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Protection against Nasopharyngeal Colonization by *Streptococcus pneumoniae* Is Mediated by Antigen-Specific CD4$^+$ T Cells$^\nabla$

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CD4$^+$ T-cell-dependent acquired immunity confers antibody-independent protection against pneumococcal colonization. Since this mechanism is poorly understood for extracellular bacteria, we assessed the antigen specificity of the induction and recall of this immune response by using BALB/c DO11.10Rag$^{-/-}$ mice, which lack mature B and T cells except for CD4$^+$ T cells specific for the OVA$^{323–339}$ peptide derived from ovalbumin. Serotype 6B *Streptococcus pneumoniae* strain 603S and unencapsulated strain Rx1Δ*lyt*A were modified to express OVA$^{323–339}$ as a fusion protein with surface protein A (PspA) (strains 603OVA^1 and Rx1Δ*lytA*OVA^1) or with PspA, neuraminidase A, and pneumolysin (Rx1Δ*lytA*OVA^3). Whole-cell vaccines (WCV) were made of ethanol-killed cells of Rx1Δ*lytA* plus cholera toxin (CT) adjuvant, of Rx1Δ*lytA*OVA^1 + CT (WCV-OVA^1), and of Rx1Δ*lytA*OVA^3 + CT (WCV-OVA^3). Mice intranasally immunized with WCV-OVA^1, but not with WCV or CT alone, were protected against intranasal challenge with 603OVA^1. There was no protection against strain 603S in mice immunized with WCV-OVA^1. These results indicate antigen specificity of both immune induction and the recall response. Effector action was not restricted to antigen-bearing bacteria since colonization by 603S was reduced in animals immunized with vaccines made of OVA-expressing strains when ovalbumin or killed Rx1Δ*lytA*OVA^3 antigen was administered around the time of challenge. CD4$^+$ T-cell-mediated protection against pneumococcal colonization can be induced in an antigen-specific fashion and requires specific antigen for effective bacterial clearance, but this activity may extend beyond antigen-expressing bacteria. These results are consistent with the recruitment and/or activation of phagocytic or other nonspecific effectors by antigen-specific CD4$^+$ T cells.

Colonization of the upper respiratory tract (URT) is a step prior to *Streptococcus pneumoniae* infection (1, 4). Most carriage episodes are asymptomatic and last on the order of weeks to a few months (1, 8). In principle, colonization may be prevented or terminated by the innate and/or adaptive immune system or by competing microbial flora (15, 33), yet the particular host and pathogen factors affecting resistance to pneumococcal colonization are still poorly understood. The successes of antipneumococcal therapy using passive transfer of serotype-specific antibodies (14) and of vaccinations that depend on anticapsular antibodies (5) showed the importance of humoral immunity as one mechanism of protection against colonization and disease from *S. pneumoniae*. For some, but not all serotypes, such immunity appears to play a role in naturally acquired protection (34). However, several lines of evidence indicate that factors other than acquisition of anticapsular antibodies play a crucial role in the development of natural protection against pneumococci. The reduction in pneumococcal disease incidence after the first birthday in the general population occurs simultaneously for many rare and common serotypes, suggesting the acquisition of one rather than many individual immune responses; in particular, it seems to precede by several years the age-related rise in anticapsular antibody (12). Similar patterns have been suggested for nasopharyngeal carriage (8). Experimental (20, 21) and observational (7, 16) studies in adults have found little or no evidence that higher anticapsular antibody concentrations are associated with greater protection from colonization.

Mouse studies have similarly shown that immunity to pneumococcal colonization acquired from prior exposure to live bacteria (31) or a killed, whole-cell vaccine (WCV) (19) is antibody independent, while other studies have shown a similar mechanism for clearance of longstanding carriage in previously unexposed animals (33). Acquired immunity was shown to be dependent on the presence of CD4$^+$ T cells at the time of challenge (19, 31).

Apart from their role in providing help for the production of antibodies, the role of CD4$^+$ T cells in acquired immunity to extracellular bacteria remains poorly understood. A basic question is whether such responses depend on classical antigen presentation to the T-cell receptor. Two types of observations particularly raised this concern. First, while wild-type mice inoculated intranasally with a strain of pneumococcus rapidly cleared bacteria from the lungs and blood within 2 days, major histocompatibility complex class II-knockout mice in the same experiment showed persistent infection in both lungs and blood over 3 days, suggesting a nonspecific role for CD4$^+$ T cells in early host defense (10). Further, the pneumococcal toxin pneumolysin caused apparently nonspecific activation and migration of CD4$^+$ T cells in vitro in the absence of antigen presentation (10). Second, we observed (data pre-

