

Protective Contributions against Invasive *Streptococcus pneumoniae* Pneumonia of Antibody and Th17-Cell Responses to Nasopharyngeal Colonisation

Jonathan M. Cohen^{1,2}, Suneeta Khandavilli¹, Emilie Camberlein¹, Catherine Hyams¹, Helen E. Baxendale^{2,3}, Jeremy S. Brown^{1*}

1 Centre for Respiratory Research, Department of Medicine, University College London, London, United Kingdom, **2** Infectious Diseases and Microbiology Unit, Child Health Institute, University College London, London, United Kingdom, **3** Department of Immunology, Royal Free Hospital Medical School Campus, University College London, London, United Kingdom

Abstract

The nasopharyngeal commensal bacteria *Streptococcus pneumoniae* is also a frequent cause of serious infections. Nasopharyngeal colonisation with *S. pneumoniae* inhibits subsequent re-colonisation by inducing Th17-cell adaptive responses, whereas vaccination prevents invasive infections by inducing antibodies to *S. pneumoniae* capsular polysaccharides. In contrast, protection against invasive infection after nasopharyngeal colonisation with mutant *S. pneumoniae* strains was associated with antibody responses to protein antigens. The role of colonisation-induced Th17-cell responses during subsequent invasive infections is unknown. Using mouse models, we show that previous colonisation with *S. pneumoniae* protects against subsequent lethal pneumonia mainly by preventing bacteraemia with a more modest effect on local control of infection within the lung. Previous colonisation resulted in CD4-dependent increased levels of Th17-cell cytokines during subsequent infectious challenge. However, mice depleted of CD4 cells prior to challenge remained protected against bacteraemia, whereas no protection was seen in antibody deficient mice and similar protection could be achieved through passive transfer of serum. Serum from colonised mice but not antibody deficient mice promoted phagocytosis of *S. pneumoniae*, and previously colonised mice were able to rapidly clear *S. pneumoniae* from the blood after intravenous inoculation. Thus, despite priming for a Th17-cell response during subsequent infection, the protective effects of prior colonisation in this model was not dependent on CD4 cells but on rapid clearance of bacteria from the blood by antibody-mediated phagocytosis. These data suggest that whilst nasopharyngeal colonisation induces a range of immune responses, the effective protective responses depend upon the site of subsequent infection.

Citation: Cohen JM, Khandavilli S, Camberlein E, Hyams C, Baxendale HE, et al. (2011) Protective Contributions against Invasive *Streptococcus pneumoniae* Pneumonia of Antibody and Th17-Cell Responses to Nasopharyngeal Colonisation. PLoS ONE 6(10): e25558. doi:10.1371/journal.pone.0025558

Editor: Dennis W. Metzger, Albany Medical College, United States of America

Received: March 24, 2011; **Accepted:** September 7, 2011; **Published:** October 7, 2011

Copyright: © 2011 Cohen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was undertaken at University College London Hospitals and University College London (UCL) which received a proportion of funding from the Department of Health's National Institute for Health Research Biomedical Research Centre's funding scheme. JMC was supported by a Clinical Research Training Fellowship from the Medical Research Council (G0700829). EC was supported by the Medical Research Council (grant G0600410). CH was supported by the Astor Foundation and with an unrestricted educational grant from GlaxoSmithKline towards the UCL MBPhD program. 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 read the journal's policy and have the following conflicts: CJH was supported by an unrestricted educational grant from GlaxoSmithKline. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

* E-mail: jeremy.brown@ucl.ac.uk

Introduction

Streptococcus pneumoniae is the second commonest cause of fatal bacterial infection worldwide. Most deaths are due to pneumonia, which when severe is often associated with septicaemia. Nasopharyngeal colonisation with *S. pneumoniae* is nearly universal in infants, with carriage rates reaching 90% [1] but then rapidly falling to 10% in late childhood and adults [2]. *S. pneumoniae* pneumonia results from aspiration of colonising bacteria from the nasopharynx into the lungs. Infants and the elderly are particularly susceptible to *S. pneumoniae* pneumonia, causing an estimated 826,000 deaths annually in children under five years of age worldwide [3] and with an incidence of at least 50 per 100,000 in the elderly in developed countries [4]. *S. pneumoniae* septicaemia is also commoner in these age groups and has a high mortality [5]. Infants often have a primary septicaemia with no associated pneumonia, whereas in adults septicaemia usually develops as a complication of pneumonia.

The reasons for the changing susceptibility to *S. pneumoniae* disease with age are not fully understood. Infants and the elderly are routinely vaccinated with capsular polysaccharide based vaccines, but these only protect against limited numbers of capsular serotypes and the unconjugated vaccine used in adults is not effective against pneumonia [6]. The conjugated vaccine used in children is effective but expensive, and has more limited serotype coverage so its efficacy could be reduced by vaccine induced changes in *S. pneumoniae* ecology. These limitations have stimulated interest in alternative *S. pneumoniae* vaccine strategies.

Although *S. pneumoniae* infections are common, the majority of colonised individuals do not develop disease suggesting there are robust natural mechanisms of immunity. These will include physical defences and innate immune responses [7], but the proportionally greater fall in *S. pneumoniae* disease rates compared to carriage rates after the first year of life suggests adaptive immune responses also have a role [1,8]. *S. pneumoniae* colonisation in humans can induce

anti-capsular antibodies, and by extension from vaccine data these were previously thought to be the main mechanism of naturally acquired adaptive immunity to invasive infection [8,9]. However, in human models *S. pneumoniae* nasopharyngeal colonisation induces mainly anti-protein rather than anti-capsular antibody responses [10]. Furthermore, recent publications have shown that mice colonised with mutant strains of *S. pneumoniae*, including unencapsulated strains, develop anti-protein antibody responses [11–13]. Colonisation-induced protection was not seen in MHCII^{-/-} mice [12], suggesting an important role for CD4+ helper T-cells. CD4 T-cells may assist protective adaptive immune responses by providing T-cell help towards B-cell antibody production and/or through memory T-cell responses recalled during infectious challenge. Recall responses to bacterial antigens by human T-cells implies priming during natural exposure [14]. Such cells include Th17-cells, capable of producing large amounts of the cytokines IL-17A and IL-22 which facilitate cellular recruitment to sites of infection and enhance the release of antimicrobial products from local epithelium [15]. Th17 responses induced through immunisation can protect against challenge with other respiratory pathogens including *Bordetella pertussis* [16] and *Mycobacterium tuberculosis* [17]. *S. pneumoniae* nasopharyngeal colonisation of mice is known to elicit a Th17-cell response that assists primary clearance of *S. pneumoniae* from the nasopharynx and inhibits recolonisation [18,19]. Furthermore, nasal immunisation with killed *S. pneumoniae* or purified pneumococcal proteins can also elicit a Th17-cell response capable of protecting against subsequent colonisation. Whether colonisation-induced Th17-cell responses are important for protective immunity against invasive disease such as *S. pneumoniae* pneumonia is not known.

We have used a murine model of nasopharyngeal colonisation with wild-type *S. pneumoniae* followed by pneumonia challenge to characterise the effects of colonisation on inflammatory and adaptive immune responses during subsequent infection, and to determine the relative contributions of antibody and Th17-cell mediated responses to protection.

Methods

Ethics statement

Experiments were approved by the UCL Biological Services Ethical Committee and the UK Home Office (Project Licence PPL70/6510). Experiments were performed according to UK national guidelines for animal use and care, under UK Home Office licence.

Bacterial strains and culture conditions

S. pneumoniae D39 was a kind gift from James Paton, University of Adelaide [20]. Bacteria were cultured on Columbia agar with 5% horse blood or in Todd-Hewitt broth with 0.5% yeast extract in 5% CO₂. Inocula for challenge experiments were prepared from mid-log phase cultures and stored at -70°C as single use aliquots.

