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RESEARCH ARTICLE

Reduction of *Streptococcus pneumoniae* Colonization and Dissemination by a Nonopsonic Capsular Polysaccharide Antibody

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ABSTRACT ***Streptococcus pneumoniae*** **colonization of the nasopharynx (NP) is a prerequisite for invasive pneumococcal disease (IPD). The marked reduction in IPD that followed the routine use of pneumococcal polysaccharide conjugate vaccines (PCVs) has been linked to reduced NP colonization with vaccine-included serotypes (STs), with the caveat that PCVs are less effective against pneumonia than against IPD. Although PCV-elicited opsonic antibodies that enhance phagocytic killing of the homologous ST are considered a key correlate of PCV-mediated protection, recent studies question this relationship for some STs, including ST3. Studies with monoclonal antibodies (MAbs) to the pneumococcal capsular polysaccharide (PPS) of ST3 (PPS3) have shown that nonopsonic, as well as opsonic, antibodies can each protect mice against pneumonia and sepsis, but the effect of these types of MAbs on NP colonization is unknown. In this study, we determined the effects of protective opsonic and nonopsonic PPS3 MAbs on ST3 NP colonization in mice. Our results show that a nonopsonic MAb reduced early NP colonization and prevented ST3 dissemination to the lungs and blood, but an opsonic MAb did not. Moreover, the opsonic MAb induced a proinflammatory NP cytokine response, but the nonopsonic MAb had an antiinflammatory effect. The effect of the nonopsonic MAb on colonization did not require its Fc region, but its antiinflammatory effect did. Our findings challenge the paradigm that opsonic MAbs are required to prevent NP colonization and suggest that further studies of the activity of nonopsonic antibodies could advance our understanding of mechanisms of PCV efficacy and provide novel correlates of protection.**

IMPORTANCE **Pneumococcal conjugate vaccines (PCVs) have markedly reduced the incidence of invasive pneumococcal disease (IPD). Vaccine-elicited pneumococcal polysaccharide (PPS) antibodies that enhance *in vitro* phagocyte killing of vaccine-included serotypes (STs) (opsonic antibodies) have been considered correlates of vaccine protection and are thought to exert their effect at the initial site of infection, the nasopharynx (NP). However, the data presented here show that this is not the necessarily the case. A nonopsonic PPS monoclonal antibody (MAb) reduced pneumococcal colonization and dissemination of its homologous ST in mice, but surprisingly, an opsonic PPS MAb to the same ST did not. These results reveal that PPS antibodies can work in different ways than previously thought, challenge the paradigm that opsonic antibodies are required to prevent IPD, and provide new insights into PCV efficacy that could lead to novel correlates of vaccine protection.**

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Colonization of the of the nasopharynx (NP) with *Streptococcus pneumoniae* (pneumococcus) is a prerequisite for the development of invasive pneumococcal disease (IPD) (1). Since the implementation of pneumococcal capsular polysaccharide (PPS) conjugate vaccine (PCV) use in infants and young children, there has been a remarkable reduction in IPD in children and adults as a result of herd immunity (2–5). However, PPS-based vaccines, including PCVs and the 23-valent unconjugated vaccine (PV23), are less effective against pneumonia than against IPD (6, 7). Nonetheless, current thought holds that PCVs protect against IPD by preventing NP colonization with PCV-included serotypes (STs) (8–10). Although definitive surrogates of PCV response have not been established, postvaccination levels of antibodies that enhance opsonophagocytic killing of homologous STs by immune cells (opsonic antibodies) have been linked to PCV efficacy and were used in their development and licensing (9, 11–14). However, recent postlicensure analyses have questioned the relationship between opsonic antibody titers and PCV13 protection against certain STs, including ST3 (15), and call for renewed investigation of the mechanisms by which PCVs work.

