



The Impact of Pneumolysin on the Macrophage Response to *Streptococcus pneumoniae* is Strain-Dependent

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Abstract

Streptococcus pneumoniae is the world's leading cause of pneumonia, bacteremia, meningitis and otitis media. A major pneumococcal virulence factor is the cholesterol-dependent cytolysin, which has the defining property of forming pores in cholesterol-containing membranes. In recent times a clinically significant and internationally successful serotype 1 ST306 clone has been found to express a non-cytolytic variant of Ply (Ply₃₀₆). However, while the pneumococcus is a naturally transformable organism, strains of the ST306 clonal group have to date been virtually impossible to transform, severely restricting efforts to understand the role of non-cytolytic Ply in the success of this clone. In this study isogenic Ply mutants were constructed in the D39 background and for the first time in the ST306 background (A0229467) to enable direct comparisons between Ply variants for their impact on the immune response in a macrophage-like cell line. Strains that expressed cytolytic Ply were found to induce a significant increase in IL-1 β release from macrophage-like cells compared to the non-cytolytic and Ply-deficient strains in a background-independent manner, confirming the requirement for pore formation in the Ply-dependent activation of the NLRP3 inflammasome. However, cytolytic activity in the D39 background was found to induce increased expression of the genes encoding GM-CSF (*CSF2*), p19 subunit of IL-23 (*IL23A*) and IFN β (*IFNB1*) compared to non-cytolytic and Ply-deficient D39 mutants, but had no effect in the A0229467 background. The impact of Ply on the immune response to the pneumococcus is highly dependent on the strain background, thus emphasising the importance of the interaction between specific virulence factors and other components of the genetic background of this organism.

Citation: Harvey RM, Hughes CE, Paton AW, Trappetti C, Tweten RK, et al. (2014) The Impact of Pneumolysin on the Macrophage Response to *Streptococcus pneumoniae* is Strain-Dependent. PLoS ONE 9(8): e103625. doi:10.1371/journal.pone.0103625

Editor: Olivier Neyrolles, Institut de Pharmacologie et de Biologie Structurale, France

Received: May 14, 2014; **Accepted:** June 28, 2014; **Published:** August 8, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This research was funded by Program Grant 565526 (to JCP and AWP) and Project Grant 627142 (to JCP) from the National Health and Medical Research Council (NHMRC) of Australia and the National Institutes of Allergy and Infectious Diseases (AI037567) to RKT. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Streptococcus pneumoniae (the pneumococcus) is responsible for almost 1 million deaths in children under 5 years of age annually, and is the leading cause of diseases such as pneumonia, bacteremia, meningitis and otitis media. A key virulence factor of the pneumococcus is the pore-forming toxin pneumolysin (Ply), which is a member of the family of cholesterol-dependent cytolysins and a potential target in future vaccine formulations [1–6]. Mutants lacking Ply have been shown to be attenuated in murine models of infection [7]. More specifically, Ply-deficient mutants have been characterised by reduced induction of pulmonary inflammation due to delayed cell recruitment into the lungs, particularly affecting neutrophil responses and the distribution of B and T lymphocytes in and around inflamed bronchioles [8,9]. The defining property of Ply is its ability to form pores in cholesterol-containing membranes, causing potent induc-

tion of inflammation and tissue damage [7,9–13]. However, Ply has a number of other properties including antibody-independent activation of the classical complement pathway and binding to toll-like receptor 4 (TLR4) [14–16]. Amongst a number of responses, pore formation activates the NLRP3 inflammasome, leading to Caspase-1 activation and subsequent processing and release of IL-1 β from immune cells [17]. There is some evidence to suggest that Ply-induced pore formation is responsible for potassium efflux and lysosomal destabilization that are detected either directly by NLRP3 or via intermediary factors leading to inflammasome assembly and Caspase-1 activation [17]. Therefore, IL-1 β release is indicative of Ply-dependent activation of the NLRP3 inflammasome [17].