Colonisation and infection models

CBA/Ca inbred mice were obtained from Charles River UK Ltd. μMT mice were a kind gift from Dr Claudia Mauri, UCL, London (UK). Mice were colonised by instillation of 10⁷ cfu *S. pneumoniae* D39 in 10 μl PBS into the nares under light halothane anaesthesia [12,21,22]. Control mice received 10 μl PBS alone. To obtain nasal washes the exposed trachea was flushed caudally with 200 μl PBS and the fluid exiting the nares collected. For the pneumonia challenge, 10⁷ cfu *S. pneumoniae* D39 in 50 μl PBS was instilled into the nares under deep general halothane anaesthesia

[21,23,24]. Animals were culled by exsanguination from the femoral artery under pentobarbital anaesthesia. BALF was collected by cannulating the exposed trachea and washing the airways three times serially with 1 ml sterile PBS. Lungs were collected aseptically after BAL had been performed into ice-cold PBS, minced and homogenised with sterile PBS as previously [21,24]. For survival experiments, animals were monitored and culled when exhibiting previously defined features of terminal disease [23]. For intravenous challenge, mice received 5 × 10⁶ cfu *S. pneumoniae* D39 in 100 μl PBS via tail vein injection. Bacterial cfu were calculated by counting colonies after plating serial dilutions of target organ preparations on blood agar plates. Sera for cytokine and flow cytometry assays were kept at 4°C before being frozen and stored as single use aliquots at -70°C. For passive transfer experiments, naïve mice received 225 μl fresh pooled serum from colonised or control mice (obtained 28 days after colonisation) by intraperitoneal injection 6 h and 30 minutes prior to pneumonia challenge. For CD4+ cell depletion, mice received 250 μg mAb GK1.5 (eBiosciences) intraperitoneal 48 and 24 h preceding pneumonia challenge; flow cytometric examination of splenocytes confirming successful depletion of >90% of CD4 cells [19].

Assessment of the inflammatory response to infection

To obtain BALF cell proportions, 300 cells from random fields of BALF cytospin preparations (Cytospin 3, Shandon) with rapid Romanowsky stain were counted according to standard morphological criteria, and absolute counts derived from haemocytometer cell counts. In selected experiments the degree of lung inflammation was assessed histologically. The entire left lung was fixed, processed to paraffin wax and stained with haematoxylin and eosin. Inflammation was assessed by a blinded observer using an established scoring system [24]. This involved assessing the percentage of overall lung involvement under ×10 magnification, followed by examination of 6 random fields under ×20 magnification. The composite score reported is the product of mean score for individual areas multiplied by percentage of lung affected. Sera and BALF supernatants cytokine levels were measured by ELISA (IL-17, IL-22 and IFN-γ, R&D Systems) or by Luminex™ bead immunoassay (Invitrogen) according to manufacturers' instructions.

ELISAs for antibody responses

Antibodies specific to *S. pneumoniae* D39 antigens were measured by whole cell ELISA using established methods as previously described [25]. Briefly, *S. pneumoniae* D39 was grown to late log-phase, washed and resuspended in PBS to OD₅₈₀ 1.0. 96-well plates were coated with this bacterial suspension, refrigerated overnight, then blocked with PBS 1% BSA prior to use. Sera were diluted in PBS 1% BSA before addition and binding to bacterial antigens detected with anti-mouse secondaries conjugated to alkaline phosphatase (Sigma).

Flow cytometry assays of phagocytosis

Phagocytosis of FAMSE (Molecular Probes) labelled *S. pneumoniae* by freshly isolated human neutrophils (MOI of 10:1) was measured using flow cytometry to obtain the MFI for cells associated with fluorescent bacteria as previously described [25]. *In vivo* phagocytosis by alveolar macrophages was measured using a flow cytometry method as previously described [26]. Briefly, mice were inoculated intranasally with FAM-SE labelled 10⁷ cfu D39, BALF obtained at 4 h and analysed using flow cytometry. Alveolar macrophages were identified based on scatter properties and bacterial phagocytosis measured as the MFI for macrophages associated with fluorescent bacteria.

Statistics

Survival of challenged mice was compared by the log rank test. Bacterial loads in BALF and lung, antibody and cytokine levels (non-parametric) were compared by Mann-Whitney U-test. Presence or absence of bacteraemia was compared by the Fischer exact test. *In vivo* phagocytosis (parametric) were assessed by unpaired Student t-test. *In vitro* neutrophil phagocytosis was compared by one-way Anova with post-hoc tests. *P* values < 0.05 were considered significant.

Results

S. pneumoniae colonisation model

To establish nasopharyngeal colonisation, CBA/Ca mice were intranasally inoculated with 10^7 cfu *S. pneumoniae* D39 suspended in 10 μ l PBS and nasopharyngeal washes and bronchoalveolar lavage fluid (BALF) collected at 2, 11, 21 and 28 days to quantify *S. pneumoniae* colonisation. *S. pneumoniae* were recovered in nasopharyngeal washes from all mice at 2 days (median 5.68×10^3 cfu/ml, interquartile range (IQR) 2.87–12.0), 80% of mice at 11 days (median 1.4×10^3 cfu/ml IQR 0.16–3.66), but from no mice on days 21 and 28 (Fig. 1). Furthermore, no bacteria were recovered from homogenates of nasopharyngeal tissue harvested on day 21 (*n* = 6). On occasion a few *S. pneumoniae* colonies were recovered from BALF (≤ 50 cfu/ml), but no bacteria were recovered from lung homogenates or blood at any timepoint.

Prior colonisation protects against lethal *S. pneumoniae* pneumonia by preventing bacteraemia

To investigate whether previous colonisation can induce adaptive mechanism(s) that protect against lethal infection, a model of rapidly developing fatal pneumonia was used in which mice were inoculated intranasally with 10^7 cfu *S. pneumoniae* D39 suspended in 50 μ l PBS. When challenged 28 days following colonisation, previously colonised mice were highly protected. All mice in the control group (which were sham colonised by intranasal administration of PBS) developed fatal infection with a median survival of 30 h whereas 94% of colonised mice survived (*P* < 0.0001) (Fig. 2A). To further characterise colonisation-induced protection, groups of colonised or control mice given a day 28 pneumonia challenge were sacrificed at 4, 9 and 18 h to quantify the bacterial load in blood, BALF and lung (Fig. 2B–2D). In

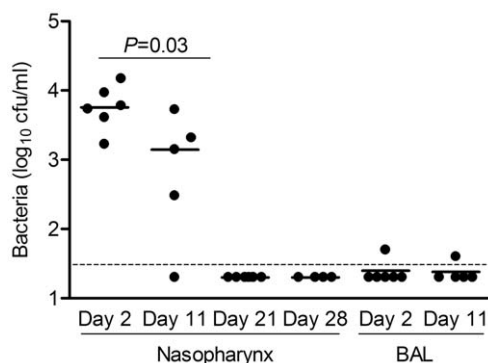


Figure 1. *S. pneumoniae* D39 colonises the nasopharynx of CBA/Ca mice. Bacterial cfu recovered from nasal washes and BALF 2, 11 and 28 days following intranasal inoculation in 10 μ l of PBS with 10^7 cfu D39 *S. pneumoniae*. Each dot represents results for an individual mouse and bars the median for each group. The dotted line is the limit of detection.

doi:10.1371/journal.pone.0025558.g001

control mice there were significant numbers of bacteria in both BALF and lungs at all timepoints, and in the blood at 9 and 18 h. In colonised mice there were similar numbers of bacteria in BALF to controls at all timepoints and in the lungs at 4 and 9 h. By 18 h, there were approximately 1-log fewer bacteria in the lungs of previously colonised mice than controls. Strikingly, at both 9 and 18 h post-challenge no bacteria were recovered from the blood of colonised mice, demonstrating that protection against fatal pneumonia in colonised mice was associated with the prevention of detectable bacteraemia.