To date, studies of PCV response have relied on an opsonophagocytic killing assay (OPKA) to identify opsonic antibodies (13). This assay is performed with sera that are heterogeneous mixtures of many antibodies with different specificities, isotypes, and functional activities. Therefore, while the OPKA provides a measurement of overall opsonic activity, it cannot separate opsonic and nonopsonic antibodies. In contrast, studies with monoclonal antibodies (MAbs) can characterize the activities of indi-

vidual antibodies. In studies with MAbs, our group discovered that PPS-based vaccines also elicit nonopsonic antibodies that are protective, including those to the PPS of ST3 (PPS3) and PPS8 that protect mice against pneumonia and sepsis (16–19). A better understanding of the activity of nonopsonic PPS antibodies may shed new light on how PCVs protect against IPD and why they might be less effective against pneumonia.

Our group has been interested in correlates of antibody immunity to ST3, a pneumococcal ST that was historically and is still associated with higher mortality than other STs (15, 20–22). We produced a human MAb to PPS3 that was highly protective in mice, but nonopsonic *in vitro* (17). We further explored the effects of nonopsonic MAbs on ST3 pathogenesis in studies with a pair of mouse IgG1 PPS3 MAbs, 1E2 and 7A9 (19). 7A9 is an opsonic MAb that promotes *in vitro* killing of ST3 by phagocytes in the standard OPKA used in the field, but 1E2 is nonopsonic and does not (19). Additionally, 7A9 requires neutrophils and FcγRIIb to protect mice against ST3 pneumonia, whereas 1E2 requires macrophages and FcγRIII (19, 23, 24). Furthermore, 1E2 can agglutinate ST3 bacteria and enhances competence, fratricide, and bacteriocin gene expression in the presence of competence-stimulating peptide 2 *in vitro* (25).

Conjugate vaccine-elicited antibodies are proposed to work against respiratory pathogens by eliminating the inoculum (26, 27), and PCV efficacy against IPD has been linked to prevention of colonization and opsonic antibody titers (8, 14). Given that the aforementioned MAb, 1E2, protected against pneumonia but was nonopsonic, we were interested in its effect on NP colonization. We determined the effects of 1E2 and an opsonic MAb, 7A9, on ST3 NP colonization in mice. On the basis of current thought, we predicted that the opsonic MAb, 7A9, would prevent colonization, but the nonopsonic MAb, 1E2, might not. Surprisingly, we found the opposite; 1E2 reduced early ST3 colonization and prevented dissemination to the lungs and blood, but 7A9 did not.

RESULTS

Effects of PPS3 MAbs on ST3 colonization. Initially, pilot experiments were performed to determine if intraperitoneally (i.p.) administered MAbs could be detected in the NP. On days 1 to 3 after i.p. MAb administration, NP lavage fluid from mice treated with 1E2 or 7A9 bound PPS3 to a similar degree at each time tested, while lavage fluid from mice that received 31B12, a control PPS8 MAb (control MAb), bound PPS8 (see Fig. S1 in the supplemental material). Thus, i.p. administered PPS3-specific MAbs reached the NP and were present up to 3 days after administration.

Next, we determined the effect of PPS3 and control MAbs on ST3 NP colonization on days 1 and 3 postinfection. Compared to the control MAb, mock treatment with phosphate-buffered saline (PBS) had no effect on the NP CFU count 1 day postinfection (see Fig. S2A in the supplemental material). Thus, only the control MAb was used in subsequent experiments. On day 1, 1E2-treated mice had significantly fewer NP CFU than 7A9- or control MAb-treated mice (Fig. 1A). On day 3, there were no differences in CFU counts between any of the MAb treatment groups (Fig. 1A). Since 1E2 can agglutinate bacteria *in vitro* (19, 25), we sought to confirm our CFU count results by quantifying the constitutively expressed DNA gyrase gene (*gyrA*) in NP lavage fluid from MAb-treated mice 1 day postinfection. This analysis showed that 1E2-treated mice had fewer genome copies than 7A9- or control MAb-treated mice (Fig. 1B). Thus, 1E2 reduced ST3 NP colonization 1 day postinfection, but 7A9 did not.