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TABLE 1. *Streptococcus pneumoniae* strains used in the study

Strain	Description[a]	Reference
CP1296	Derivative of laboratory strain R6; *cbp3*::*kan-rpsL*$^+$ Km^r Sm^s	29
TIGR4		30
TIGR4S	Derivative of clinical strain TIGR4 but Sm^r by selection of mutants; Km^s	32
TIGR4Δ*pspA*	TIGR4S but *pspA*::*kan-rpsL*$^+$ by transformation with ligation product of PCR fragments amplified with primer pairs TTM053-TTM066 in TIGR4, DAM406-DAM351 in CP1296, and TTM067-TTM069 in TIGR4, and prior to ligation digested with BamHI, BamHI, and ApaI and ApaI restrictases, respectively; Km^r Sm^s	This study
TIGR4OVA^1	TIGR4Δ*pspA* derivative, *kan-rpsL*$^+$::*pspA*OVA by transformation with PspA-OVA fusion protein cassette constructed using TIGR4 genomic DNA; Km^s Sm^r	This study
R6		9
Rx1		28
Rx1S		25
Rx1Δ*lytA*J	Derivative of streptomycin-resistant mutant of laboratory strain Rx1; *lytA*::*kan-rpsL*$^+$ Km^r Sm^s	25
Rx1Δ*lytA*	Rx1Δ*lytA*J derivative, *kan-rpsL*$^+$ negative by transformation with truncated *lytA* fragment constructed using R6 genomic DNA; Km^s Sm^r	This study
Rx1Δ*lytA*Δ*pspA*	Rx1Δ*lytA* derivative, *pspA*::*kan-rpsL*$^+$ by transformation with primer pair TTM053-TTM069 PCR product of TIGR4Δ*pspA*; Km^r Sm^s	This study
Rx1Δ*lytA*OVA^1	Rx1Δ*lytA*Δ*pspA* derivative, *kan-rpsL*$^+$::*pspA*OVA by transformation with primer pair TTM053-TTM069 product of TIGR4OVA^1; Km^s Sm^r	This study
Rx1Δ*lytA*Δ*ply*	Rx1Δ*lytA*OVA^1 derivative, ply::*kan-rpsL*$^+$ by transformation with ligation product of PCR fragments amplified with primer pairs TTM171-TTM173 in R6, DAM406-DAM351 in CP1296, and TTM176-TTM178 in R6, and prior to ligation digested with BamHI, BamHI, and ApaI and ApaI restrictases, respectively; Km^r Sm^s	This study
Rx1Δ*lytA*OVA^2	Rx1Δ*lytA*Δ*ply* derivative, *kan-rpsL*$^+$::*ply*OVA by transformation with Ply-OVA fusion protein cassette constructed using R6 genomic DNA, strain expressing two OVA fusion proteins; Km^s Sm^r	This study
Rx1Δ*lytA*Δ*nanA*	Rx1Δ*lytA*OVA^2 derivative, Δ*nanA*::*kan-rpsL*$^+$ by transformation with ligation product of PCR fragments amplified with primer pairs TTM186-TTM188 in R6, DAM406-DAM351 in CP1296, and TTM191-TTM193 in R6, and prior to ligation digested with BamHI, BamHI, and ApaI and ApaI restrictases, respectively; Km^r Sm^s	This study
Rx1Δ*lytA*OVA^3	Rx1Δ*lytA*Δ*nanA* derivative, *kan-rpsL*$^+$::*nanAOVA* by transformation with NanA-OVA fusion protein cassette constructed using R6 genomic DNA, strain expressing three OVA fusion proteins; Km^s Sm^r	This study
603	Serotype 6B clinical isolate; Km^s Sm^s	19
603S	603 derivative, Sm^r by selection of mutants	This study
603Δ*pspA*	603S derivative, *pspA*::*kan-rpsL*$^+$ by transformation with primer pair TTM053-TTM069 PCR product of TIGR4Δ*pspA*; Km^r Sm^s	This study
603OVA^1	603Δ*pspA* derivative, *kan-rpsL*$^+$::*pspA*OVA by transformation with primer pair TTM053-TTM069 PCR product of TIGR4OVA^1; Km^s Sm^r	This study

[a] Km, kanamycin; Sm, streptomycin.

sented below) that immunization of mice with very small quantities of killed whole pneumococci could protect them against subsequent intranasal challenge. These data are in accord with the results of a recent report by Roche et al. (26) showing that, in mice, a single exposure to a live attenuated pneumococcal strain conferred resistance against colonization and invasive disease and raise concern about the antigenic specificity of the observed protection.

