

Effect of Serotype on Pneumococcal Competition in a Mouse Colonization Model

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ABSTRACT Competitive interactions between *Streptococcus pneumoniae* strains during host colonization could influence the serotype distribution in nasopharyngeal carriage and pneumococcal disease. We evaluated the competitive fitness of strains of serotypes 6B, 14, 19A, 19F, 23F, and 35B in a mouse model of multiserotype carriage. Isogenic variants were constructed using clinical strains as the capsule gene donors. Animals were intranasally inoculated with a mixture of up to six pneumococcal strains of different serotypes, with separate experiments involving either clinical isolates or isogenic capsule-switch variants of clinical strain TIGR4. Upper-respiratory-tract samples were repeatedly collected from animals in order to monitor changes in the serotype ratios using quantitative PCR. A reproducible hierarchy of capsular types developed in the airways of mice inoculated with multiple strains. Serotype ranks in this hierarchy were similar among pneumococcal strains of different genetic backgrounds in different strains of mice and were not altered when tested under a range of host conditions. This rank correlated with the measure of the metabolic cost of capsule synthesis and *in vitro* measure of pneumococcal cell surface charge, both parameters considered to be predictors of serotype-specific fitness in carriage. This study demonstrates the presence of a robust competitive hierarchy of pneumococcal serotypes *in vivo* that is driven mainly, but not exclusively, by the capsule itself.

IMPORTANCE *Streptococcus pneumoniae* (pneumococcus) is the leading cause of death due to respiratory bacterial infections but also a commensal frequently carried in upper airways. Available vaccines induce immune responses against polysaccharides coating pneumococcal cells, but with over 90 different capsular types (serotypes) identified, they can only target strains of the selected few serotypes most prevalent in disease. Vaccines not only protect vaccinated individuals against disease but also protect by reducing carriage of vaccine-targeted strains to induce herd effects across whole populations. Unfortunately, reduction in the circulation of vaccine-type strains is offset by increase in carriage and disease from nonvaccine strains, indicating the importance of competitive interactions between pneumococci in shaping the population structure of this pathogen. Here, we showed that the competitive ability of pneumococcal strains to colonize the host strongly depends on the type of capsular polysaccharide expressed by pneumococci and only to a lesser degree on strain or host genetic backgrounds or on variation in host immune responses.

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Streptococcus pneumoniae (pneumococcus) is a commensal inhabitant of the human upper respiratory tract (URT). Colonization of the airways generally precedes disease, whose incidence peaks in very young children and in the elderly (1, 2). The worldwide burden of invasive pneumococcal disease (IPD) in children under 6 years of age was estimated to be at least 400,000 deaths in 2010, despite the existence of effective pneumococcal vaccines (3). A critical virulence factor in *S. pneumoniae* is the polysaccharide capsule, as unencapsulated strains are greatly attenuated in their virulence (4) and thus virtually absent from IPD (5, 6). Pneumococcus is characterized by extensive antigenic diversity in its capsule, resulting in more than 90 recognized serotypes (7, 8). Serotype distributions of pneumococci were consistent across space and time prior to the introduction of vaccination. For example,

strains of a few specific capsular types dominated in carriage (9, 10) and to some extent also in disease (11–13) both in Europe and in the United States. Available preventive strategies against pneumococcal disease are based on induction of immune responses against polysaccharides of the serotypes most prevalent in IPD (14). Reductions in carriage of vaccine serotypes (VTs) targeted by current polyvalent polysaccharide-conjugate vaccines (PCVs) have been largely offset by increases in nonvaccine serotype (NVT) carriage (15, 16), indicating that one factor shaping the population structure of *S. pneumoniae* might be serotype-dependent competitive interactions between pneumococci within the respiratory niche (17, 18). Because this “serotype replacement” phenomenon has led to increases in pneumococcal disease from the NVT strains (although usually not enough to

offset the vaccine-type disease prevented) (19, 20), understanding these competitive interactions has relevance for public health. Our knowledge about the extent of and the mechanisms involved in this interference is still limited.

Studies in humans have shown that simultaneous carriage of multiple pneumococcal strains is common, with approximately half of carriers among children cocolonized simultaneously with two or more strains of *S. pneumoniae* (21–23). Carriage of multiple strains is also a necessary condition for these highly recombinogenic bacteria to encounter and incorporate genetic material from other members of the species, providing raw material for evolution (24). The optimal settings to investigate the direct interaction between serotypes would require repeated sampling of the pneumococcal population colonizing or infecting an individual host. Such studies of human carriage are difficult to conduct and rarely performed. The few available studies conducted in humans have documented the presence of serotype-dependent competitive interactions among pneumococci (25–28). The alternative is to use animal models, which permit the study of carriage in a relatively controlled *in vivo* environment (29). Moreover, because bacteria in natural populations differ genetically in many ways besides their serotypes, experimental studies are required to isolate the contribution of serotype to differences in competitive ability.

Here, we studied serotype-dependent interactions between pneumococci in a mouse model of *S. pneumoniae* multistrain carriage. We hypothesized that the ability of a pneumococcal strain to outcompete other cocolonizing *S. pneumoniae* in the upper airways is determined by the type of capsular polysaccharide expressed. To test our hypothesis, we developed a protocol allowing repeated sampling of a single animal for pneumococci present in the URT and used quantitative PCR (qPCR) to track shifts in serotypes colonizing the host (30). We used both clinical strains of *S. pneumoniae* and sets of isogenic capsular variants of the clinical isolate TIGR4. We considered these variants to be genetically identical to the parent TIGR4 strain, except for the type of capsule expressed (10, 31).