Previous colonisation primes for greater mucosal and systemic IL-17 responses

To characterise the effect of previous colonisation on the inflammatory response during *S. pneumoniae* pneumonia, BALF cytokine levels were measured at 4 and 18 h after pneumonia challenge (Figure 3A, 3B). Compared to controls, in previously colonised mice there were modest but statistically significant increases in BALF levels of several innate cytokines, including TNF- α and IL-6, at 4 h but not at 18 h. In addition, at 18 h, IL-17 and IL-22 were markedly higher in BALF from previously colonised mice compared to controls, and IL-17 was also significantly higher in the serum (Figure 3C). Colonisation also primed for greater BALF neutrophil recruitment compared to controls at 4 h (mean $2.2 \pm 0.4 \times 10^5$ /ml versus $1.1 \pm 0.73 \times 10^6$ /ml, *P* = 0.02) but not at 9 h (mean $1.94 \pm 1.57 \times 10^6$ /ml versus $1.33 \pm 0.94 \times 10^6$ /ml, *P* = 0.43) (Fig. 4A, 4B). Hence, in previously colonised mice there was a modest increase in the strength of the early inflammatory response to subsequent *S. pneumoniae* lung infection and potentially significant mucosal and systemic Th17 responses. However, histological assessment of lung sections obtained 18 h after inoculation demonstrated there were no detectable differences in lung inflammation scores between colonised and control mice in established pneumonia (mean score 2.10 ± 0.77 versus 1.61 ± 0.59 , *P* = 0.24) (Fig. 4C).

CD4 cells were not required for colonisation induced protection against bacteraemia

Th17-cell mediated immunity assists nasopharyngeal clearance of *S. pneumoniae* [19], and the enhanced IL-17 response to pneumonia in previously colonised mice suggest a Th17 response might be contributing towards protection against invasive disease. To investigate this possibility, colonisation and subsequent *S. pneumoniae* pneumonia challenge experiments were repeated in mice depleted of CD4+ cells immediately before challenge using anti-CD4 antibody. CD4 depletion (>90% efficacy) abolished the colonisation-induced enhancement of BALF IL-17 levels at 18 hours in response to *S. pneumoniae* pneumonia in previously colonised mice (Figure 5A). Furthermore, in the absence of CD4 cells, no IL-17 was detected in the serum following infection of either colonised or control mice (data not shown). These data suggest that colonisation-induced Th17-cells were the source of the enhanced mucosal and systemic IL-17 responses observed in colonised mice.

In the absence of CD4 cells, 42% of challenged control mice developed significant bacteraemia by 18 h following challenge (Figure 5B). Nevertheless, even in the absence of Th17 cell responses, colonised mice were still fully protected against bacteraemia (*P* = 0.037). In addition, there were still fewer bacteria in the lungs of colonised than control mice despite the absence of CD4 T-cells. Thus, the enhanced CD4-cell dependant IL-17 response to subsequent pneumonia in colonised mice does not seem to be required for protection in this model.

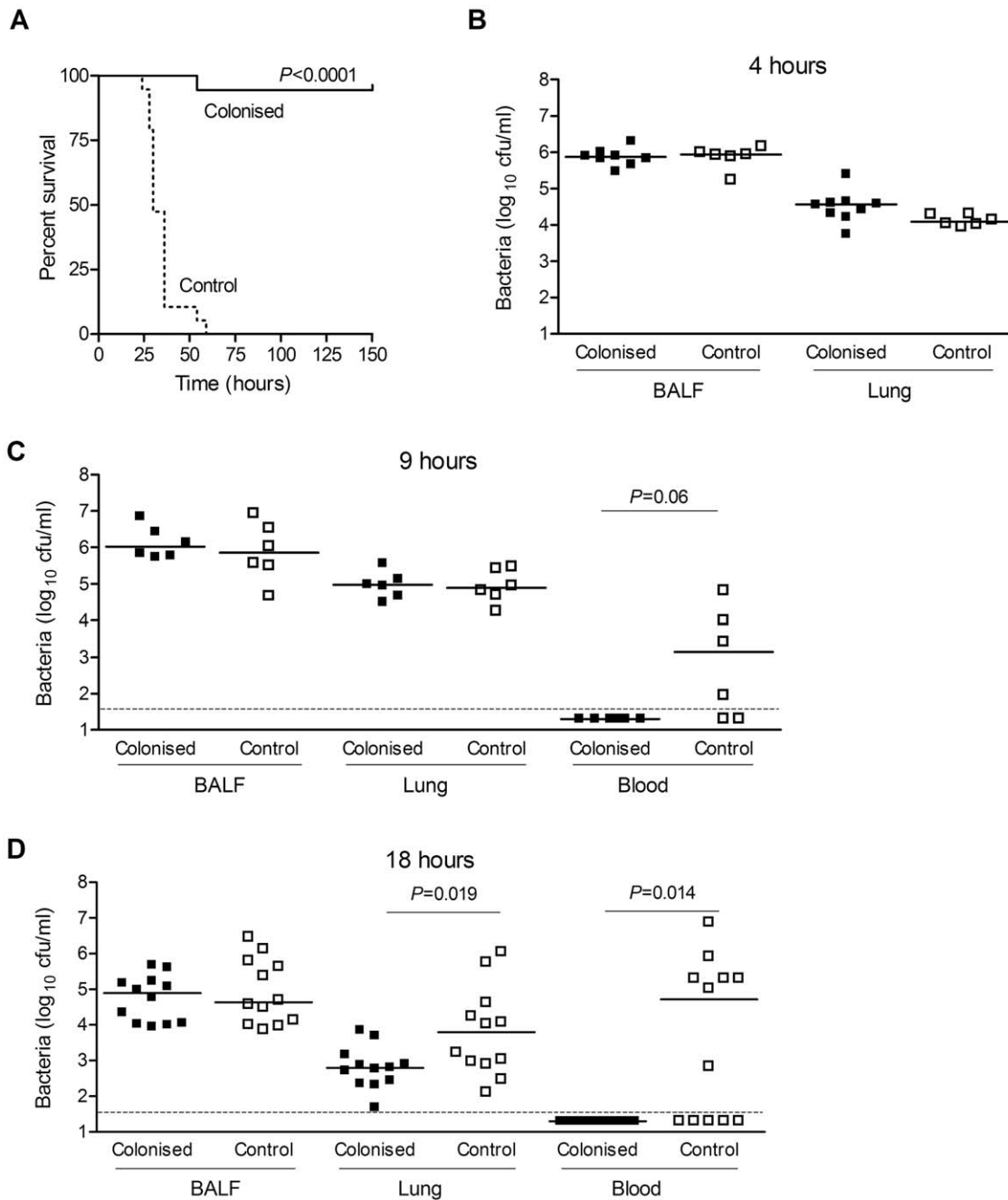


Figure 2. Effects of prior colonisation on progression of subsequent *S. pneumoniae* pneumonia. (A) Kaplan-Meier survival curves of previously colonised or control CBA/Ca mice following pneumonia challenge with 10^7 cfu D39 *S. pneumoniae* in 50 μ l PBS on day 28 ($n = 18$ or 19). (B–D) Bacterial cfu in BALF, lung or blood of colonised or control mice at (B) 4 h, (C) 9 h or (D) 18 h following pneumonia challenge. Each symbol represents results for an individual mouse and bars the median for each group. The dotted line is the limit of detection. Significance of the difference in bacterial numbers recovered from lungs of colonised and control mice was assessed by Mann-Whitney U-test. Significance of differences in presence or absence of bacteraemia was assessed by Fisher exact test. Data at 18 h are pooled from two experiments with similar results. doi:10.1371/journal.pone.0025558.g002

Colonisation induces a protective antibody response

The continued colonisation-induced protection in CD4 depleted mice demonstrated there must be alternative mechanisms of protection to Th17 responses, suggesting a role for colonisation-induced antibody. We therefore assessed antibody responses to *S. pneumoniae* colonisation in our model. Whole cell ELISAs detected high levels of serum IgG against D39 antigens in nearly all colonised mice 28 days after colonisation (Fig. 6A). Only a small

number of colonised mice had serum IgA or IgM responses greater than controls (Fig. 6B, 6C). There were strong IgG and IgA responses to D39 evident in BALF (Fig. 6D, 6E).