We therefore sought to test the hypothesis that exposure to killed pneumococci with cholera toxin adjuvant induces CD4$^+$ T-cell-dependent immunity in an antigen-specific fashion. We approached this question by using BALB/c DO11.10 Rag$^{-/-}$ mice, which lack mature B and T cells except for CD4$^+$ T cells specific for the OVA$^{323-339}$ peptide derived from ovalbumin (22). By varying the presence of this peptide in the immunizing and challenge doses, we assessed (i) whether the induction of CD4$^+$ T-cell-dependent immunity was antigen specific; (ii) whether the memory immune response required antigen-specific stimulation of CD4$^+$ T cells; and (iii) whether the effector function, once stimulated by cognate antigen, was limited to cells bearing that antigen. We measured the effectiveness of this immunity by evaluating the impact of vaccination with killed bacterial cells on subsequent *S. pneumoniae* colonization.

MATERIALS AND METHODS

***Streptococcus pneumoniae* strains.** Pneumococcal isolates were maintained as previously described (32). Strains were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (Becton Dickinson, Sparks, MD) or on blood agar base no. 2 medium (Acumedia Manufacturers, Lansing, MI) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO) (SBA). An autolysin-negative mutant of unencapsulated strain Rx1 (Rx1Δ*lytA*) (25, 28), serotype 4 strain TIGR4 (30), and serotype 6B strain 603 (17) were modified to express the OVA$^{323–339}$ peptide on the bacterial surface as a fusion protein with pneumococcal surface protein A (PspA). To construct these, the *pspA* locus in TIGR4 was replaced with a Janus-type cassette (29) by using the transformation protocol described by Pozzi et al. (24). The cassette was then replaced with a DNA fragment coding for PspA with OVA$^{323–339}$, generated in a two-step PCR as described by Park et al. (23), to create strain TIGR4OVA^1. Strains Rx1Δ*lytA*OVA^1 and 603OVA^1 were created by transforming parental strains with PCR products generated in TIGR4 mutants. Rx1Δ*lytA*OVA^1 was further modified to express fusion proteins of OVA$^{323–339}$ with pneumolysin and neuraminidase A (Rx1Δ*lytA*OVA^3) by using cassettes constructed in strain R6 (9). See Table 1 for a detailed description of strains and Table 2 for primers used in the course of the study. Fusion protein integrity was confirmed by sequencing open reading frames. PspA-OVA expression was confirmed by Western blotting using antisera to both chicken egg albumin (Sigma) and PspA (gift of Susan