We observed a reproducible hierarchy of capsular types developing within days after animal inoculation. The hierarchy was relatively consistent when assessed in clinical or isogenic strain sets and whether in inbred or outbred mice. Finally, manipulations of particular components of innate or acquired immunity did not substantially alter the hierarchy. The overall experiment's organization in the study is depicted in Fig. S1 in the supplemental material.

(This study was presented in part at the 7th International Symposium on Pneumococci and Pneumococcal Disease, Tel-Aviv, Israel, 14 to 18 March 2010 [32].)

RESULTS

Competitive rank of clinical strains. First, we tested clinical strains of serotypes 6B, 14, 19F, and 23F, four capsular types targeted by PCVs that were relatively common prior to introduction of the 7-valent vaccine (PCV7), and strains of non-PCV7 serotypes 19A and 35B representing types reported to increase in frequency in carriage after PCV7 implementation (9, 10, 16, 33, 34). There were no statistically significant differences in *S. pneumoniae* CFU recovered from C57BL/6 mice 5 days after inoculation with each strain alone (data not shown) (Kruskal-Wallis, $P > 0.05$).

Following intranasal inoculation with a mixture of all six clin-

ical strains, there was significant variation in strain frequency determined using serotype-specific, quantitative PCRs (qPCRs) in live samples on days 1 and 6 and in postmortem samples on day 15 (Kruskal-Wallis, $P < 0.0001$ within any sampling time point) (Fig. 1). At all of these time points, serotype 19A and 19F strains had the highest frequencies, and serotype 6B, 14, and 35B strains had the lowest; however, individual serotype frequencies on day 1 did not correlate with later time points ($P > 0.05$), whereas median strain frequencies on day 6 were closely correlated with those on day 15 ($\rho = 0.89$, $n = 6$, $P = 0.03$) (see Fig. S2A in the supplemental material), as were numbers of animals carrying particular strains on those days ($\rho = 0.88$, $n = 6$, $P = 0.03$). Based on this result, later experiments were terminated by day 9 or earlier.

Role of serotype in *in vivo* competitive fitness. The clinical strains tested differ genetically in many loci besides that which determines the serotype. To test the causal role of serotype in the competitive differences, C57BL/6 mice were inoculated with a mixture of six otherwise isogenic variants of *S. pneumoniae* stain TIGR4. Variants were constructed by transforming TIGR4 with DNA from clinical strains tested in the previous experiment, using back-crossing to reduce cotransformation of noncapsular loci (10, 31). In postmortem samples on day 9, the same two groupings of variants with high (serotype 19A, 19F, and 23F strains) and low (serotype 6B, 14, and 35B strains) frequencies were observed as with the clinical strains but with different orderings within the groups (Fig. 2). Correlations between postmortem frequencies of TIGR4 variants and frequencies of clinical strains at any of three sampling time points, although strongly positive, were not statistically significant (see Fig. S2B, C, and D in the supplemental material).

Effect of removing the top competitor. To assess whether the hierarchy was robust with the removal of the most successful serotype, we measured the rank of serotypes in an identical experiment, but leaving out the serotype 19F strain from the inoculum (Fig. 3). In the absence of the strain previously topping the hierarchy, the frequencies of serotype 23F and 6B variants significantly increased, whereas serotype 14 and 35B variants remained with the lowest relative abundance and colonized the fewest animals. Removal of the top competitor maintained a similar, but not identical, ordering of the remaining serotypes.

Effect of mouse genetic background. In order to determine if the outcomes of competition depended on the mouse genetic background, we assessed the ranks of serotypes in outbred CD1 mice inoculated with a mixture of six capsular variants of TIGR4 (strains of serotypes 6B, 14, 19A, 19F, 23F, and 35B). Overall, the pattern was similar to the findings in C57BL/6 mice: by day 7, the serotype 19F strain significantly increased its frequency, whereas the frequencies of strains of serotypes 6B, 14, and 35B had all declined (Fig. 4). The serotype 19A strain, in contrast, was much less successful in CD1 mice than in C57BL/6 mice. This single exception led to a nonsignificant correlation between results in CD1 and C57BL/6 mice (see Fig. S2E in the supplemental material), while the other ranks were well preserved between host backgrounds. Notably, the outlier in CD1 mice, the serotype 19A strain, showed a bimodal distribution of rankings, with the median frequency falling in the lower cluster of mice. This suggests the possibility that the large difference in medians with the serotype 19A variant between CD1 and C57BL/6 mice may have occurred by chance. In summary, it appears that the success of the