To determine whether protection was dependant on the presence of antibody, further colonisation and challenge experiments were performed in μ MT mice which congenitally lack antibody. All colonised μ MT mice developed bacteraemia by 18 h following challenge (Fig. 7A). To identify whether the antibody

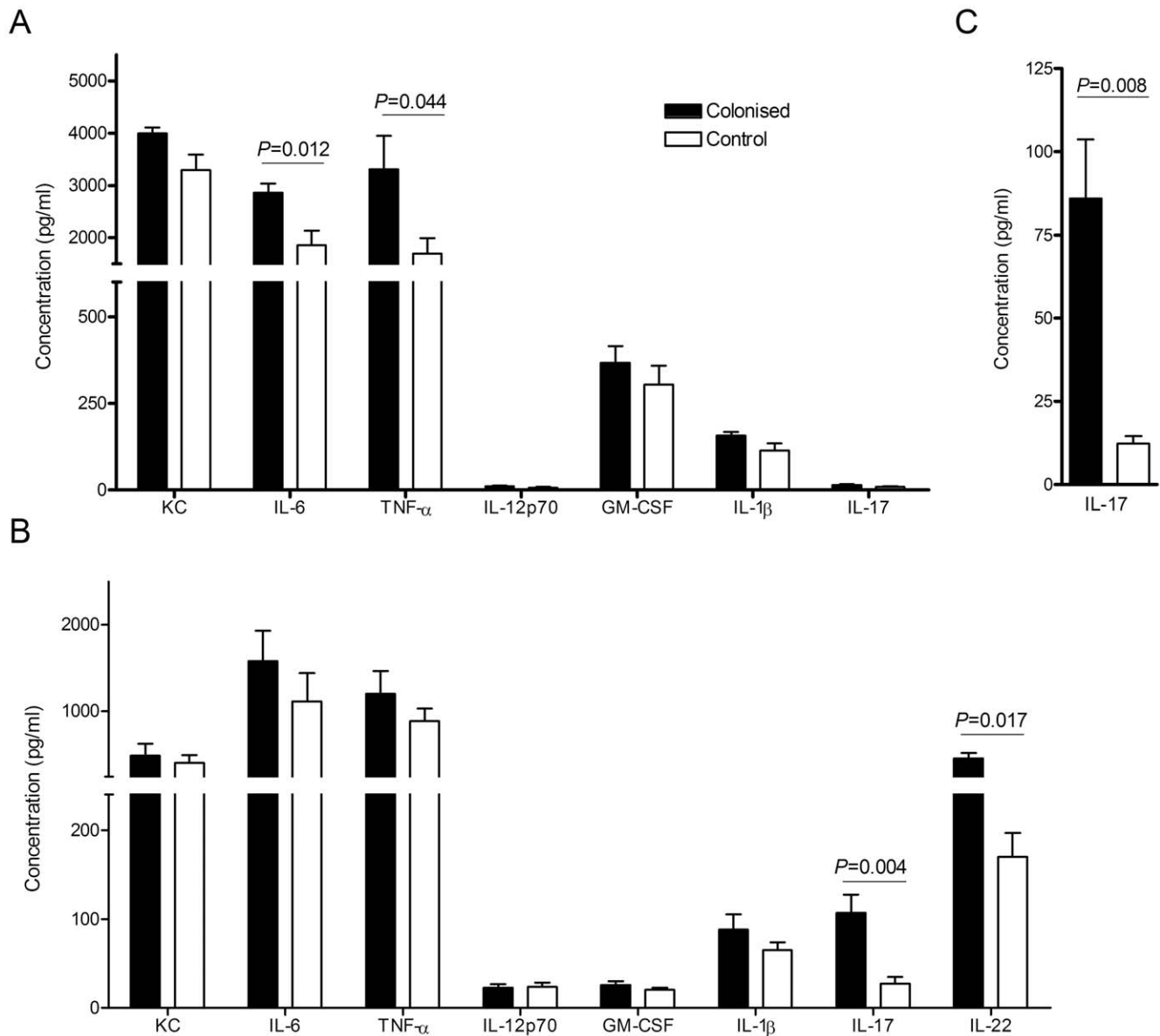


Figure 3. Effect of prior colonisation on cytokine levels during subsequent pneumonia. BALF samples were collected at either (A) 4 h or (B) 18 h and serum (C) at 18 h following pneumonia challenge of previously colonised (black bars) or control (white bars) CBA/Ca mice ($n=6$ per group). Cytokine levels were measured by either Luminex bead assay or ELISA. Group mean + SEM are displayed. Dotted line represents limit of detection.

doi:10.1371/journal.pone.0025558.g003

response to colonisation was sufficient to protect against subsequent pneumonia, naïve CBA/Ca mice were passively vaccinated by intraperitoneal injection with fresh pooled sera obtained from colonised or control CBA/Ca mice, then given a pneumonia challenge. The anti-D39 whole cell IgG titre of donor serum was 1/2260, and achieved a mean titre in the serum of passively immunised mice of $1/309 \pm 1/110$. Compared to recipients of control serum, passively immunised mice had significantly fewer bacterial CFU recovered from the lungs at 18 h. Furthermore, there was a strong trend towards prevention of bacteraemia, with only 1 of 6 recipients of immune serum developing bacteraemia compared to 5 of 6 recipients of control serum by 18 h post-pneumonia challenge (Fig. 7B), similar to the data obtained with actively colonised mice (Fig. 2B–2D). Overall, these data demonstrate that the systemic antibody response to *S. pneumoniae* colonisation is both

necessary and sufficient to protect, and that whilst colonisation induces a Th17 cell response, this is not required.

Enhanced *S. pneumoniae* phagocytosis in serum from previously colonised mice

To further characterise the mechanism of antibody mediated protection in colonised mice, flow cytometry assays were used to assess the impact of colonisation-induced serum antibody on neutrophil association with live *S. pneumoniae*. Incubation of 6-carboxyfluorescein succinimidyl ester (FAMSE) labelled D39 with serum from colonised CBA/Ca mice resulted in enhanced association with human neutrophils (known to be mainly due to phagocytosis [27]) compared to serum from control CBA/Ca mice (Fig. 8A). No differences in phagocytosis were observed when bacteria were incubated in sera from colonised or control μ MT