Serotype 1 strains of *S. pneumoniae* are a significant cause of disease worldwide and are frequently responsible for outbreaks of IPD in otherwise healthy individuals [18–23]. In the last 20 years

the ST306 serotype 1 clone has seen a dramatic expansion across the globe responsible for more than 80% of cases of serotype 1 disease in many jurisdictions [24–28]. Interestingly the ST306 clone expresses a non-cytolytic Ply variant that retains the ability to bind to cholesterol-containing membranes and oligomerise, but cannot form pores [25].

Previous work has also found that a hypervirulent serotype 1 strain 4496 of the clonal cluster 615 produced a Ply variant (Ply₄₄₉₆) with very low specific hemolytic activity, 1.6% of Ply_{D39} [29,30]. However, when D39 was constructed to express Ply₄₄₉₆ the resultant mutant was similarly virulent to wild-type D39 in mice in terms of survival and was significantly more virulent than Ply-deficient D39 [29]. In addition, D39 constructed to express an engineered Ply toxoid PdT (D385N/C428G/W433F) that retains only 0.001% of Ply_{D39} hemolytic activity, was similar in virulence to D39 in terms of survival, but was significantly more virulent than the Ply-deficient strains [7]. The apparent importance of Ply to pathogenesis even in the absence of hemolytic activity highlights the lack of understanding of the relative importance of cytolytic activity to pneumococcal pathogenesis as a whole.

However, efforts to characterise the significance of this phenomenon have been frustrated by the genetically intractable nature of a number of serotype 1 strains such as those of the ST306 clonal group. While attempts to characterise low and non-cytolytic variants, such as Ply₄₄₉₆ and Ply₃₀₆, respectively, have been performed [25,29,31], these studies have either relied on comparisons between different wild-type strains that express different Ply variants in the context of numerous other known and unknown strain-specific differences, or performed comparisons between isogenic strains, but in the context of an alternative strain background that is easily transformable. In both cases the significance of the interaction between the cytolytic activity of Ply and the clinically relevant strain background is not considered, representing a significant shortcoming in understanding the role of Ply in clinically important and internationally successful strains.

The current studies are the first to detail mutations made in a strain of the ST306 clonal group and the first to detail comparisons between the immune response to isogenic Ply mutants in either the D39 or ST306 (strain A0229467) backgrounds. Ply-deficient mutants (D39ΔPly & A0229467ΔPly) and mutants expressing engineered non-cytolytic derivatives that cannot bind to mammalian cell membranes, oligomerise or form pores (L460D [32]) were constructed in the D39 and A0229467 backgrounds. In this work the cytolytic activity of Ply was confirmed to be responsible for activation of the NLRP3 inflammasome in a strain-independent manner. However, more broadly the immune response of a macrophage-like cell line [33,34] to different Ply variants was found to be highly dependent on the strain background and appeared to suggest that the naturally non-cytolytic strain had adapted to the lack of cytolytic Ply. In addition, this work highlights the significant interaction between specific virulence factors and other strain-specific components of the organism's genetic composition.

Materials and Methods

Ethics statement

This study was conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition 2004) and the South Australian Animal Welfare Act 1985. All animal experiments were approved by the Animal Ethics Committee of the University of Adelaide. Ethics approval was obtained for molecular analyses of the A0229467

strain from the Central Australian Human Ethics Committee of the Northern Territory Department of Health.

Strains and media

The virulent serotype 2 strain, D39 (NCTC 7466) was used in this study [35]. The serotype 1 blood isolate belonging to the ST306 clonal group (A0229467) was obtained from the Alice Springs Hospital. Serotyping and multi locus sequence typing were performed by the Public Health Microbiology Laboratory at Queensland Health Scientific Services. Opaque-phase variants of all strains were selected on Todd-Hewitt broth supplemented with 1% yeast extract (THY)-catalase plates [36], and these were used in all animal experiments. Before infection, the bacteria were grown in serum broth (SB) (nutrient broth [10 g/l peptone (Oxoid), 10 g/l Lab Lemco powder (Oxoid) and 5 g/l NaCl] and 10% [v/v] donor horse serum) to 1×10^8 CFU/ml, and then diluted to the relevant challenge dose. For all other experiments, strains were grown at 37°C in a CO₂-enriched atmosphere in a casein-based semisynthetic liquid medium (C+Y) [37] to 1×10^8 CFU/ml. Bacteria were pelleted, washed and resuspended in PBS to 1×10^8 CFU/ml.