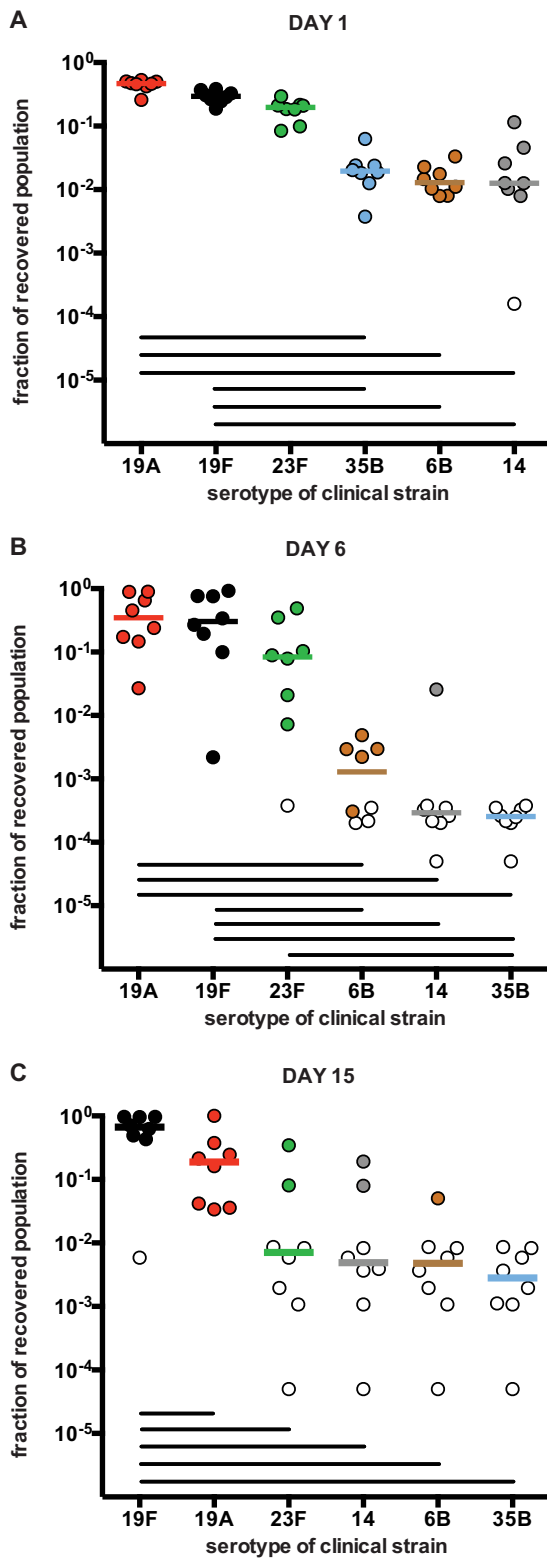


FIG 1 Changes in the relative abundance of *Streptococcus pneumoniae* clinical strains of different capsular types within populations recovered from C57BL/6 mice ($n = 8$) over the course of experimental multistrain carriage. Animals were inoculated intranasally with a mixture of six pneumococcal strains of serotypes 6B, 14, 19A, 19F, 23F, and 35B (fractions range from 0.13 to 0.19). Samples recovered from live animals 1 day (A) and 6 days (B) postinoculation and postmortem on day 15 (C) were tested in serotype-specific qPCRs to

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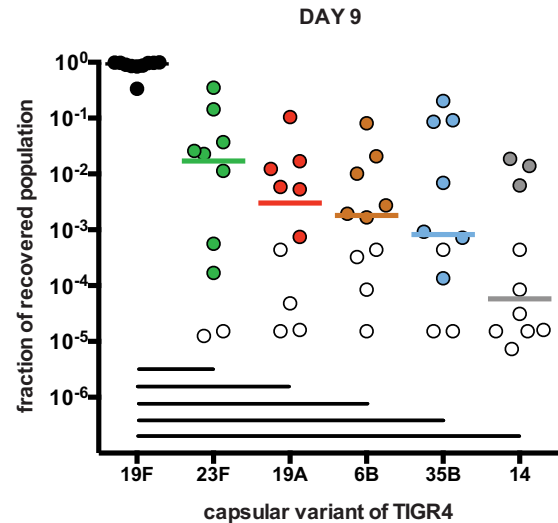


FIG 2 Relative abundance of serotypes in postmortem samples from C57BL/6 mice inoculated with capsular variants of *Streptococcus pneumoniae* strain TIGR4. Animals ($n = 10$) were inoculated intranasally with a mixture of six TIGR4 variants (fractions range from 0.11 to 0.20) of the same serotypes as clinical strains in the experiment depicted in Fig. 1. Samples recovered from animals on day 9 were tested in serotype-specific qPCRs to determine the fraction of the population represented by a variant of a particular capsular type. Results are coded as described in the legend to Fig. 1.

individual serotype was not strongly influenced by mouse genotype.

Effects of innate immunity on serotype ranking. Because we (35) and others (36) reported a contribution of neutrophils to control of *S. pneumoniae* colonization, and we also reported on serotype-related variation in *S. pneumoniae* resistance to opsonin-independent killing by human neutrophils (10), we tested in our model the effect of systemic depletion of neutrophils on rankings among TIGR4 capsular variants. In this experiment, C57BL/6 mice were either systemically depleted of neutrophil-like cells (here, neutrophils) with Ly6G antibodies (Fig. 5A) (35) or treated with control rat IgG (Fig. 5B). This resulted in over a 95% reduction in the number of neutrophil-like cells detected in peripheral blood of animals injected with Ly6G antibodies compared to mice treated with rat IgG (see Fig. S3 in the supplemental material). In postmortem samples collected on day 7, the serotype median frequency ranks were nearly identical in neutrophil-depleted versus control mice (see Fig. S2F in the supplemental material), indicating that neutrophil-like cells do not play a crucial role in deter-

Figure Legend Continued

determine the fraction of the population represented by a particular capsular type. Dots present the ratio of a given variant in samples as depicted on the y axis. Results are color coded per serotype: brown for 6B, gray for 14, red for 19A, black for 19F, green for 23F, and blue for 35B. Each dot depicts the ratio of a particular variant in the individual animal. Color-filled dots represent samples positive for a serotype. Blank dots represent samples qPCR negative for a serotype and the lower limit of detection according to total number of CFU recovered per sample. Colored horizontal lines represent medians of serotype fractions in samples, and strains are ranked from left to right according to the descending order of medians. Black horizontal lines depict significant differences (Kruskal-Wallis test, $P < 0.05$) between fractions of particular serotypes.