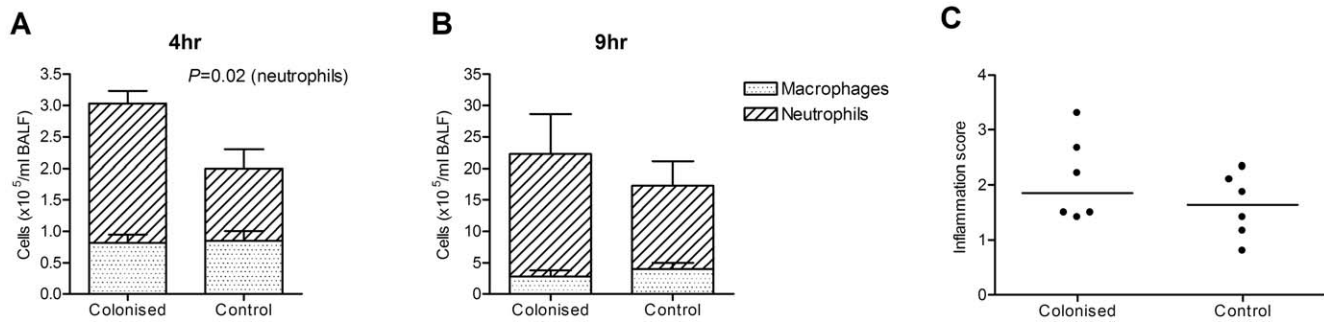


Figure 4. Effect of prior colonisation on inflammation during subsequent pneumonia. (A–B) Numbers of neutrophils (hatched region) and macrophages (dotted region) present in BALF of previously colonised or control CBA/Ca mice at (A) 4 h and (B) 9 h following pneumonia challenge. Bars represent mean + SEM (n=6 mice per group). (C) Lung inflammation scores assessed by blinded histological examination of lung sections collected 18 h following challenge of colonised or control mice. Dots represent individual mice, bars represent medians. doi:10.1371/journal.pone.0025558.g004

mice (Fig. 8B), confirming that serum antibody from colonised CBA/Ca mice bound to the *S. pneumoniae* surface was responsible for the enhanced phagocytosis in this serum. In contrast, when colonised and control mice were challenged with FAM-SE-

labelled D39 there were no differences in the association of fluorescent *S. pneumoniae* with alveolar macrophages recovered from BALF 4 h post-inoculation (Fig. 8C). Hence colonisation resulted in improved antibody dependent phagocytosis in serum

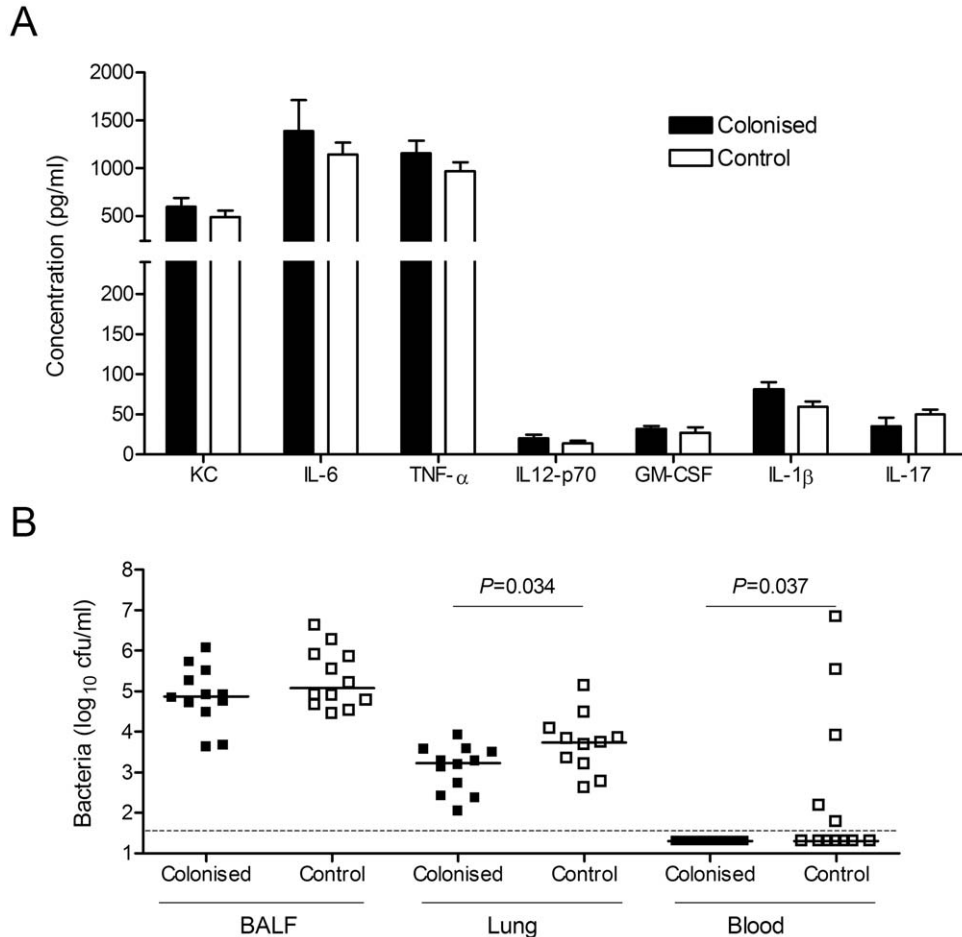


Figure 5. Role of CD4⁺ cells in the effect of prior colonisation on subsequent pneumonia. Cytokine levels (A) and bacterial CFU (B) recovered from colonised or control CBA/Ca mice 18 h following pneumonia challenge in the absence of CD4⁺ cells. Mice were depleted of CD4⁺ cells using GK1.5 prior to challenge. (A) Cytokine levels in BALF of colonised mice (black bars) or control mice (white bars) measured by Luminex or ELISA. Group mean + SEM are displayed. (B) Bacterial cfu in BALF, lung and blood of colonised or control CBA/Ca. Each symbol represents results for an individual mouse and bars the median for each group. The dotted line is the limit of detection. Significance of the difference in bacterial numbers recovered from lungs of colonised and control mice was assessed by Mann-Whitney U-test. Significance of differences in presence or absence of bacteraemia was assessed by Fisher exact test. Data are pooled from two experiments with similar results. doi:10.1371/journal.pone.0025558.g005

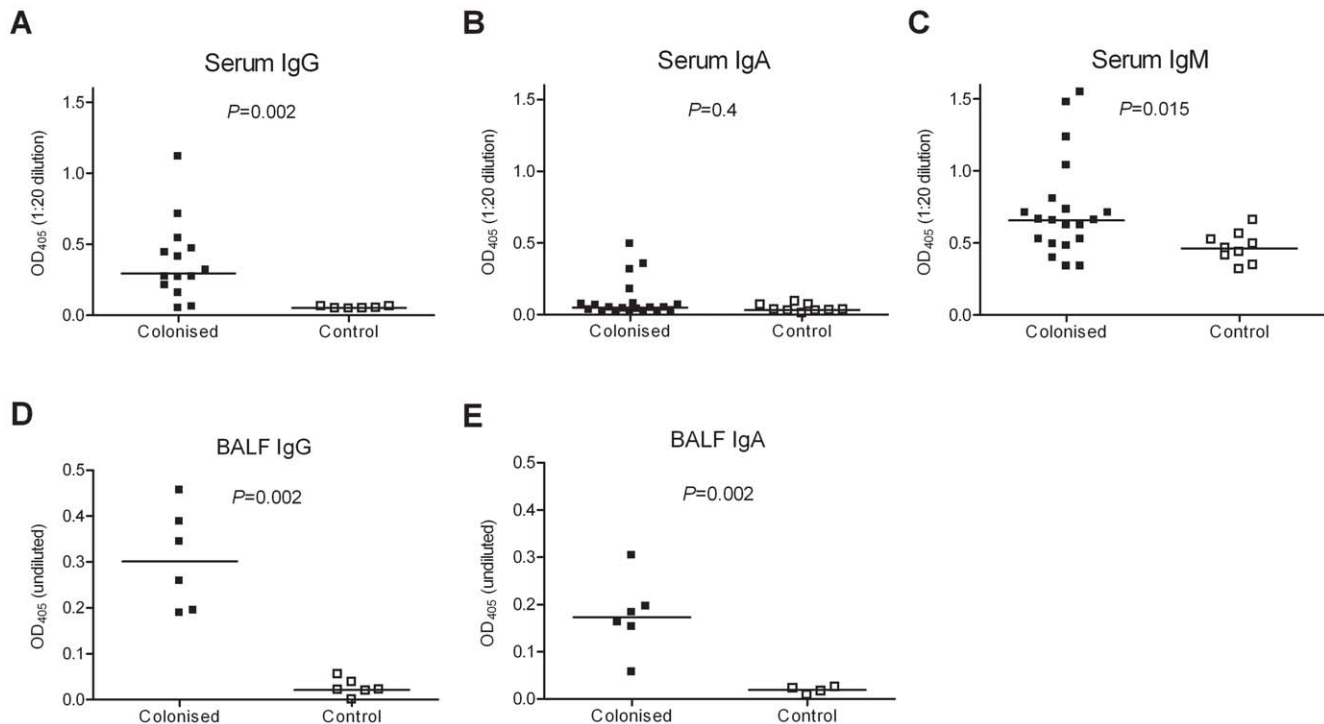


Figure 6. Systemic and mucosal antibody responses to colonisation. IgG (A and D), IgA (B and E) and IgM (C) responses to D39 in serum (A–C) or BALF (D–E) obtained from colonised or control mice measured using whole cell ELISAs. doi:10.1371/journal.pone.0025558.g006

but had no detectable effect on alveolar macrophage phagocytosis *in vivo*. This was consistent with prior colonisation having no effect on the number of bacteria recovered from BALF following pneumonia, but marked protection against pneumonia-associated bacteraemia (Fig. 2B–2D).