PCR

PCR reactions were performed using a Mastercycler Nexus thermal cycler (Eppendorf). Reactions were performed using the Phusion-Flash PCR system (Thermo Scientific). Overlap-extension PCR was carried out as previously described [38,39].

Transformation of pneumococci

For *S. pneumoniae* D39, preparation of competent cells and subsequent transformation was performed using the complete transformation medium (CTM) method [40,41]. A0229467 was transformed by diluting an overnight culture in C+Y medium in a 1:1 mixture of C+Y medium and Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (FCS). After 2 h at 37°C, 50 ng/ml competence stimulating peptide 1 (CSP-1) was added, followed 15 min later by donor DNA (approximately 1 µg). After a further 2 h incubation at 37°C, transformation mixes were plated on blood agar supplemented with the appropriate antibiotics.

Construction of Pneumolysin mutants

D39ΔPly was constructed by replacing the *ply* open reading frame with the gene encoding chloramphenicol acetyltransferase (*cml^R*) and subsequent selection of chloramphenicol resistant recombinants. D39::Ply₃₀₆ was constructed by replacing *cml^R* in D39ΔPly with *ply₃₀₆*, which was amplified from A0229467. D39::Ply₃₀₆ recombinants were selected for the loss of chloramphenicol resistance as previously described [42]. D39::Ply_{L460D} was constructed by replacing *cml^R* in D39ΔPly with the *ply_{L460D}* allele, which was generated by site-directed mutagenesis and overlap-extension PCR using primers RHPLYF₍₅₎ (GGT GGT GCT TAT GCT TTG TCG), RHPLYR₍₅₎ (GTG GGC AAT GAC AAA GGA TGT G), RHPLYL460DF (CCT GAG GAT AAT CAG TTG TTC C) and RHPLYL460DR (GGA ACA GAT GAT TAT CCT CAG G). D39::Ply_{L460D} recombinants were selected for loss of chloramphenicol resistance, as described above for D39::Ply₃₀₆.

Ply mutants in the A0229467 background were constructed by replacing the *ply* ORF of a ST306 derivative with a *rpsL* mutation with the Janus cassette [43] (designated A0229467*rpsLply*:Janus). Insertion of the Janus cassette suppresses the *rpsL* Strep^R phenotype and confers kanamycin resistance. This construct was

then transformed with genomic DNA from D39 Δ Ply and transformants were counter-selected by plating on BA+Strep. Genomic DNA was then extracted from a confirmed Strep^R/Kan^S transformant that produced no Ply protein, as judged by western blotting and used to transform A0229467*rpsLply*::Janus. Deletion of the *ply* gene from one of the resultant second generation Strep^R/Kan^S transformants (designated A0229467 Δ Ply) was confirmed by western blotting and DNA sequencing. This two-step procedure essentially eliminates co-transformation of other genetic markers from the donor DNA into the A0229467 background. An analogous procedure was used to replace the Janus cassette in A0229467*rpsLply*::Janus with the *ply* alleles from wild type D39 and D39::Ply_{L460D}, generating A0229467::Ply_{D39} and A0229467::Ply_{L460D}, respectively.

Quantitative Western Blotting

Relative expression of Ply was determined by quantitative western blotting on whole cell lysates using the Odyssey Infrared Imaging System and associated software package (LI-COR Biosciences), as previously described [29].

Hemolysis Assay

The hemolytic activity of pneumococcal lysates was determined as described previously [5], using PBS-washed human erythrocytes. Approximately 50 μ g of total lysate supernatant protein was used for each sample in triplicate. The hemolytic activity was defined as the dilution factor at which 50% hemolysis was detected.