TABLE 2. Primers used in the study

Primer	Description	Reference
DAM351	CTAGGGCCCCTTTCCTTATGCTTTTGGAC	29
DAM406	TCTATGCCTATTCCAGAGGAAATGGAT	29
TTM051	CTTTGGGCAGTAGTGGTAAC; corresponds to position 853–834 upstream of *pspA* in TIGR4	This study
TTM053	GCGATAAGCCTAATAAGCACAAC; corresponds to position 910–888 upstream of *pspA* in TIGR4	This study
TTM059	GGTCTGATCCTTGCCATTGTC; corresponds to position 859–839 downstream of *pspA* in TIGR4	This study
TTM069	TGCGTACTTCAGGCTTTTC; corresponds to position 941–923 downstream of *pspA* in TIGR4	This study
TTM066	AAAGGATCCTTAAGCCTGCGGACAATC; position 9–27, corresponds to position 269–287 upstream of *pspA* in TIGR4	This study
TTM067	TTTGGGCCCATGGGTTGTTTTATTTATTAT; position 10–30, corresponds to position 174–194 downstream of *pspA* in TIGR4	This study
TTM072	ATTACTTGGAGGGGCTGATTCTGA; within *pspA* of TIGR4, position 1206–1229, sequencing primer	This study
TTM073	AGTGGCTGGTTTTTTCTGGTTGAGT; within *pspA* of TIGR4, position 1569–1546, sequencing primer	This study
TTM061	TGATTTCTGCATGTGCTGCATGGACAGCTTGAGATATAGTTTTTTCCAATTCAGC; position 38–55, corresponds to position 1290–1273 in *pspA* of TIGR4, sequence-coding fragment of OVA peptide underlined	This study
TTM062	GCAGCACATGCAGAAATCAATGAAGCAGGCAGACAAAAAGAATTAGATGCAGC; position 34–53, corresponds to position 1291–1310 in *pspA* of TIGR4, sequence-coding fragment of OVA peptide underlined	This study
TTM171	CTACCTGTCGCCCTTGCTCTGG; corresponds to position 1008–987 upstream of *ply* in TIGR4	This study
TTM172	CTCAAGGCTAACCAAGAAGTA; corresponds to position 776–756 upstream of *ply* in TIGR4	This study
TTM173	TTTGGATCCATTTACTGCTTTATTTGCCATCT; position 9–32, corresponds to position 22 to 2 upstream of *ply* in TIGR4	This study
TTM174	TGATTTCTGCATGTGCGGCATGGACAGCTTGAGATATCATCTTCTACCTCCTAATAA; position 38–57, corresponds to position 23–4 within *ply* in TIGR4, sequence-coding fragment of OVA peptide underlined	This study
TTM175	GCCGCACATGCAGAAATCAATGAAGCAGGCAGAGCAAATAAAGCAGTAAATGA; position 53–34, corresponds to position 17 upstream of *ply* to position 3 within *ply* in TIGR4, sequence-coding fragment of OVA peptide underlined	This study
TTM176	TTTGGGCCCTCTTGCCTAGCGATTTCCATT; position 9–30, corresponds to position 9–30 downstream of *ply* in TIGR4	This study
TTM177	CATTGGCTGCTGTACGGTTGAC; position 1054–1035 downstream of *ply* in TIGR4	This study
TTM178	CCATGTTTCCGCCGTTTTTACC; position 1090–1071 downstream of *ply* in TIGR4	This study
TTM186	CGTCTGACCTGCGATAAAACC; corresponds to position 1493–1473 upstream of *nanA* in R6	This study
TTM187	AACAGGACAAATCGATCAGGACAG; corresponds to position 1251–1228 upstream of *nanA* in R6	This study
TTM188	TTTGGATCCCGATTTCTGAAATAAGA; position from 6–26, corresponds to position 24–4 in *nanA* of R6	This study
TTM189	TGATTTCTGCATGTGCGGCATGGACAGCTTGAGATATAGAAGGCTGGCTCTTTTCTGT; position 38–58, corresponds to position 252–232 in *nanA* of R6, sequence-coding fragment of OVA peptide underlined	This study
TTM190	GCCGCACATGCAGAAATCAATGAAGCAGGCAGATCAGAGACTGAACTTTCTGGC; position 34–54, corresponds to position 253–273 in *nanA* of R6, sequence-coding fragment of OVA peptide underlined	This study
TTM191	TTTGGGCCCTCTTGCTCAGCAGGCACT; position 8–27, corresponds to position 2989–3008 in *nanA* of R6	This study
TTM192	GAAGGACATAAAAACACTACTCTC; corresponds to position 941–918 downstream of *nanA* in R6	This study
TTM193	TCTTCATCATATTCTTGGGTAACG; corresponds to position 1310–1287 downstream of *nanA* in R6	This study

Hollingshead, University of Alabama at Birmingham). Pneumolysin and neuraminidase activity in Rx1Δ*lytA*OVA[3] was confirmed in a hemolysis assay as described by Benton et al. (3) and in a neuraminidase A assay developed by Lock et al. (13) and modified by King et al. (11).

Animals. Wild-type C57BL/6J mice were ordered from Jackson Laboratory, BALB/c mice from Jackson Laboratory or Taconic Farms, and BALB/c DO11.10 RAG$^{-/-}$ transgenic mice from Taconic Farms (22). In all experiments, mice aged 6 to 7 weeks with sexes equally distributed among experimental and control groups were used unless stated otherwise. All animals were kept in sterile microisolator settings in a BL2 facility. Procedures that involved mice were approved by the Institutional Animal Care and Use Committee.

Immunization and challenge. In all experiments, a single dose of the WCV was composed of ethanol-killed cells equivalent to 10^8 CFU and 1 μg of cholera toxin (CT) mucosal adjuvant (List Biological Laboratories, Campbell, CA) in a 10-μl dose volume unless stated otherwise (17). The exception was the evaluation of WCV dose responses, in which four groups of C57BL/6J female mice were immunized as previously described (17) twice, one week apart, with the regular dose of Rx1Δ*lytA* WCV (group 1), a dose composed of a 1/100 or 1/10,000 dilution of this CFU dose (groups 2 and 3, respectively), or CT alone (group 4). Four weeks after the last immunization, all animals were challenged intranasally with 2×10^6 CFU of strain 603. One week after the challenge, animals were euthanized and URT washes were collected and counted following serial dilution and culturing on SBA supplemented with gentamicin (2.5 mg/liter) to evaluate the presence of the challenge strain in the URT. The same protocol was followed in other experiments unless stated otherwise.