To investigate whether previous colonisation improved systemic clearance of *S. pneumoniae*, known to be largely dependent on phagocytosis [28], mice were inoculated intravenously with 5×10^6 cfu of D39. At 2 and 5 h post-intravenous inoculation there were over 10^4 fewer bacteria in the blood of previously colonised mice compared to control mice (Fig. 8D), demonstrating that colonisation promoted rapid clearance of *S. pneumoniae* from the blood consistent with previous colonisation resulting in a marked augmentation of serum-mediated phagocytosis.

Discussion

Recent data obtained using mouse models of colonisation have emphasised the importance of Th17 responses for protection against subsequent re-infection of the nasopharynx, and have shown that colonisation also protects against subsequent invasive infection. The development of protection against invasive infection required both CD4 cells and antibody but the precise mechanisms involved have not been clearly defined [12]. In this study, we have investigated in detail potential mechanisms of protection against subsequent pneumonia associated with *S. pneumoniae* nasopharyngeal colonisation. Mortality due to *S. pneumoniae* is mainly related to severe infections with septicaemia. Hence, to identify protective mechanisms that are effective against severe infection we have deliberately chosen a disease model of fulminant infection requiring a large inoculum with rapid spread of bacteria from the lungs to the blood and a high mortality. Several mechanisms through which colonisation may impact on subsequent disease progression were identified.

Firstly, prior nasopharyngeal colonisation was associated with a stronger mucosal inflammatory response 4 h after pneumonia challenge, with higher levels of some cytokines and a more rapid influx of neutrophils. The rapidity of onset of this difference between colonised and control mice suggests that colonisation may lead to alteration of the ‘innate immune rheostat’, priming for a more robust response to subsequent pneumonia challenge [29]. During the time that the nasopharynx remains colonised, small numbers of bacteria are likely to be aspirated into the lungs. These could have effects on innate immune cells such as macrophages and gamma-delta T-cells as shown for non-bacteraemic pneumonia [30]. This could lead to more robust cytokine production early in subsequent infection. Alternatively, mucosal antibody induced through prior colonisation may facilitate the interaction between bacteria and host cells such as alveolar macrophages during early pneumonia challenge and enhance cytokine responses. However, the effects of prior colonisation on the early inflammatory response in the lungs did not limit the development of disease in this model. There were no significant differences between colonised and control mice in *in vivo* phagocytosis of bacteria by alveolar macrophages, in bacterial CFU in BALF or lung, or in the histological severity of pneumonia. Potentially, the effects of colonisation on early inflammatory responses may have protective effects with other less fulminant models of pneumonia using lower inocula and / or less virulent *S. pneumoniae* strains.

Nasopharyngeal colonisation also primed for an enhanced lung and systemic IL-17 response following *in vivo* challenge. This was dependant on CD4-cells, strongly suggesting that it derives from a Th17-cell response to colonisation similar to that found in the nasopharynx following re-colonisation [19,31]. However in contrast to the importance of a Th17-cell response for preventing re-colonisation of the nasopharynx, colonised mice depleted of CD4 cells were still protected against bacteraemia and still had reduced lung CFU at 18 hours. Hence, Th17 responses are not

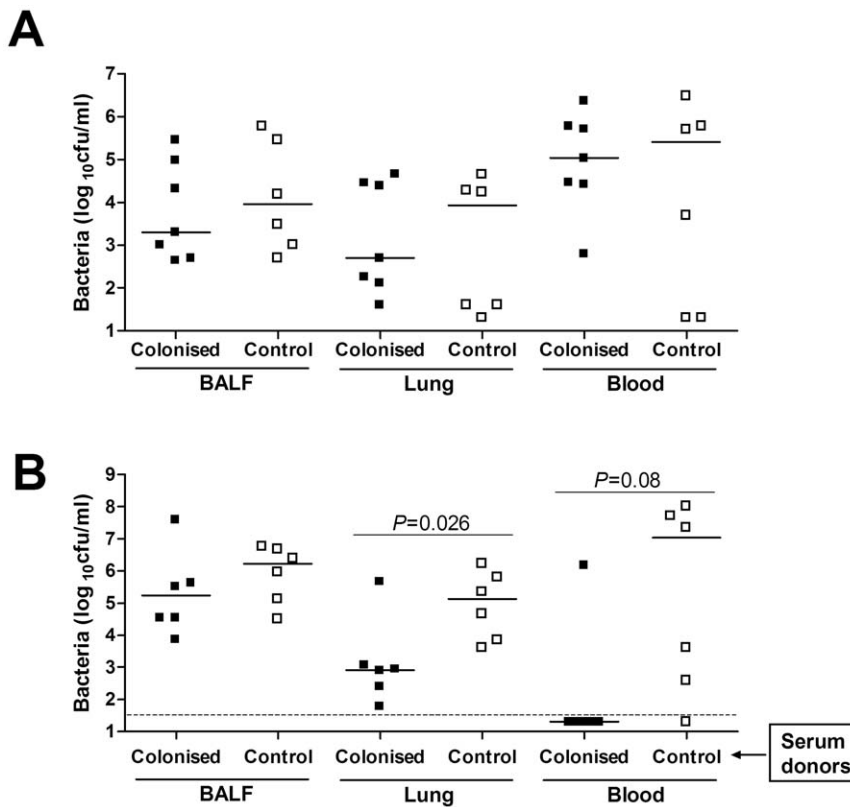


Figure 7. Role of antibody in the effect of prior colonisation on subsequent pneumonia. (A) Bacterial cfu in BALF, lung and blood of colonised or control μ MT mice 18 h following pneumonia challenge. (B) Bacterial cfu in BALF, lung and blood of passively immunised CBA/Ca mice 18 h following pneumonia challenge. Mice received pooled serum collected from either colonised or control CBA/Ca mice prior to challenge. Each symbol represents results for an individual mouse and bars the median for each group. The dotted line is the limit of detection. Lung cfu are compared between groups by Mann-Whitney U-test. Presence or absence of bacteraemia was assessed by Fisher exact test. doi:10.1371/journal.pone.0025558.g007

required for colonisation-induced protection in this model. The inability of previous colonisation to protect MHCII deficient mice against lethal challenge [12] is likely to reflect a need for CD4 T-cells in supporting the development of a mature antibody response to colonisation, rather than an effector role at the time of challenge. Rapid neutrophil recruitment occurs within the lungs even in uncolonised control mice, and bacteraemia is established relatively early in infection, probably eclipsing any benefits from a Th17-cell mediated increase in phagocyte recruitment. In contrast, recruitment of phagocytes to the nasopharynx during *S. pneumoniae* colonisation is delayed unless supported by an adaptive Th17 cell response induced by a previous colonisation event [19]. Hence a Th17 response is critical for adaptive immunity against colonising *S. pneumoniae* but not for the rapidly invasive model of pneumonia described in this manuscript.