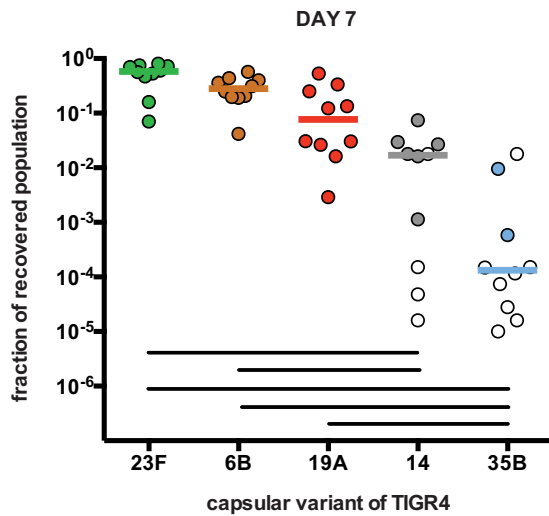


FIG 3 Effect of removing the top competitor on the hierarchy of pneumococcal strains *in vivo*. C57BL/6 mice ($n = 10$) were inoculated intranasally with a mixture of capsular variants of the TIGR4 strain as in Fig. 2 (fractions range from 0.17 to 0.26), except for the variant of serotype 19F removed from the inoculum. Samples recovered postmortem on day 7 were tested in serotype-specific qPCRs in order to determine the fraction of the population represented by a particular capsular type. Results are coded as described in the legend to Fig. 1.

mining the competitive ability of serotypes in colonization of naive mice.

Impact of noncapsular acquired immune responses on serotype rank. Immunity to pneumococcal carriage depends in part on acquisition of CD4⁺ Th17 cell-mediated immunity to antigens other than the capsule (9, 35, 37). Immunization with adjuvanted, unencapsulated whole pneumococci produces such an immune response in a controlled fashion (35). To test the impact of such immunity on the competitive interactions of capsular types, C57BL/6 mice were immunized twice intranasally with either ethanol-killed cells of unencapsulated *S. pneumoniae* strain RM200 with cholera toxin (CT) as the mucosal adjuvant (whole-cell vaccine [WCV]) ($n = 30$) or CT alone ($n = 20$). Two instead of the usual three doses of WCV were given in order to generate a suboptimal response and slow down the pneumococcal clearance described previously in WCV-immunized mice (38). Four weeks after the second immunization, all animals were inoculated intranasally with a mixture of serotype 14 and 19F variants of the TIGR4 strain at a ratio of 100 to 1. We tested two instead of six variants per experiment to reduce the variation observed in previous experiments (39). Because neutrophils are thought to be effectors of the Th17-mediated response in the immune mouse (35, 40), we further divided the WCV group into two subgroups: one ($n = 10$) that was depleted of neutrophil-like cells as described above and one ($n = 20$) that received control rat IgG. All in the CT group received control rat IgG. Postmortem samples were collected 5 days postinoculation because earlier experiments suggested clearance might have occurred by day 7 (38). There was a trend toward fewer CFU recovered from mice immunized with WCV (Fig. 6A) compared with mice immunized with CT alone, yet there were no differences in the ratios between strains of serotypes 14 and 19F in the pneumococcal populations recovered from mice still colonized between any of experimental groups

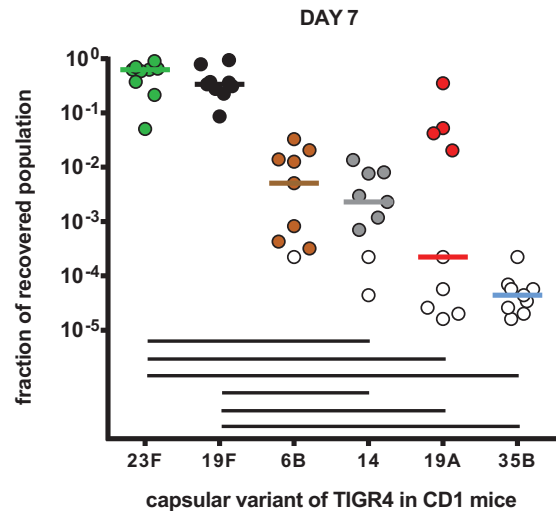


FIG 4 Effect of mouse genetic background on the outcome of competition between pneumococcal strains. CD1 outbred mice ($n = 9$) were intranasally inoculated with a mixture of six capsular variants of *S. pneumoniae* strain TIGR4 (fractions range from 0.14 to 0.21). Samples recovered postmortem on day 7 were tested in serotype-specific qPCRs to determine the fraction of the population represented by a variant of a particular capsular type. Results are coded as described in the legend to Fig. 1.

(Fig. 6B). We therefore concluded that the competitive relationships between at least this pair of serotypes were not altered in the presence of Th17-based immunity.