Animal studies

Outbred 5- to 6-week old female CD1 (Swiss) mice were used in all challenge experiments. Mice were challenged intraperitoneally with 100 μ l of bacterial suspension containing approximately 1×10^4 CFU in SB. The challenge dose was confirmed retrospectively by serial dilution and plating of the inocula on blood agar. For survival experiments, mice were closely monitored for signs of illness over 14 days and were humanely euthanised when terminal infection was reached. Heart blood was taken from euthanised mice and plated on blood agar to confirm the presence of *S. pneumoniae*.

Cell Culture and differentiation of THP-1 cells into a macrophage-like cell type

All tissue culture media and reagents were obtained from Gibco. THP-1 cells (ATCC TIB-202) were grown in 95% air-5% CO₂ at 37°C in complete RPMI medium (RPMI with phenol red, supplemented with 10% FCS, 10 mM HEPES, 50 IU/ml penicillin and 50 μ g/ml streptomycin). 10 cm dishes were seeded with 3.5×10^6 THP-1 cells and differentiated by adding phorbol 12-myristate 13-acetate (PMA) to a final concentration of 10 ng/ml and incubating for three days at 37°C in 95% air-5% CO₂. Differentiated cells attach to the plastic surface. Following the three day incubation cells were washed twice with complete RPMI and then rested at 37°C in 95% air-5% CO₂ in complete RPMI for a further 3 days.

Co-incubation of differentiated THP-1 cells with *S. pneumoniae*

Differentiated THP-1 cells were washed twice with PBS before adding 5 ml of RPMI without antibiotics, FCS, HEPES or phenol red. Bacteria (100 μ l) were added to each dish at a 10:1 (bacteria:THP1 cells) ratio (100 μ l PBS was added to each resting control) and incubated at 37°C in 95% air-5% CO₂ for 6 h before

RNA extraction, or 16 h if collecting supernatants for enzyme-linked immunosorbent assay (ELISA).

ELISAs

Following 16 h co-incubation, the culture medium was collected and centrifuged at 16,000 \times g to pellet the bacteria. IL-1 β in the resultant supernatants was quantified using the BD OptEIA Human IL-1 β ELISA Kit II (BD Biosciences) and scanned on a SPECTROstar Omega microplate reader (BMG Labtech), according to the manufacturer's instructions.

PCR Arrays

Following 6 h co-incubation, RNA was extracted from infected THP-1 cells using the PureLink RNA Mini Kit (Life Technologies), including on-column DNase digestion, according to the manufacturer's instructions. Purified RNA was analysed on a LightCycler 480 II (Roche) by qRT-PCR using the RT² Profiler PCR Array Human Innate and Adaptive Immune Responses kit (Qiagen), according to the manufacturer's instructions. Data were generated from quadruplicate samples.

Statistical Analyses

The one-way ANOVA with the Tukey's multiple comparisons test was used to compare the mean survival times of mice, the mean relative Ply expression by quantitative Western Blot and the mean relative amount of IL-1 β release by ELISA. The One-way ANOVA and Kruskal-Wallis multiple comparisons test were performed using GraphPad Prism 6 for Mac. Statistical analyses for PCR Array data were performed according to the manufacturer's instructions. Changes in expression >2 fold and $P < 0.05$ were considered statistically significant.

Results

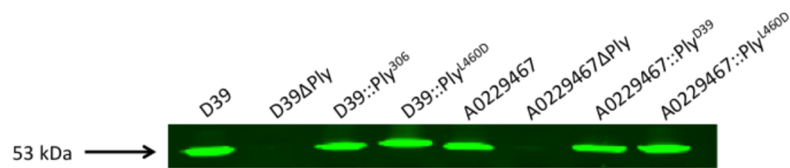
Loss of the Ply cytolytic property increases mouse survival

D39 was constructed to express the naturally occurring non-cytolytic Ply₃₀₆ variant. The virulence of D39, D39 Δ Ply and D39::Ply₃₀₆ was compared to determine whether the lack of cytolytic activity exhibited by Ply₃₀₆ would lead to increased survival when expressed in D39. The survival times of groups of 12 mice challenged intraperitoneally with 10^4 CFU of each strain D39, D39 Δ Ply or D39::Ply₃₀₆ were compared (Fig. 1). The median survival time of mice challenged with D39 Δ Ply was significantly longer than both the wild type ($P < 0.001$) and D39::Ply₃₀₆ ($P < 0.05$), but there was a much smaller but nevertheless statistically significant difference in median survival times between the wild type and D39::Ply₃₀₆ ($P > 0.05$). Thus, we confirm that while the cytolytic properties of Ply do contribute the virulence of wild type D39, the non-cytolytic properties appear to be much more important in this model.

