). We did not detect proliferation in cells stimulated with PLYh or PLYa.

**Effect of PLYa and PLYh on IL-17A CD4+ T-Cell Memory Responses**

Since PLYa expression led to a diminished innate (cytokine and costimulatory) response, we determined its ability to induce IL-17A-expressing memory CD4+ T-cell responses. The frequency of IL-17A-expressing CD4+ T cells was significantly higher after infection with PLYh strains, compared with PLYa-expressing strains, irrespective of capsule type \((P < .05; \text{Figure 6B})\).

IL-17A-expressing cells were derived from both CD45RA+ and CD45RA- CD4+ T-cell populations (Figure 6A). Although the memory phenotype is generally associated with a loss of CD45RA, effector CD4+ T cells producing cytokines can reacquire CD45RA \([32]\). Neither PLYh nor PLYa induced detectable specific IL-17A-expressing CD4+ T-cell memory responses (data not shown).

**DISCUSSION**

Our interest in PLYa switches stems from known genetic exchange between pneumococcal strains \([23, 24]\) and the possibility that PLY exchange could occur in the setting of PCV and/or antibiotic use. We chose to examine ST8 and ST3 PLYs because ST8, a non-PCV ST that often expresses PLYa, has been linked to invasive disease in vulnerable patients \([17, 18]\) and because ST3, a PCV13 ST, is a major cause of sepsis, necrotizing pneumonia, and empyema \([19–22]\). Our data show that colonization densities were markedly higher for PLYa-expressing strains than for PLYh-expressing strains, irrespective of capsule type. Colonization phenotype has been linked to capsule biology and growth phenotype \([33, 34]\). However, our data add another layer of complexity to our understanding of colonization by establishing that PLY hemolytic status can govern colonization independent of capsule type, albeit in mice. Consistent with the role of murine macrophages in early nasopharyngeal pneumococcal clearance \([28]\), mice infected with PLYh-expressing strains had more nasal macrophages than those infected with...
PLYα-expressing strains. After infection with a higher inoculum, mortality among A66.1-PLYα–infected mice was higher than that among A66.1-infected mice and 6308-PLYh–infected mice and similar to that of 6308-infected mice. Thus, our data support previous work showing that ST8 is a cause of invasive disease [33,35,36] but also suggest that, for ST3, acquisition of PLYα could...
enhance invasiveness. Although, to our knowledge, PLY variants have not been identified in clinical ST3 isolates, coinfection with ST1 (which can express PLYa) and ST5 in children with emphyema [22] leads us to wonder whether PLYa acquisition could lead to enhanced ST3 invasiveness in the post-PCV7 era.

It has been shown that ST1 and ST7F strains do not activate the NLRP3 inflammasome or induce IL-1β in mice [4]. While IL-1β–mediated inflammation was implicated in tissue damage in mouse models of pneumonia and meningitis [37, 38], impaired IL-1β secretion led to prolonged pneumococcal colonization in aged mice [39]. Our finding that PLYa-expressing strains induced less IL-1β than PLYh-expressing strains in human mDCs suggests that the propensity of PLYa-expressing ST1 and ST8 to cause invasive disease [17, 18, 40, 41] stems from their inability to stimulate an adequate innate immune response, rather than capsule type. However, PLYh-expressing and PLYa-expressing ST8, but not ST3, induced more IL-10–producing mDCs. At present, we cannot explain this difference. Lower levels of IL-6 in mDCs infected with PLYa-expressing strains could be due to what our data show is a reduced ability of PLYa to trigger TLR-4–mediated IL-6 production. However, as in another report [42], our data show that PLY-induced IL-6 is only partially TLR-4 dependent. Nonetheless, IL-6 plays a key role in inducing T-helper 17 (Th17) responses [43, 44], which mediate pneumococcal clearance in mice [27, 45].

Our data show that human in vitro–derived DCs exhibited more uptake of PLYh-expressing strains than PLYa-expressing strains. This confirms and extends to ST3 and ST8 previous work that showed less uptake of PLYa-expressing ST1 than PLYh-expressing ST1 [30]. Our results also show that expression of maturation markers HLA-DR and CD86 was reduced on mDCs infected with PLYa-expressing strains, compared with PLYh-expressing strains. Consistent with the role of these markers in providing costimulation for T-cell activation [46], PLYa-expressing strains induced less CD4+ T-cell proliferation than PLYh-expressing strains. Notably, a previous report found that a PLYh-expressing acapsular strain induced less interleukin 8, interleukin 12, and CD86 expression than a PLY-deficient mutant [30]. We did not evaluate acapsular strains. However, based on our data, the possibility that PLYh-induced proliferative responses could require capsule expression requires further study. Nonetheless, our data show that PLYh-expressing strains elicited a significantly higher frequency of IL-17A–expressing memory CD4+ T cells than PLYa-expressing strains, irrespective of capsule type. This is consistent with a report showing that domain 4 of PLYh elicited CD4+ T-cell proliferative and memory Th17 responses in nasal-associated lymphoid tissues, but a non-cholesterol binding domain 4 mutant did not [42]. Thus, our data suggest that PLY switches between common clinical strains could have an impact on naïve and memory T-cell responses.

Clinical ST1 strains expressing PLYa induced more DC apoptosis than PLYh-expressing strains [30], and PLYh-dependent T-cell apoptosis has been proposed to limit T-cell activation and prevent pneumococcal invasiveness [47]. Our data show that PLYa-expressing strains reduced less PMBC and DC apoptosis than PLYh-expressing strains, irrespective of capsule type. Thus, our findings indicate that apoptosis phenotype is likely to be governed by PLY status, not capsule type. Apoptosis of pneumococcus-infected cells responsible for nonopsonic [48] and opsonic [49, 50] bacterial clearance is mediated by capsule-specific antibody. Our data support the hypothesis that an inability of PLYa-expressing strains to induce apoptosis could enhance their invasive potential either by failing to limit the inflammatory response to pneumococcal infection or by promoting evasion of innate responses that govern clearance.

Together, our data suggest that pneumococcal strains expressing PLYa induce less bacterial uptake, innate and CD4+ T-cell activation, and apoptosis than PLYh-expressing strains. Although PLYa expression has been linked to less robust innate and T-cell activation previously, which has been hypothesized to increase invasiveness, our data establish that this is the case in mice for clinically important STs and show that the aforementioned effects on innate and acquired immune responses are independent of capsule type. We think that our findings have important implications for pneumococcal epidemiology and pathogenesis in the current PCV era. They raise the concern that absence of a ST8 moiety in current PCVs could increase its prevalence as a replacement ST and the chance of exchange of its PLYa with other STs. They also lead us to wonder whether the current propensity of ST3 to cause sepsis and empyema could stem from PLYa acquisition. These questions are under investigation in our laboratory.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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