Specificity of immune induction. To test the antigen specificity of immune induction, three groups of DO11.10 RAG$^{-/-}$ mice were immunized twice with WCV derived from strain Rx1Δ*lytA*, WCV-OVA[1] derived from Rx1Δ*lytA*OVA[1], or CT alone and challenged with 603OVA[1]. The presence of the challenge strain in the URT was evaluated by using SBA plates supplemented with streptomycin (150 mg/liter). Two groups of 8 BALB/c female mice were immunized with either WCV-OVA[1] or CT alone and challenged with a streptomycin-resistant mutant of strain 603 (603S) to test if WCV-OVA[1] elicits protection in normal mice similar to that previously observed for unmodified WCV.

Specificity of recall responses. To test whether the recall immune responses require antigen-specific stimulation of CD4$^+$ cells, two groups of DO11.10 RAG$^{-/-}$ mice were immunized with either WCV-OVA[1] or CT alone and challenged with strain 603S.

Specificity of effector action. To test whether the effector function, once stimulated by cognate antigen, is limited to bacteria bearing the antigen, two groups of DO11.10 RAG$^{-/-}$ mice were immunized with either CT alone or WCV-OVA[1]. All mice were challenged intranasally with strain 603S administered with 100 μg of albumin from chicken egg white (ovalbumin; Sigma-Aldrich, St. Louis, MO). The treatment with ovalbumin alone was repeated 24, 48, and 120 h after challenge.

The same effect was also tested by immunizing two groups of DO11.10 RAG$^{-/-}$ mice with Rx1Δ*lytA*OVA[3]-derived WCV-OVA[3] and challenging them intranasally with strain 603S 4 weeks after the second vaccine dose. For four consecutive days (beginning a day after challenge), animals in the first group received a dose of Rx1Δ*lytA* dead cells (whole-cell antigen [WCA]) and those in

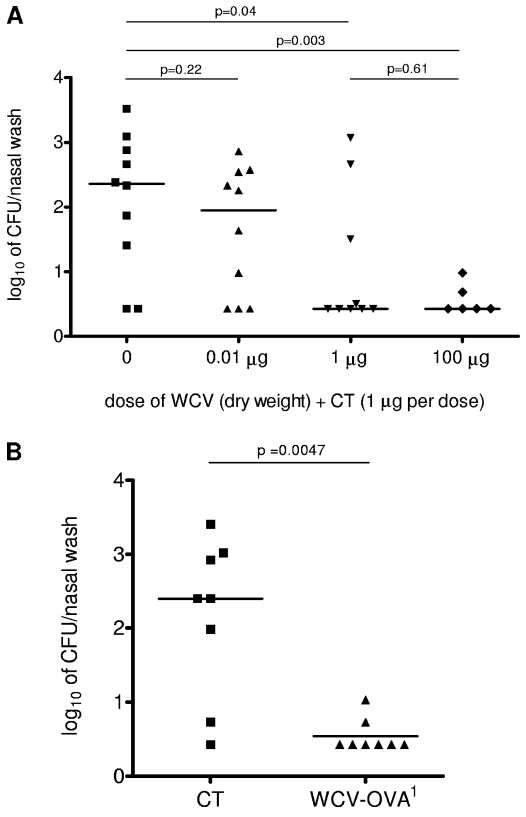

FIG. 1. Intranasal colonization of immunocompetent mice with *S. pneumoniae* strain 603 (A) and its streptomycin-resistant mutant 603S (B) after immunization with WCVs. (A) Comparison of the colonization density of C57BL/6J mice immunized with various doses of WCV made of Rx1Δ*lytA* strain (0.01, 1, or 100 μg) or CT alone (0). (B) Protection of BALB/c mice after immunization with WCV-OVA^1 vaccine made of Rx1Δ*lytA*OVA^1 strain in comparison with mice immunized with CT alone. Solid lines indicate group medians. *P* values refer to results of the Mann-Whitney test for differences in the distribution of CFU per nasal wash between groups.