Induction of IL-1 β release is dependent on the cytolytic activity of Ply but independent of the strain background

Table 1 lists the isogenic Ply mutants that were used in this study and include mutants in both the D39 and A0229467 backgrounds. All 8 strains were cultured in C+Y broth, and aliquots were taken for western blot and hemolytic activity analyses. Quantitative western blotting of equal quantities of total lysate supernatant protein confirmed that there were no significant differences in the amounts of Ply protein produced by D39, D39::Ply₃₀₆, D39::Ply_{L460D}, A0229467, A0229467::Ply_{D39} and A0229467::Ply_{L460D} (Fig. 2B). Ply was not detected in either the

A



B

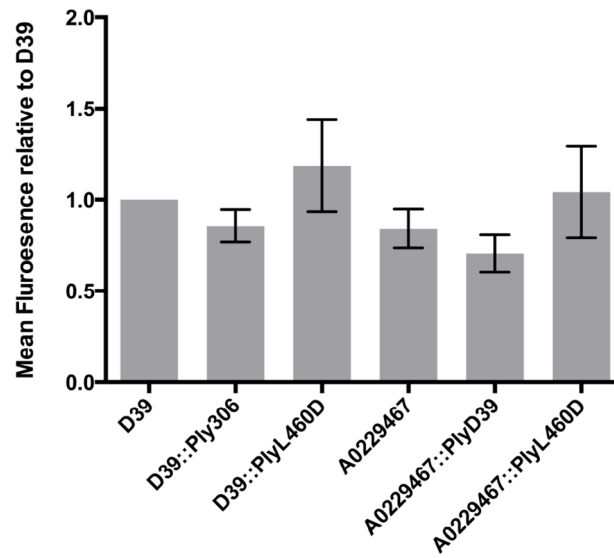


Figure 2. Ply expression. Ply expression analysis. Ply expression was compared between D39, D39 Δ Ply, D39::Ply₃₀₆, D39::Ply_{L460D}, A0229467, A0229467 Δ Ply, A0229467::Ply_{D39} and A0229467::Ply_{L460D} by quantitative western blot using Ply-specific mouse antisera (A) The size of Ply (53 kDa) is indicated. The mean fluorescence units relative to D39 from biological triplicates are shown (B). Error bars indicate the SEM. Relative expression was compared between strains using the one-way ANOVA and Kruskal-Wallis and Dunn's multiple comparisons test, where $P < 0.05$ would indicate statistical significance.

doi:10.1371/journal.pone.0103625.g002

pression was increased by 5.99, 10.5 and 4.51 fold ($P < 0.05$) in the presence of A0229467 Δ Ply, A0229467::Ply_{D39} and A0229467::Ply_{L460D} relative to the resting group, respectively.

Ply-independent changes in gene expression

Table 3 lists the genes that exhibited changed expression between the resting groups and the challenge groups in a Ply-independent manner. In particular, the genes encoding complement component 3 (*C3*), the CD86 molecule (*CD86*), myeloperoxidase (*MPO*) and nucleotide-binding oligomerisation domain-containing protein 2 (*NOD2*) were significantly changed in the presence of D39, but not A0229467 (Table 3). In contrast to D39, 9 genes exhibited reduced expression in the presence of A0229467 compared to the resting group but were unaltered in the presence of D39 (Table 3). These included the genes encoding chemokine receptor 5 (*CCR5*), the CD4 molecule (*CD4*), CD40 ligand (*CD40LG*), CD11b (*ITGAM*), lymphocyte antigen 96 (*LY96*), nucleotide-oligomerisation domain containing protein 1 (*NOD1*) and toll-like receptor 1 (*TLR1*). Ply-independent changes in expression that were similar between the two strain backgrounds are listed in the supplementary section (File S1).