Hierarchy of serotypes in mouse model of multistrain carriage is consistent with other predictors of serotype-specific fitness. It has been previously reported that several traits associated with the fitness of pneumococcal serotypes in human hosts, including an epidemiological measure of competitive ability to colonize, mean duration of carriage in young children, and resistance to phagocytosis *in vitro*, correlate negatively with biochemical predictors, such as cell surface charge (41) and the number of carbons per repeat unit of the capsular polysaccharide (10). Similar negative correlations with number of carbons (Fig. 7A and B) and surface charge (Fig. 7C and D) were observed for our *in vivo* measure of the competitive ability of clinical strains or isogenic capsular variants of the TIGR4 strain tested in C57BL/6 mice, although these correlations with only 6 data points were statistically significant only in some cases.

DISCUSSION

Capsular serotype has been associated with a variety of phenotypes in *S. pneumoniae* that are likely to contribute, to some extent, to serotype-specific variation in fitness for transmission. Biochemical properties of the capsule, such as negative surface charge (41) and simple capsular sugar repeat unit structure, have been associated with large capsular size and resistance to *in vitro* surface phagocytosis by human neutrophils (10), as well as better *in vitro* growth in nutrient-limited medium (42). Serotypes with these properties tend to be successful in human colonization, showing evidence of strong competitive ability, high incidence, high prevalence, and long duration of carriage in epidemiological studies (28). Experimental studies have the advantage of being controllable and of being able to use isogenic strains to isolate the effect of capsule from that of linked polymorphism in natural populations

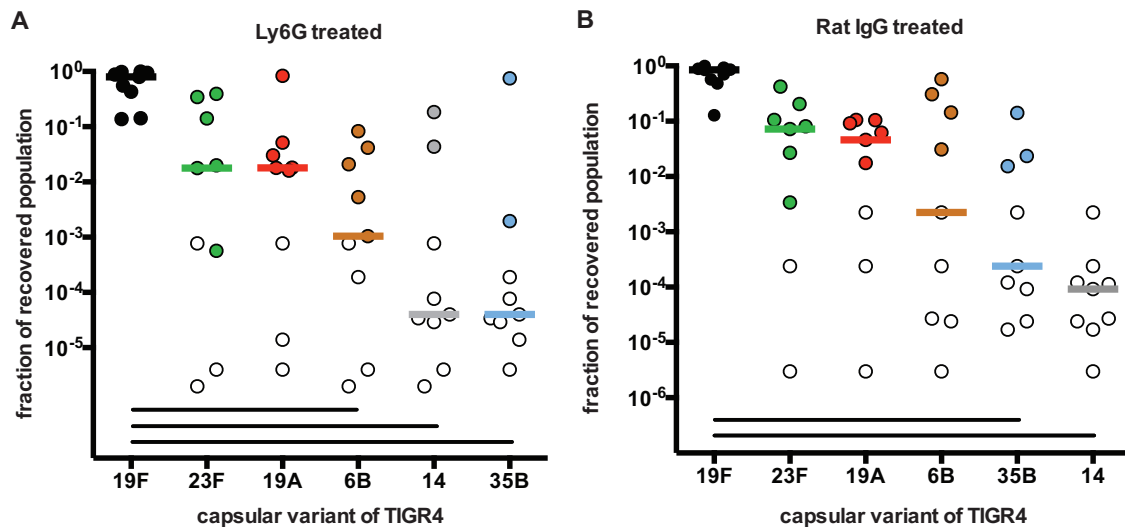


FIG 5 Impact of *in vivo* depletion of neutrophil-like cells on competitive interaction between capsular variants of *Streptococcus pneumoniae* strain TIGR4. C57BL/6 mice were intranasally inoculated with a mixture of six variants (fractions range from 0.14 to 0.21) and either depleted of neutrophil-like cells with Ly6G antibodies (A) or treated with control rat IgG (B) ($n = 9$ per group). Samples recovered postmortem on day 7 were tested in serotype-specific qPCRs to determine the fraction of the population represented by a variant of a particular capsular type. Results are coded as described in the legend to Fig. 1.

but have been rarely performed *in vivo* (30, 43). Epidemiological studies have the clear advantage of being able to suggest the relevance of capsular differences in humans, the host of interest, but cannot prove causality of the capsule itself as they are observational and involve strains that differ in many loci apart from the capsule. We have undertaken this study to combine experimental manipulation of capsule alone with an *in vivo* system, allowing us to test several hypotheses about the role of capsule in an important *in vivo* phenotype—competitive ability in multiple colonization (21, 44).

To accomplish these experiments required development of a qPCR assay for detection of multiple populations of isogenic

pneumococci that differed in serotype, as well as a live-sampling protocol for sequential samples from the upper respiratory tract of mice. A culture step was introduced in sample processing prior to genomic DNA purification to facilitate detection of all serotypes present in a sample as we were aware that DNA templates generated directly from samples may result in the loss of a signal due poor reproducibility of the DNA purification protocol when low-copy-number samples are processed (results not shown) but also due to possible differences in molecular assays' efficiencies (45), both leading to false-negative results.