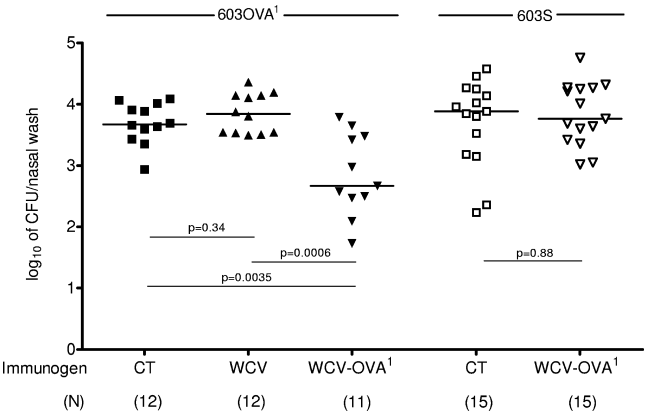

FIG. 2. Comparison of the density of intranasal colonization by *S. pneumoniae* strains 603OVA^1 and 603S in DO11.10 RAG$^{-/-}$ mice after immunization with CT alone (CT), WCV made of the Rx1Δ*lytA* strain (WCV), or WCV made of Rx1Δ*lytA*OVA^1 (WCV-OVA^1) as indicated below the *x* axis. The number of animals in each group is depicted at the bottom. Solid lines indicate group medians. *P* values refer to results of the Mann-Whitney test for differences in the distribution of CFU per nasal wash between groups.

the second group a dose of Rx1Δ*lytA*OVA^3 dead cells (WCA-OVA^3); in both cases the equivalent of 10^8 CFU per dose was used.

Statistical analysis. The significance of differences between animal groups in levels of pneumococcal colonization was evaluated by using the two-sided Mann-Whitney test. All calculations were made by using Prism (GraphPad Software, Inc., San Diego, CA). The lower limit of detection was established at 1 CFU per 30 μl of nasal wash sample. For all comparisons, a *P* value of <0.05 was considered significant.

RESULTS

Protection by low doses of WCV and by WCV-OVA^1 in normal mice. Previously we showed that immunization of immunocompetent mice with intranasally applied vaccine made of ethanol-killed whole cells of Rx1Δ*lytA*, an autolysin (LytA)-negative mutant of Rx1 (100 μg of the vaccine, dry weight, equivalent to 10^8 CFU per dose), and CT mucosal adjuvant protected animals against subsequent colonization by heterologous pneumococcal strains (17, 19). In the present study, we observed similar protection after immunization with a dose of WCV 100× lower than that previously used (Fig. 1A), indicating that a vaccine containing a small amount of antigen is sufficient to stimulate efficient mucosal immunity. This raised the question of the antigen specificity of the protection observed. Before proceeding to assess this question in DO11.10 Rag$^{-/-}$ mice, we verified that *S. pneumoniae* strains expressing OVA$^{323–339}$ were still protective in normal mice. WCV was made from strain Rx1Δ*lytA*OVA^1 (WCV-OVA^1), a variant of Rx1Δ*lytA* expressing the OVA$^{323–339}$ peptide as a fusion protein with pneumococcal surface protein A (PspA-OVA). WCV-OVA^1 proved to be protective in immunocompetent BALB/c mice (Fig. 1B).

Induction of CD4$^+$ T-cell-mediated immunity is antigen specific. Immunization with WCV-OVA^1 protected DO11.10 RAG$^{-/-}$ mice against subsequent colonization with the unrelated *S. pneumoniae* strain 603OVA^1 expressing PspA-OVA (Fig. 2). The level of colonization in the group of 11 animals immunized with WCV-OVA^1 (median $\log_{10}$ of CFU recovered per nasal wash, 2.67; interquartile range, 2.47 to 3.48) was significantly lower than the levels in groups of 12 animals either vaccinated with WCV made of the OVA-negative variant of Rx1Δ*lytA* (3.84; 3.53 to 4.14 [$P = 0.0006$]) or immunized with CT alone (3.67; 3.51 to 3.96 [$P = 0.0035$]). These results show that the induction of CD4$^+$ T-cell-mediated immunity to pneumococcal colonization was antigen specific.

Recall responses to WCV-OVA^1 are antigen specific. Immunization of DO11.10 RAG$^{-/-}$ mice with WCV-OVA^1 had no detectable effect on subsequent colonization with the non-OVA-expressing strain 603S (Fig. 2). The number of 603S bacteria recovered from the URT of animals immunized with WCV-OVA^1 was nearly identical to the number of cells recovered from mice immunized with CT alone (3.76; 3.42 to 4.26 versus 3.88; 3.18 to 4.25 [$P = 0.88$]). This result confirmed that recall of the response observed in the previous experiment also requires antigen-specific CD4$^+$ T-cell stimulation. Altogether, the expression of vaccine-induced T-cell immunity in these mice which can only express T-cell immunity to OVA$^{323–339}$ peptide depends upon the presence of OVA in both the vaccine and the colonizing bacteria.