Discussion

While the importance of Ply as a pneumococcal virulence factor has been known for decades, it is becoming increasingly clear that the role Ply plays in disease is more complex than originally thought. The apparent success of clonal types that express non-cytolytic variants of Ply suggest that cytolytic activity may not be an essential property in certain contexts. Furthermore, Ply variants with low (Ply₄₄₉₆ [29]) and absent (Ply₃₀₆ [this study]) cytolytic activity suffer only small reductions in virulence compared to the fully cytolytic wild-type D39 [29]. Given that the Ply₃₀₆-expressing D39 strain was shown to be significantly more virulent than Ply-deficient D39, it is possible to conclude that the non-cytolytic properties of Ply are essential for full virulence within the host. In previous work it has been suggested that strains expressing non-cytolytic Ply may have an advantage over cytolytic strains as they do not activate the NLRP3 inflammasome and may be less vulnerable to clearance from the host [17,31]. One such study, which compared the ability of the cytolytic and non-cytolytic clinical isolates to trigger IL-1 β release, suggested a role for the cytolytic activity of Ply in NLRP3 inflammasome activation [31]. However, given that the study was performed between different isolates it is difficult to resolve the contribution to NLRP3 inflammasome activation made by Ply from that of other strain-specific differences in genetic background. In contrast, the use of

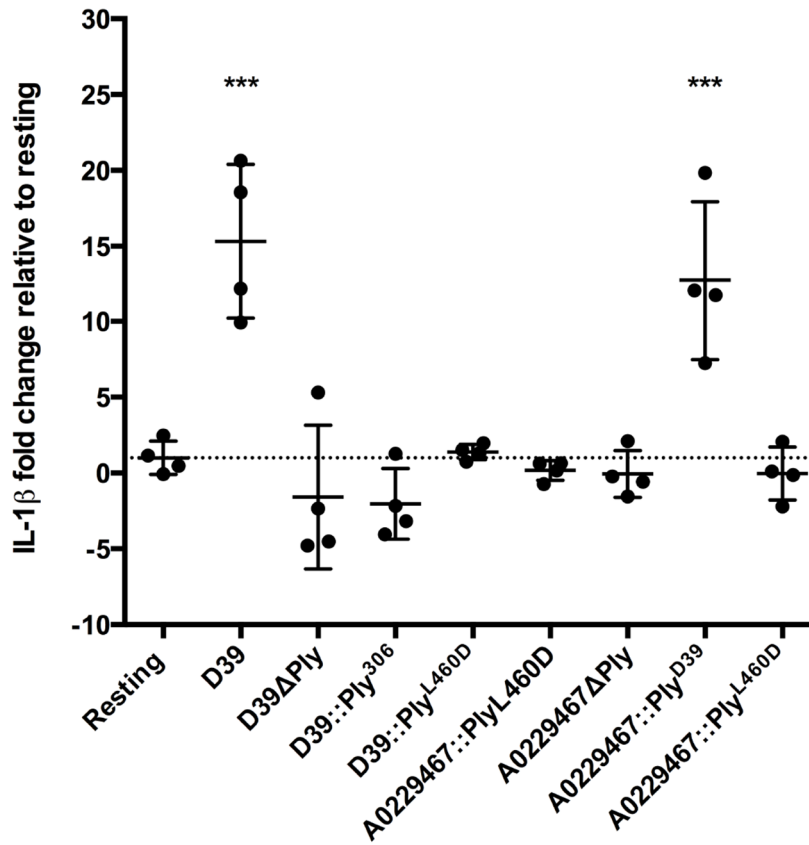


Figure 3. Ply-dependent induction of IL-1β release requires cytolytic activity. Differentiated THP-1 cells were co-incubated with bacteria at a 1:10 ratio for 16 h. Supernatants were collected and IL-1β levels measured by ELISA. The mean quantity of IL-1β detected from resting samples is set at a relative value of 1 (dashed line), with all data points plotted as fold-change from this value. Error bars indicate the mean with standard deviation. Significant increases in IL-1β levels are highlighted, *** = $P < 0.001$, $n = 4$. doi:10.1371/journal.pone.0103625.g003

isogenic Ply mutants in the present study was able to confirm that only bacteria expressing the cytolytic Ply variant, PlyD39, were capable of triggering IL-1β release from cultured human macrophage-like cells, irrespective of the strain background.