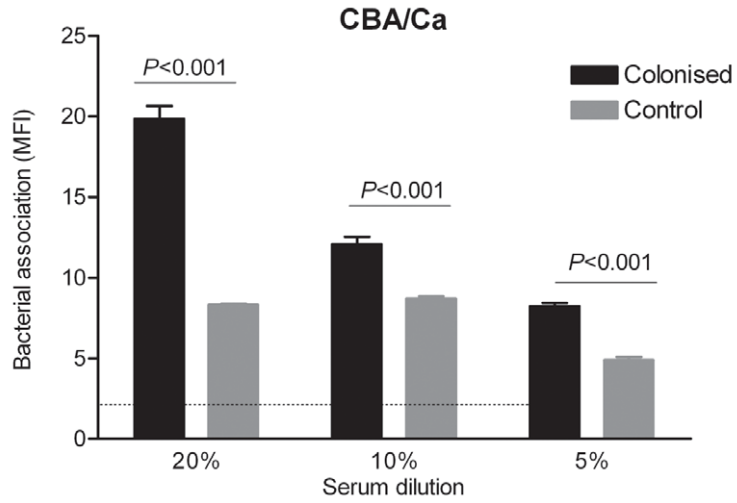
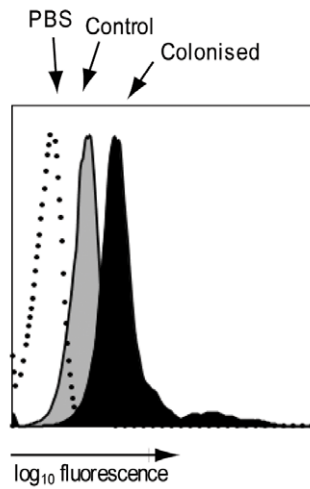
In addition, there is some evidence that Th17 cells actually might be deleterious during *S. pneumoniae* pneumonia [32,33], possibly explaining why we found CD4 depletion was associated with a trend towards fewer bacteria in the blood at 18 h. Vaccination-induced Th17-cell responses may still be beneficial for protection against a less fulminant pneumonia in which bacteria remain within the lung.

The strongest effect of colonisation observed during challenge in our model was the protection against bacteraemia. This suggests protective responses either prevent spread of *S. pneumoniae* from the lungs to the blood or that on reaching the blood bacteria are rapidly cleared. Nearly all mice developed serum IgG responses to

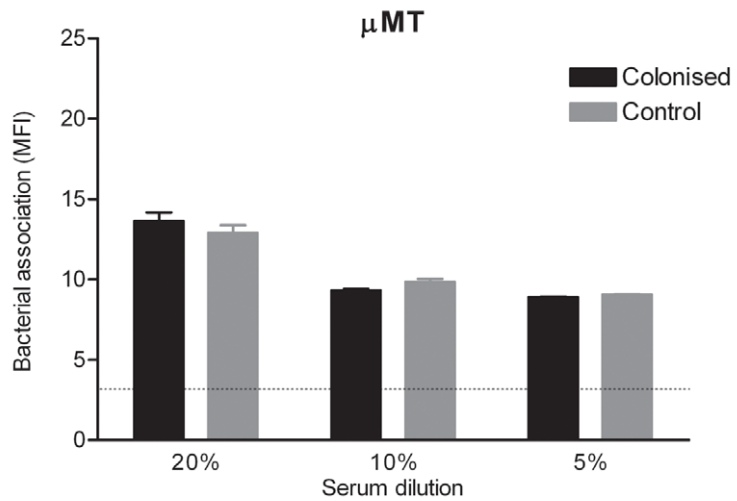
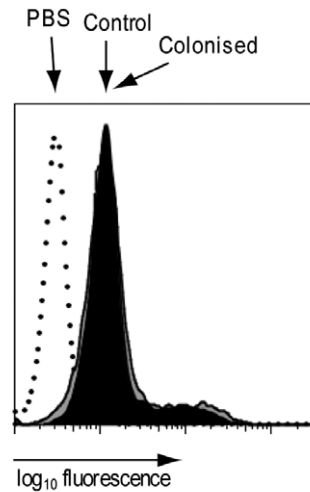
S. pneumoniae D39 antigens, and experiments with antibody-deficient mice demonstrated that protection against bacteraemia was highly dependent on antibody to *S. pneumoniae*. Following passive transfer of serum by intraperitoneal injection, there was a significant reduction in the number of bacteria present in the lungs 18 h following infection. This suggests that systemic antibody can assist control of infection within the lungs as well. Antibody to *S. pneumoniae* is mainly thought to cause protection by opsonising bacteria for phagocytosis, and *in vitro* phagocytosis assays and *in vivo* IV clearance data confirmed that colonisation induced antibody responses improved *S. pneumoniae* phagocytosis. The strength of the effect on IV clearance was particularly striking, reducing bacterial CFU by a factor of 3 to 4 log₁₀, readily explaining why colonised mice do not develop septicaemia. The observation that colonisation prevents the development of systemic infection provides one potential explanation for the rapid fall in the incidence of *S. pneumoniae* septicaemia in older children after a period of recurrent colonisation as infants.

Which *S. pneumoniae* antigens are the targets for protective responses in our model is not clear. Both capsular and protein antigens may induce antibody responses after murine nasopharyngeal colonisation [11,13]. However, colonisation with *S. pneumoniae* mutant strains not able to express the capsule or seemingly immunodominant protein antigens were still able to induce protective responses [13] suggesting antigens inducing protective antibody responses are partially redundant. We are currently identifying which antibodies dominate the immune response to colonisation in

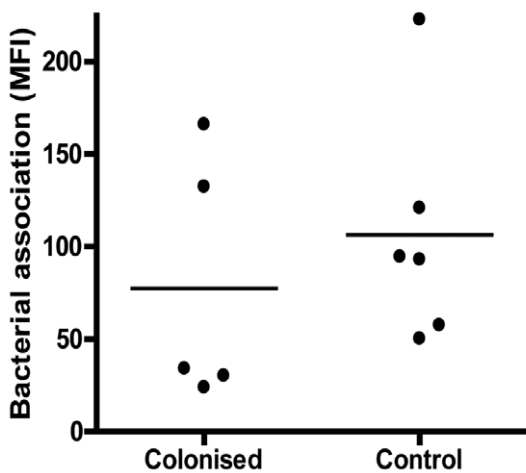
A



B



C



D

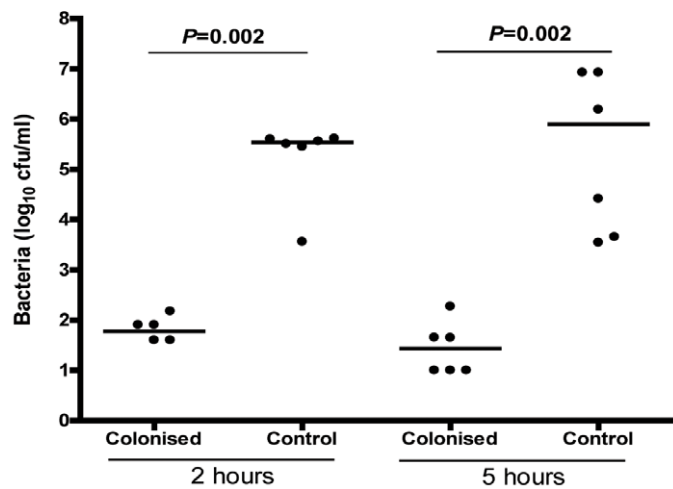


Figure 8. Colonisation induces serum opsonophagocytic antibody. (A–B) Flow cytometry assays of neutrophil phagocytosis of FAM-SE labelled D39 after incubation in serum from either (A) colonised or control CBA/Ca mice or PBS, or (B) colonised or control μ MT mice or PBS. A representative histogram is shown on the left (clear PBS, black 20% control sera, grey 20% colonised sera), and mean MFI \pm SEM ($n = 4$ replicates) for each condition in the graph on the right. (C) MFI of alveolar macrophages present in BALF of previously colonised or control CBA/Ca mice 4 h following challenge with 10^7 cfu FAM-SE-labelled D39 *S. pneumoniae*. Dots represent individual mice, bars represent medians. (D) Numbers of cfu of *S. pneumoniae* recovered from blood of colonised or control mice inoculated intravenously with 5×10^6 cfu *S. pneumoniae* D39 ($n = 6$ per group) at 2 and 5 h. Dots represent data from individual mice, bars represent group medians.
doi:10.1371/journal.pone.0025558.g008

this model, and which are the important antibodies in effecting protection.