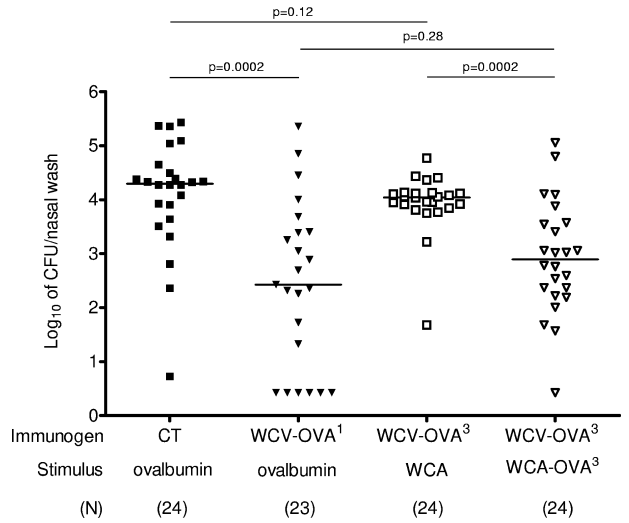

FIG. 3. Comparison of the density of intranasal colonization with *S. pneumoniae* strain 603S in DO11.10 RAG$^{-/-}$ mice immunized either with CT alone (CT), WCV made of Rx1Δ*lytA*OVA^1 (WCV-OVA^1), or WCV made of Rx1Δ*lytA*OVA^3 (WCV-OVA^3) as indicated below the *x* axis and stimulated during colonization intranasally with either ovalbumin, killed cells of Rx1Δ*lytA* (WCA), or killed cells of Rx1Δ*lytA*OVA^3 (WCA-OVA^3) for 4 days after challenge with 603S. The number of animals in each group is depicted at the bottom. Solid lines indicate group medians. *P* values refer to results of the Mann-Whitney test for differences in the distribution of CFU per nasal wash between groups.

Effector action is not limited to antigen-bearing bacteria. Next, we evaluated whether the effector action was limited to bacteria expressing the OVA antigen or whether protection can be seen in *trans* by exposure to the challenge strain without OVA$^{323–339}$ but accompanied by soluble OVA antigen. Among mice challenged with the OVA-negative strain 603S and stimulated at the time of challenge with soluble ovalbumin, prior immunization with WCV-OVA^1 significantly reduced colonization (2.43; 0.43 to 3.40) compared to the colonization in a group that had been immunized with CT alone (4.30; 3.77 to 4.57 [$P = 0.0002$]) (Fig. 3). This observation strongly suggested that the clearance activity may extend beyond bacteria presenting the antigen. However, we could not exclude the possibility that ovalbumin applied at the time of challenge attached noncovalently to the surface of pneumococcal cells and the cell-ovalbumin complexes were recognized and processed by antigen-presenting cells in a manner similar to that for *S. pneumoniae* expressing the PspA-OVA fusion protein. To address the problem, in the following experiment, instead of ovalbumin, we applied as stimulus at the time of challenge killed cells of strain Rx1Δ*lytA*OVA^3, a variant of Rx1 *S. pneumoniae* expressing OVA$^{323–339}$ peptide on the two surface proteins PspA and neuraminidase A (NanA) and on the cytolytic toxin pneumolysin (Ply). These two additional OVA fusions (with NanA and Ply) were introduced to increase the opportunity for peptide presentation at the time of immunization and to induce stronger responses at the time of challenge. After immunization with WCV made of Rx1Δ*lytA*OVA^3 cells (WCV-OVA^3), the colonization of DO11.10 RAG$^{-/-}$ mice by strain 603S was significantly lower ($P = 0.0002$) in the group stimulated at the time of challenge with dead cells of strain Rx1Δ*lytA*OVA^3 (2.90; 2.29 to 3.56) than in the group stimulated with killed cells of the OVA-negative variant of the same strain (4.04; 3.88 to 4.12), a result similar to that observed with soluble ovalbumin as a stimulating factor (Fig. 3).