Construction of isogenic Ply mutants in the D39 and A0229467 backgrounds provided an opportunity to broadly characterise the differences in the induced immune response associated with the different Ply variants in two backgrounds. This study examined the

effects of strain background and Ply variant at the transcriptional level, which is one of the earliest measurable parameters of the cellular response to pathogens. It focuses on a select subset of genes known to be involved in the host immune response against bacteria [44]. Firstly the ability of Ply to bind to the cell surface was not associated with any consistent changes in gene expression in either background, as the Ply₃₀₆ v Ply_{L460D} and Ply_{D39} v Ply_{L460D} comparisons showed. In the D39 background, the

Table 1. Hemolytic activity of the strains used in this study.

Strain	Specific Hemolytic Activity (HU/mg) ^a	Mutant Source (Ref)
D39	3×10^3	^c
D39ΔPly	^b	[29]
D39::Ply ₃₀₆	-	This study
D39::Ply _{L460D}	-	This study
A0229467	-	^c
A0229467ΔPly	-	This study
A0229467::Ply _{D39}	2×10^3	This study
A0229467::Ply _{L460D}	-	This study

^aHemolytic units per mg of cell lysate supernatant protein.

^bHemolytic activity below the limit of detection of 20 HU/mg.

^cWild type strains.

doi:10.1371/journal.pone.0103625.t001

Table 2. Ply-dependent changes in expression in the D39 background.

	D39 v D39ΔPly	D39 v Ply ₃₀₆	D39 v Ply _{L460D}	D39ΔPly v Ply _{L460D}
Gene				
<i>CSF2</i>	-6.94**	-9.15**	-13.99**	-2.01**
<i>IFNB1</i>	-6.18**	-5.35*	ns	ns
<i>IL23A</i>	-3.22***	-4.06***	-4.46***	ns
<i>CXCL10</i>	ns	2.15*	ns	ns
<i>IL8</i>	ns	-2.07**	-2.41	ns
<i>IRF7</i>	ns	-2.05*	ns	ns
<i>IL10</i>	ns	ns	-2.65**	ns
<i>IL1A</i>	ns	ns	-2.56*	ns

Significant changes in expression are ≥2 fold and $P < 0.05$. (** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$). ns denotes changes in expression that are not significant. doi:10.1371/journal.pone.0103625.t002

cytolytic activity of Ply induces increased expression of genes encoding pro-inflammatory cytokines, such as GM-CSF and IL-23α, as well as IFNβ1. *CSF2*, which encodes GM-CSF, remained either unchanged or was weakly up-regulated in the presence of D39 strains expressing no or non-cytolytic Ply, but was significantly induced upon expression of cytolytic Ply. GM-CSF stimulation of macrophages in culture has been shown to induce an activated/pro-inflammatory “M1” phenotype, leading to increased production of other pro-inflammatory cytokines upon exposure to bacterial components [45]. *In vivo* it is involved in recruitment of granulocytes, including neutrophils, and macrophages to sites of inflammation, as well as promoting survival and activation of these cell types within the tissue [45]. All D39 strains induced a significant increase in *IL23A* expression. However, the expression of cytolytic Ply in this background induced a much greater increase in *IL23A* expression than the other variants, which may at least in part be related to the induction of *CSF2*. IL-23 also induces expression of pro-inflammatory cytokines such as TNF and IL-6. It has been identified as an important factor in the maintenance and function of Th17 cells [46,47], which have been shown to be key players in the host response against the

pneumococcus [48,49]. *IFNB1* expression was also only induced by D39 expressing cytolytic Ply, possibly due to the detection of bacterial nucleic acids in the cytoplasm of the THP-1 cells that may have entered via the Ply pore [50]. *CXCL10* was the only gene that exhibited increased expression in the presence of any one of the non-cytolytic variants versus the wild-type, which occurred in the presence of D39::Ply₃₀₆ and may point to unknown properties of Ply.