These experiments clearly demonstrated that different strains of pneumococci compete with one another during colonization

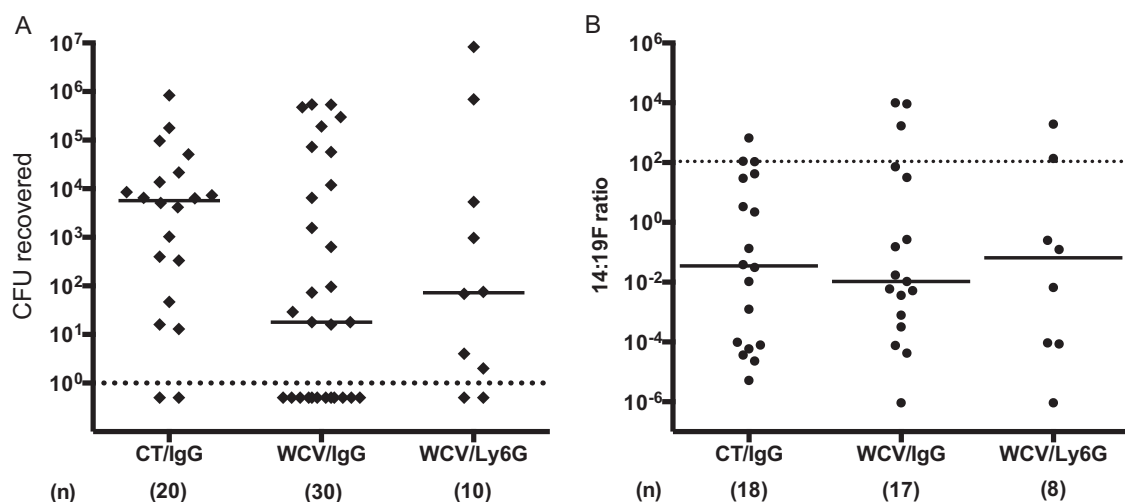


FIG 6 Impact of acquired, serotype-independent immunity to pneumococcal carriage on competitive interaction between serotypes 14 and 19F in C57BL/6 mice. C57BL/6 mice were immunized intranasally with either whole-cell vaccine (whole killed cells of unencapsulated *S. pneumoniae* strain with cholera toxin as an adjuvant [WCV]) or cholera toxin alone (CT), challenged with serotype 14 and 19F capsular variants of *S. pneumoniae* strain TIGR4 mixed at a ratio of 109:1 (represented by the dotted horizontal line in panel B), and either treated with control rat IgG or depleted of neutrophil-like cells with Ly6G antibodies. Postmortem samples collected 5 days after intranasal inoculation with mixture of pneumococcal strains were tested for the total number of CFU recovered per animal (A) and the ratio of serotype 14 to the 19F variant (B). None of the differences between groups was of significance.

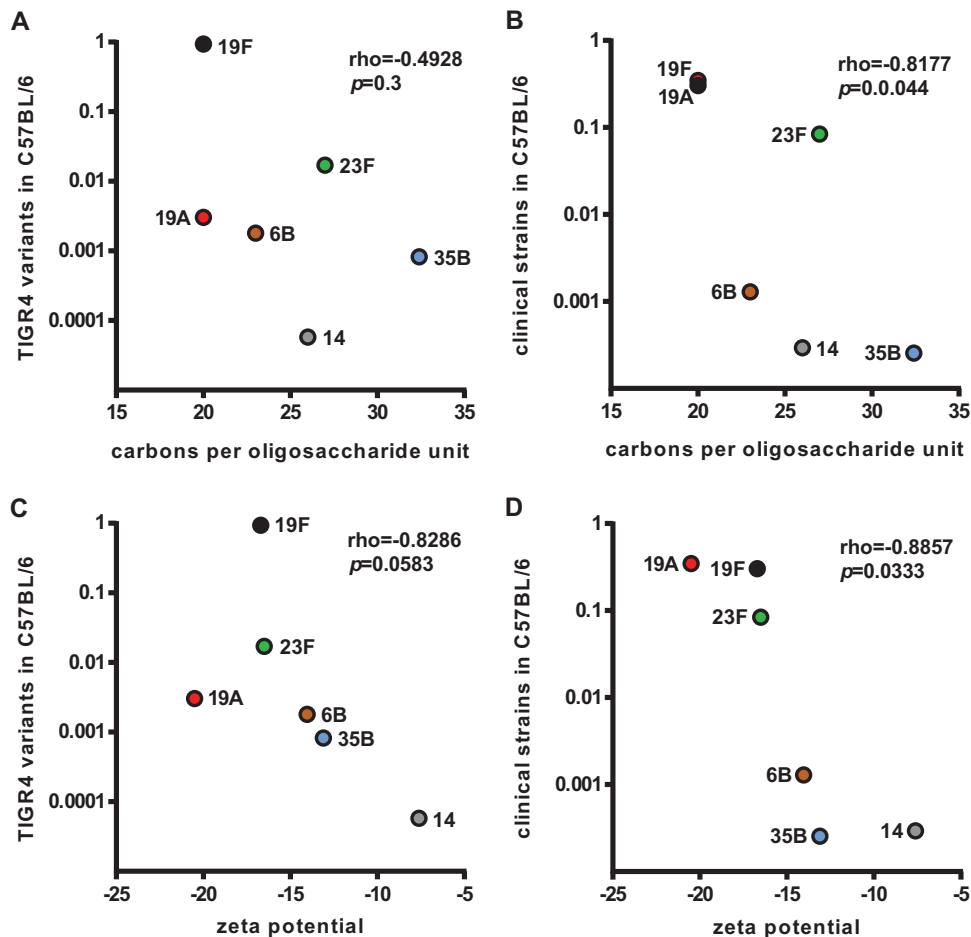


FIG 7 Correlations between *in vivo* hierarchical ranks of serotypes among *Streptococcus pneumoniae* strains tested in this study and other predictors of serotype-specific fitness. Median serotype frequencies of a capsular variant of TIGR4 in postmortem samples collected 9 days postinoculation (A and C) or of clinical strains detected with live sampling on day 6 postinoculation (B and D) were tested for the presence of correlation with the number of carbons per repeat unit of the capsular polysaccharide (A and B) as reported by Weinberger et al. (10) or the cell surface charge reported for corresponding serotype variant of the TIGR4 strain by Li et al. (41). Spearman's rank correlation coefficient (ρ) and the associated *P* value (*p*) are shown.