DISCUSSION

The existing pediatric vaccine against *S. pneumoniae*—the 7-valent pneumococcal conjugate vaccine—has been remarkably successful in preventing disease in vaccinated children and also has reduced colonization in these children and thereby protected unvaccinated individuals against pneumococcal disease (5). Indeed, it is estimated that these indirect (herd immunity) effects have prevented more than twice as many cases as have been directly prevented in vaccinated persons (5). As second- and later-generation vaccines are developed, with the goals of increasing the number of pneumococcal serotypes covered and reducing vaccine cost, a better understanding of mechanisms of immunity to colonization could lead to the development of novel strategies against this pathogen and, potentially, against other nasopharyngeal pathogens. Previous work by our group and others (19, 31, 33) demonstrated the critical role of CD4$^+$ T cells in resistance to pneumococcal carriage. Our initial observation (Fig. 1) that very low doses of a killed WCV could confer immune protection, combined with observations by others of nonspecific roles for CD4$^+$ T cells recruited by pneumococci (10), raised the possibility that protection by this strategy may be nonspecific. Using DO11.10/Rag$^{-/-}$ mice, we found that both the induction and recall of the protective response require specific antigen seen by the CD4$^+$ T cells but that clearance of pneumococci, once set in motion by specific antigen, could work in *trans* against pneumococci not expressing the antigen. Elsewhere, we have shown that the Th17 subset of CD4$^+$ T-helper cells is critical to this protective activity and that neutrophil-like cells are also required (Y. J. Lu, J. Gross, D. Bogaert, A. Finn, L. Bagrade, Z. Qibo, J. Kolls, A. Srivastava, A. Lundgren, S. Forte, C. M. Thompson, K. F. Harney, P. W. Anderson, M. Lipsitch, and R. Malley, submitted for publication), consistent with our previous findings on the importance of Th17 cells in response to the conserved cell wall polysaccharide antigen of pneumococcus (18) and to pneumococcal proteins (2). Thus, it is tempting to speculate that the in *trans* effect observed here reflects the recruitment and activation of neutrophils by Th17 cells following antigen-specific stimulation and that these neutrophils kill pneumococci regardless of whether they bear the relevant antigen.

Our experiments show that *S. pneumoniae* is highly susceptible to this nonspecific effector response. It remains to be seen whether these same mechanisms of immunity could also impact colonization by other respiratory pathogens. In this regard, Lysenko et al. (15) described a key role of complement-dependent phagocytic killing of pneumococci by neutrophils in a mouse carriage model in which neutrophils were recruited and activated, not by *S. pneumoniae*, but through innate recognition of another respiratory pathogen that occupies the same niche, *Haemophilus influenzae.* Interestingly, their findings suggest that while *H. influenzae* is required for the recruitment of these neutrophils, they are much more efficient at clearing *S. pneumoniae* than at clearing *H. influenzae*. It is thus

possible in principle that the type of immunity elicited by immunization with WCV expressing OVA is particularly effective at clearing pneumococci from the respiratory tree but may not be as potent against other organisms. If there are other organisms in the respiratory tree that are similarly susceptible to the action of these clearance mechanisms, then the action observed in *trans* against non-antigen-bearing pneumococci might also serve as a mechanism of interspecific bacterial antagonism, mediated by antigen-specific responses.

The approach described here, in which a whole organism is used in transgenic mice to stimulate an immune response to a defined antigen, may provide a useful basis for future work to understand the mechanisms by which whole organisms can be more immunogenic than individual, soluble moieties (6). By separating the antigenic function from possible adjuvant or other effects of the whole organism on the immune system, it should be possible to elucidate the identity and effect of other, immunogenicity-affecting components of the whole organism.

There is growing evidence that various naturally acquired mechanisms of immunity to pneumococcal carriage and infection are antibody independent. Rosseau et al. (27), in a comparative study on transcriptional profiling of the mouse lung during pneumococcal pneumonia, revealed a simultaneous down-regulation of B-cell-mediated responses and increase in the expression of T-cell-specific genes during inflammation caused by *S. pneumoniae*. The inflammatory responses were dominated by interleukin-1 (IL-1) family cytokines, IL-6, and tumor necrosis factor alpha. Interestingly, there was also significant up-regulation of IL-17 expression (27). In a study of elderly adults with chronic obstructive pulmonary disease, we have shown that systemic antipneumococcal antibodies did not predict resistance to the acquisition of a new pneumococcal strain, suggesting that other mechanisms, possibly including T cells, may be responsible (16). In a recent study, significantly lower proliferative and cytokine peripheral blood T-cell responses to pneumolysin were observed in children who were colonized with *S. pneumoniae* than in noncolonized children, raising the intriguing hypothesis that T-cell responses to this antigen may be associated with increased resistance to pneumococcal colonization (35).

In conclusion, we have shown that CD4$^+$ T-cell-mediated WCV-induced protection against pneumococcal colonization is antigen specific and can be triggered even in the absence of antigen-bearing pneumococci, provided that the target antigen is present at the time of challenge. While many aspects of the immune response to this antigen remain to be analyzed, these data provide additional support for a strategy based on mucosal immunization with a killed pneumococcal antigen.

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