Unlike D39, the A0229467 strains did not induce any consistent changes in gene expression that were associated with cytolytic activity. Interestingly, in contrast to the D39 strains, the overall effect of A0229467 strains seemed to be a general down-regulation of immune genes, independent of Ply expression. Whereas D39 induced up-regulation of a number of genes not induced by A0229467 (including *C3*, *MPO* and *NOD2*), A0229467-specific Ply-independent differential gene regulation was almost universally the down-regulation of genes, including *CCR5*, *CD4*, *ITGAM*, *NOD1* and *TLR1*. This might suggest a mechanism by which A0229467 can compensate for a lack of cytolytic activity by inducing a general suppression of the immune response in this cell line. A notable exception was the increased *CSF2* expression

Table 3. Strain-dependent Ply-independent changes in expression relative to resting.

Gene	D39	D39ΔPly	D39::Ply ₃₀₆	D39::Ply _{L460D}
<i>C3</i>	6.04*	4.01*	ns	3.54*
<i>CD86</i>	-2.51*	-2.49*	ns	-2.28*
<i>MPO</i>	2.16*	2.10*	2.63*	2.44**
<i>NOD2</i>	2.33**	2.87**	2.30***	2.51**
Gene	A0229467	A0229467ΔPly	A0229467::Ply _{D39}	A0229467::Ply _{L460D}
<i>CCR5</i>	-3.43*	-3.12*	-3.05*	-3.95*
<i>CD4</i>	-2.13*	ns	-3.05*	-3.08*
<i>CD40LG</i>	-3.25**	-3.23**	-3.39**	-2.97*
<i>ITGAM</i>	-2.80**	-2.42*	-3.28**	-2.43**
<i>LY96</i>	-2.64**	-2.61**	-2.33**	-2.08**
<i>NOD1</i>	-3.00*	-2.01*	-2.66*	-2.48*
<i>TLR1</i>	-2.37***	-2.50***	-3.77***	-3.75***

Significant changes in expression are ≥2 fold and $P < 0.05$. (** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$). ns denotes changes in expression that are not significant. doi:10.1371/journal.pone.0103625.t003

consistently associated with the A0229467 background. Contrary to the cytolytic Ply-dependent increase in expression seen in the D39 background, A0229467 appears to cause an up-regulation of *CSF2*, with only a slight increase in expression between cytolytic and non-cytolytic Ply-expressing strains. The dramatic increase in *IL8* expression observed for all strains is consistent with detection of bacterial cell components by pattern-recognition receptors and secondary stimulation by proinflammatory cytokines as IL-1 and TNF [51–54]. The implications of these Ply-independent changes in gene expression merit further investigation.

In summary, the ability to genetically manipulate A0229467 made it possible to perform the first direct comparisons between the immune response to isogenic Ply mutants in the naturally non-cytolytic A0229467 background. This work confirmed that the cytolytic activity of Ply is responsible for IL-1 β release in a strain-independent manner. Furthermore, this work showed that the cytolytic activity of Ply was important for the immune response to the naturally cytolytic D39, but had very little impact on the response to the naturally non-cytolytic A0229467, suggesting that A0229467 has adapted to the absence of Ply cytolytic activity.

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Supporting Information

File S1 Ply-independent changes in expression that were similar between the two strain backgrounds.
(DOCX)

Acknowledgments

The authors would like to thank James MacLeod from the Alice Springs Hospital for providing the A0229467 strain and Helen Smith from Queensland Health for the multi-locus sequence typing data and serotyping data.

Author Contributions

Conceived and designed the experiments: RMH AWP JCP. Performed the experiments: RMH CEH CT. Analyzed the data: RMH CEH JCP CT. Contributed reagents/materials/analysis tools: RKT. Contributed to the writing of the manuscript: RMH CEH JCP.

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