and that the serotype of the strain is a major, though not the only, determinant of competitive success. Strains of serotypes with the highest prevalence of carriage in human populations almost always did well in these competitions (19A, 19F, and 23F), while strains of serotypes less prevalent in carriage (35B and 14) did less well, suggesting that competitive success in the mouse may be correlated with the properties of fitness in human populations. Biochemical predictors of a serotype's human success also predicted success in these competition assays. The intermediate position in our competition assays of serotype 6B, the most common serotype in many populations (9), suggests that such correlations are not complete.

Combining the findings from different experiments provides some insight into the mechanisms involved in competitive success. The nearly identical rankings of serotypes in experiments involving clinical (genetically diverse) strains (Fig. 1) and isogenic strains (Fig. 2) varying only in capsular type suggest a strong role for capsule in determining competitive success *in vivo*, although variation in other genes surely plays a role. Indeed, as we discovered serendipitously in the course of these experiments and have reported in a separate publication, genetic traits apart from sero-

type determinants also affect competitive success *in vivo* (30). The similar rankings of different serotypes on day 1 and subsequent days (Fig. 1) suggested that a significant part of the difference in serotype success was due to differences in growth rate, attachment, or other intrinsic factors that produced variation in success prior to the induction of a strong immune response. This was further confirmed by our findings that depletion of neutrophil-like cells (Fig. 5) or the induction of partial, serotype-transcending acquired CD4⁺ T cell immunity (Fig. 6) had little effect on the outcome of competition. Given that macrophages are thought to play a part in clearance of carriage in previously naive mice (46) and given that one advantage of highly fit capsule-type strains seems to be their ability to evade phagocytosis (10), one should not rule out the possibility that part of the fitness variation is due to variation in resistance to macrophage-mediated killing, a hypothesis we did not test. The similar findings when the top competitor was removed (Fig. 3) or when the experiment was performed in outbred mice (Fig. 4) reinforce the robustness of our findings.

In summary, we have shown that a robust competitive hierarchy exists among pneumococcal serotypes in a mouse model of

upper respiratory tract carriage, which persists relatively unchanged under a range of host conditions and which is driven mainly, but not exclusively, by the capsule itself. Given the similarity to serotype rankings in human carriage and other likely components of pneumococcal fitness, further *in vitro* and *in vivo* work to uncover the mechanistic basis of this interaction with the host would be valuable.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All *S. pneumoniae* strains used in this study are listed in Table S1 in the supplemental material. All bacterial stocks were prepared as follows. Cells harvested from overnight culture on Trypticase soy agar supplemented with 5% sheep blood (TSA-SB [Becton Dickinson, Sparks, MD]) were used to inoculate Todd-Hewitt broth supplemented with 5% yeast extract (THY [Becton Dickinson]) at an optical density at 620 nm (OD_{620}) of 0.05. These cultures were further diluted 2.5×10^{-1} , incubated at 37°C until they reached OD_{620} of 0.2 to 0.25, supplemented with 10% glycerol, aliquoted to 1 ml, and stored frozen at -70°C . Prior to use, cell stocks were thawed on ice and washed by centrifugation at $12,000 \times g$ for 2 min, and the pellet was resuspended in 1 ml of ice-cold phosphate-buffered saline (PBS). The wash step was repeated, with the exception that cells were suspended in a predetermined volume of PBS to adjust the CFU concentration to that targeted in a particular experiment. All strains were individually tested for their ability to colonize C57BL/6 mice for at least 5 days, and only mouse-passaged isolates were used in experiments that followed (31, 47).

Animal strains. Inbred C57BL/6 and outbred CD1 mice were obtained from Jackson ImmunoResearch Laboratories, (Bar Harbor, ME). Animals (all females) were 5 to 6 weeks old at the start of experiments. Mice were housed and experiments were conducted in compliance with Institutional Animal Care and Use Committee protocols of Harvard University. Unless stated otherwise, all experiments were performed in groups of 10 animals.

Mouse model of multiserotype carriage. Mice were inoculated intranasally with a mixture of up to six *S. pneumoniae* strains at $\sim 10^6$ CFU in 10 μl of PBS delivered to a single nostril (48). Samples from live animals were collected by applying 10 μl of ice-cold PBS to each nostril of a mouse immobilized in an adapted 50-ml Falcon tube and collecting droplets discharged by the animal into an empty petri dish. Plates were immediately rinsed with 0.5 ml of PBS harvested to 1.5-ml Eppendorf tubes pre-filled with 50 μl of PBS (49). Postmortem upper respiratory tract samples were collected from retrotracheal washes as described by Wu et al. (48), except 30 instead of 6 drops were collected per animal (49). All samples collected from animals were kept on ice prior to further processing. If not stated otherwise, animals were sampled alive the day after inoculation and postmortem 7 days after inoculation.