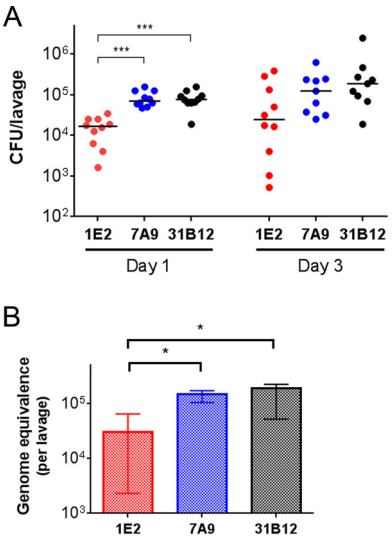

FIG 1 1E2 reduces ST3 bacterial colonization. (A) NP CFU counts per milliliter of lavage fluid at 1 and 3 days postinfection are shown for mice treated i.p. with the MAbs indicated 2 h before i.n. infection with 10^5 CFU of ST3 bacteria. Data represent median values from three independent experiments, with data for individual mice shown as circles. There were nine or more mice per group. (B) ST3 bacterial genome equivalence 1 day postinfection was determined by qPCR and is shown for the MAbs indicated. Data represent median values ± interquartile ranges from three independent experiments. There were six mice per group. The overall P value is <0.05 by one-way analysis of variance for day 1 CFU counts and qPCR. ***, $P < 0.001$; *, $P < 0.05$ by Dunn's multiple comparison posttest.

Effects of PPS MAbs on ST3 dissemination. To gain insight into antibody action in the NP, we examined ST3 localization in NP tissue sections obtained 1 day postinfection from MAb-treated mice (Fig. 2A). There were no differences in the amount of lumen- or epithelial surface-associated ST3 bacteria in any of the MAb treatment groups (Fig. 2B). However, 1E2-treated mice had fewer ST3 bacteria in deeper tissue than did 7A9-treated mice or control MAb-treated mice (Fig. 2B). Next, we determined CFU counts in the lungs and blood of MAb-treated, ST3-colonized mice. At 1 day postinfection, there were no detectable CFU in the lungs in any treatment group (Fig. 3A). At 2 days postinfection, 40% of 7A9- and control MAb-treated mice had 10^1 to 10^4 CFU/g of lung tissue, but 1E2-treated mice had no detectable CFU. At 3 days postinfection, 60% of 7A9-treated mice and 79% of control MAb-treated mice had 10^2 to 10^5 CFU/g, but 1E2-treated mice still had no detectable CFU (Fig. 3A). There was a similar trend in the blood. On day 4 postinfection, 67% of 7A9-treated mice and 83% of control MAb-treated mice had 10^2 to 10^4 CFU/ml of blood, but none were detectable in 1E2-treated mice. On day 6, all control MAb-treated mice had 10^2 to 10^4 CFU/ml, and 33% of 7A9-treated mice, had 10^2 to 10^3 CFU/ml, but 1E2-treated mice still had no detectable CFU (Fig. 3B). Thus, 1E2 prevented ST3 dissemination to the lungs and blood in ST3-colonized mice, but 7A9 did not.

Effect of PPS MAbs on NP inflammation. Inflammation during NP colonization can promote pneumococcal dissemination. We determined levels of proinflammatory (interleukin-1β [IL-1β], IL-6, and CXCL1 [KC]) and antiinflammatory (IL-10) cyto-