To conclude, we have investigated the mechanisms of protection against subsequent pneumonia induced by nasopharyngeal colonisation with *S. pneumoniae*. In our model although colonisation results in a more rapid inflammatory response during early lung infection and a significant CD4-dependent IL-17 response, neither was necessary for the powerful protection against fulminant pneumonia provided by prior colonisation. Instead, protection was due to serum antibody responses that promoted rapid clearance of *S. pneumoniae* from the blood.

References

- Bogaert D, de Groot R, Hermans PW (2004) *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* 4: 144–154.
- Granat SM, Ollgren J, Herva E, Mia Z, Auranen K, et al. (2009) Epidemiological evidence for serotype-independent acquired immunity to pneumococcal carriage. *J Infect Dis* 200: 99–106.
- O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Oria-Knoll M, et al. (2009) Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* 374: 893–902.
- Fedson DS, Scott JA (1999) The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. *Vaccine* 17 Suppl 1:S11–8. S11–S18.
- Trotter CL, Waight P, Andrews NJ, Slack M, Efstratiou A, et al. (2010) Epidemiology of invasive pneumococcal disease in the pre-conjugate vaccine era: England and Wales, 1996–2006. *Journal of Infection* 60: 200–208.
- Jackson LA, Janoff EN (2008) Vaccines: pneumococcal vaccination of elderly adults: new paradigms for protection. *Clin Infect Dis* 47: 1328–1338.
- van der PT, Opal SM (2009) Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet* 374: 1543–1556.
- Lipsitch M, Whitney CG, Zell E, Kajjalainen T, Dagan R, et al. (2005) Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? *PLoS Med* 2: e15.
- Musher DM, Groover JE, Rowland JM, Watson DA, Struwing JB, et al. (1993) Antibody to capsular polysaccharides of *Streptococcus pneumoniae*: prevalence, persistence, and response to revaccination. *Clin Infect Dis* 17: 66–73.
- McCool TL, Cate TR, Tuomanen EI, Adrian P, Mitchell TJ, et al. (2003) Serum immunoglobulin G response to candidate vaccine antigens during experimental human pneumococcal colonization. *Infect Immun* 71: 5724–5732.
- Richards L, Ferreira DM, Miyaji EN, Andrew PW, Kadioglu A (2010) The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. *Immunobiology* 215: 251–263.
- Roche AM, King SJ, Weiser JN (2007) Live attenuated *Streptococcus pneumoniae* strains induce serotype-independent mucosal and systemic protection in mice. *Infect Immun* 75: 2469–2475.
- Roche AM, Weiser JN (2010) Identification of the Targets of Cross-Reactive Antibodies Induced by *Streptococcus pneumoniae* Colonization. *Infect Immun* 78: 2231–2239.
- Zhang Q, Bagrade L, Bernatoniene J, Clarke E, Paton J, et al. (2007) Low CD4 T Cell Immunity to Pneumolysin Is Associated with Nasopharyngeal Carriage of Pneumococci in Children. *J Infect Dis* 195: 1194–1202.
- Khader SA, Gaffen SL, Kolls JK (2009) Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal Immunol* 2: 403–411.
- Higgins SC, Jarnicki AG, Lavelle EC, Mills KHG (2006) TLR4 Mediates Vaccine-Induced Protective Cellular Immunity to Bordetella pertussis: Role of IL-17-Producing T Cells. *J Immunol* 177: 7980–7989.
- Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, et al. (2007) IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat Immunol* 8: 369–377.
- Lu YJ, Gross J, Bogaert D, Finn A, Bagrade L, et al. (2008) Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog* 4: e1000159.
- Zhang Z, Clarke TB, Weiser JN (2009) Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Invest* 119: 1899–1909.
- Morona J, Miller D, Morona R, Paton J (2004) The effect that mutations in the conserved capsular polysaccharide biosynthesis genes cpsA, cpsB, and cpsD have on virulence of *Streptococcus pneumoniae*. *J Infect Dis* 189: 1905–1913.
- Basavanna S, Khandavilli S, Yuste J, Cohen JM, Hosie AHF, et al. (2009) Screening of *Streptococcus pneumoniae* ABC transporter mutants demonstrates that LivJHMGF, a branched-chain amino acid ABC transporter, is necessary for disease pathogenesis. *Infect Immun* 77: 3412–3423.
- Wu HY, Virolainen A, Mathews B, King J, Russell MW, et al. (1997) Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. *Microb Pathog* 23: 127–137.
- Brown JS, Gilliland SM, Holden DW (2001) A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Mol Microbiol* 40: 572–585.
- Yuste J, Botto M, Bottoms SE, Brown JS (2007) Serum Amyloid P Aids Complement-Mediated Immunity to *Streptococcus pneumoniae*. *PLoS Pathog* 3: e120.
- Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS (2010) The *Streptococcus pneumoniae* Capsule Inhibits Complement Activity and Neutrophil Phagocytosis by Multiple Mechanisms. *Infect Immun* 78: 704–715.
- Arredouani MS, Yang Z, Imrich A, Ning Y, Qin G, et al. (2006) The macrophage scavenger receptor SR-AI/II and lung defense against pneumococci and particles. *Am J Respir Cell Mol Biol* 35: 474–478.
- Yuste J, Sen A, Truedsson L, Jonsson G, Tay LS, et al. (2008) Impaired opsonization with C3b and phagocytosis of *Streptococcus pneumoniae* in sera from subjects with defects in the classical complement pathway. *Infect Immun* 76: 3761–3770.
- Holdsworth RJ, Neill GD, Irving AD, Cuschieri A (1989) Blood clearance and tissue distribution of ^{99}Tc -labelled pneumococci following splenectomy in rabbits. *Br J Exp Pathol* 70: 669–677.
- Hussell T, Cavanagh MM (2009) The innate immune rheostat: influence on lung inflammatory disease and secondary bacterial pneumonia. *Biochem Soc Trans* 37: 811–813.
- Kirby AC, Newton DJ, Carding SR, Kaye PM (2007) Evidence for the involvement of lung-specific gamma delta T cell subsets in local responses to *Streptococcus pneumoniae* infection. *Eur J Immunol* 37: 3404–3413.
- Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, et al. (2005) CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *PNAS* 102: 4848–4853.
- LeMessurier K, Hacker H, Tuomanen E, Redecke V (2010) Inhibition of T Cells Provides Protection against Early Invasive Pneumococcal Disease. *Infect Immun* 78: 5287–5294.
- Weber SE, Tian H, Pirofski LA (2011) CD8+ Cells Enhance Resistance to Pulmonary Serotype 3 *Streptococcus pneumoniae* Infection in Mice. *J Immunol* 186: 432–442.

Acknowledgments

The authors are grateful to Dr Claudia Mauri and Dr Natalie Carter for the kind gift of μ MT mice, and to the staff at the UCL Biological Services Unit for assistance with animal maintenance and procedures.

Author Contributions

Conceived and designed the experiments: JMC HEB JSB. Performed the experiments: JMC SK EC CH. Analyzed the data: JMC HEB JSB. Wrote the paper: JMC JSB.