Samples were cultured and titers determined on plates containing blood agar base 2 medium (Becton Dickinson) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO) and 2.5 mg/liter gentamicin (GENT-BAB plates) (48). Neat cultures were produced by inoculating GENT-BAB plates with 0.5 ml of either live-sampled material or retrotracheal wash. After overnight incubation at 37°C in 5% CO_2 and colony counting, bacterial cells were harvested by pouring 2 ml of THY-10% glycerol over the plate and scratching the bacterial growth with a glass spreader. One milliliter of cell suspension was recovered and stored frozen at -70°C for determination of sample serotype composition by a molecular method. In all experiments, a 10- μl volume of cell suspension used to inoculate the animals was also cultured and bacterial growth harvested as described above. In the majority of experiments, postmortem samples revealed that up to 2 animals per group of 10 were negative for *S. pneumoniae* after inoculation with a mixture of pneumococcal strains. Unless stated otherwise, results for these animals were excluded from analysis.

Neutrophil-like cell depletion. To deplete mice of neutrophil-like cells, 1 day before and 1 and 4 days after intranasal inoculation with

pneumococci, animals were injected with rat anti-mouse gG2B antibody RB6-8C5 (Bio Express, Lebanon, NH) against the Ly6G molecule expressed on mouse myeloid cells (35). Control rat immunoglobulin was used at the same time and dose to inject all remaining animals in a particular experiment. Up to five animals per group were bled retro-orbitally 2 days after last antibody injection in order to assess the decline in number of neutrophil-like cells in peripheral blood based on morphotypes of nucleated cells (150 to 300 leukocytes per sample) in a blood smears stained with Dip Quick stain (Jorgensen Laboratories, Loveland, CO).

Immunization with whole-cell vaccine. Animals were intranasally immunized twice (with a week apart) with the whole-cell antigen (WCA) derived from strain RM200 (38). The dose of 10- μl vaccine volume was made of 100 μg (dry weight) of WCA and 1 μg cholera toxin (CT) (List Biological Laboratories, Campbell, CA) as a mucosal adjuvant in PBS.

Determination of sample serotype composition by qPCR. Genomic DNA was purified from harvests of GENT-BAB plate cultures of specimens collected from animals and cultures of the inoculum using the DNeasy blood and tissue kit (Qiagen, Valencia, CA). The DNA concentration was measured in all samples using a NanoDrop spectrophotometer (Wilmington, DE) and diluted in $1 \times$ Tris-EDTA (TE) buffer to 1 ng/ μl . SYBR green chemistry quantitative PCR (qPCR) was applied to determine serotype composition. Serotype-specific primers (see Table S2 in the supplemental material for a description of the oligonucleotides) were designed with Primer Express 3.0 software (Applied Biosystems, Foster City, CA) based on published capsule locus sequences (http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/) (7). A total reaction volume of 25 μl was composed of $1 \times$ SYBR green PCR master mix (Applied Biosystems), 2.5 μl of DNA template, and a pair of primers (each at a 400 nM concentration) specific for a particular serotype, all in a molecular-biology-grade water (VWR). PCRs were performed on 7300 real-time PCR system (Applied Biosystems). The quantity of target sequences in a sample was determined based on a standard curve buildup with 10-fold dilutions of the DNA extracted from harvests of GENT-BAB cultures inoculated with the cell stock used to colonize mice in a particular experiment (DNA range from 25 to 0.025 ng per reaction). Strains' relative abundances were calculated after normalization of the frequency of serotype-specific sequence for strain frequency in the inoculum based on CFU counts prior to inoculum mixing. The lower limits of detection were set up for each sample as $1/(2n)$, where n is the number of CFU recovered from a neat culture plate of that sample.

Ranking of *S. pneumoniae* strains in experimental mouse carriage. To characterize serotype-specific competitive ability, within each experiment, we ranked the serotypes by the median adjusted frequency at a given time point. Adjusted frequencies were obtained by dividing each observed serotype frequency from *ex vivo* samples by that serotype's measured frequency in the inoculum, to compensate for sampling variation when the inoculum was prepared.

Statistical analysis and data presentation. Results were analyzed and graphed using GraphPad Prism v5.0 (GraphPad Software, San Diego, CA). The nonparametric Kruskal-Wallis test was used to test variation in serotype relative abundance or in *S. pneumoniae* colonization densities. Planned single comparisons were also tested with a Kruskal-Wallis test and correlations with Spearman's rank correlation for nonparametric data. Statistical significance was defined by a P value of ≤ 0.05 .

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00902-15/-/DCSupplemental>.

Figure S1, PDF file, 0.05 MB.

Figure S2, PDF file, 0.2 MB.

Figure S3, PDF file, 0.1 MB.

Table S1, PDF file, 0.1 MB.

Table S2, PDF file, 0.04 MB.

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K.T. and M.L. conceived and designed the experiments, K.T., Y.L., C.T.M., D.C., and A.B. performed the experiments, K.T., Y.L., D.M.W., and M.L. analyzed the data, K.T., Y.L., D.M.W., D.C., and R.M. contributed reagents, materials, and analysis tools, and K.T. and M.L. wrote the paper. All authors read and approved the final manuscript.

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