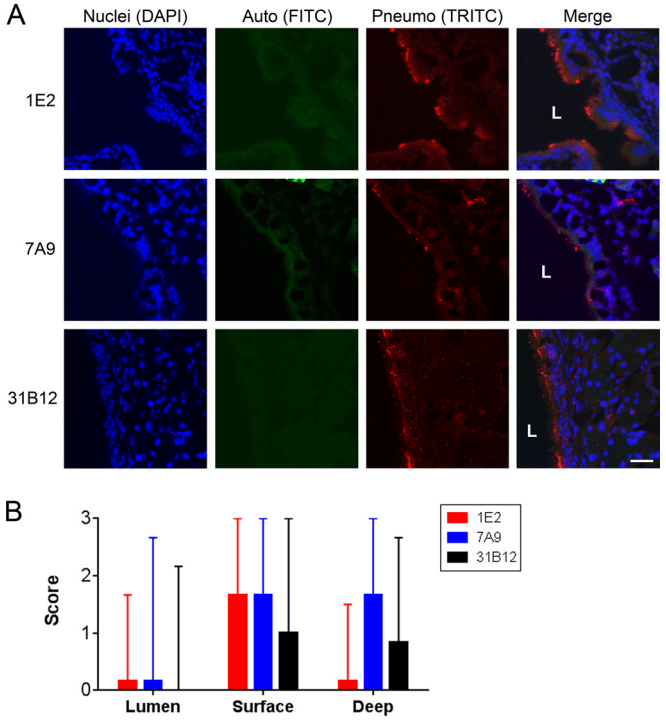

FIG 2 ST3 bacterial localization in the NPs of MAb-immunized mice. (A) Immunofluorescent images of NP tissue sections stained with an anti-*S. pneumoniae* polyclonal antibody (red) and 4′,6-diamidino-2-phenylindole (blue) collected 1 day postinfection from mice treated i.p. with 1E2, 7A9, or 31B12 2 h before i.n. infection with 10^5 CFU of ST3 bacteria. Tissue autofluorescence was also detected and is shown for orientation (green). L, NP lumen. Shown are representative images from experiments with two mice per MAb. Images were taken at ×400 magnification; scale bar, 30 μm. (B) Images randomly selected from sections in panel A were scored on a scale of 0 to 3 based on the amount of bacteria present in the NP lumen, at the epithelial surface, or in deeper NP tissue. Bars represent median scores with the range shown as error bars. Scoring was done in a blind fashion by two independent scorers. There were 15 images per MAb.

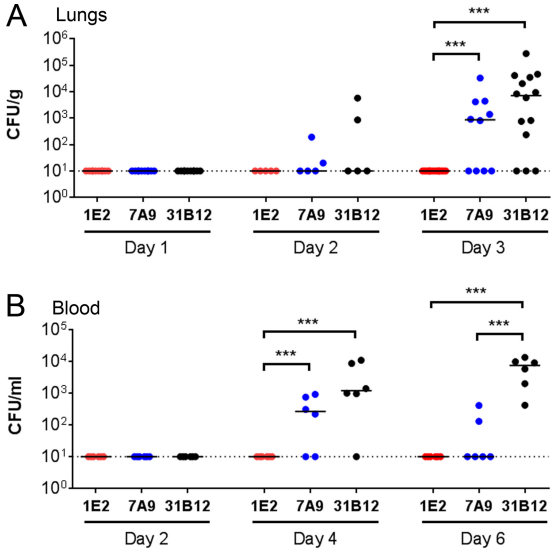

FIG 3 1E2 prevents ST3 bacterial dissemination. Mice were treated i.p. with 1E2, 7A9, or 31B12 2 h before i.n. infection with 10^5 CFU of ST3 bacteria. (A) Lung CFU counts per gram 1, 2, or 3 days postinfection are shown for the MAbs indicated. Bars represent median values from one (day 2) or three (days 1 and 3) independent experiments, with data for individual mice shown as circles. There were nine or more mice per group (days 1 and 3) or five mice per group (day 2). (B) CFU counts per milliliter of blood 2, 4, or 6 days postinfection are shown for the MAbs indicated. Bars represent median values from two independent experiments, with data for individual mice shown as circles. There were six mice per group. Limits of detection, 10 CFU/g (A) and 10 CFU/ml (B). The overall *P* value is <0.05 by one-way analysis of variance for days where comparisons are shown. ***, $P < 0.001$ by Dunn's multiple comparison posttest.

kines in NP lavage fluid from MAb-treated mice 1 day postinfection. Compared to the control MAb, mock treatment with PBS had no effect on IL-6 or IL-1β levels and was not used in subsequent experiments (see Fig. S2B in the supplemental material). Levels of IL-1β, IL-6, and KC in 1E2-treated mice were significantly lower than those in 7A9- or control MAb-treated mice and similar to those in uninfected mice (Fig. 4). In contrast, levels of IL-10 were significantly higher in 1E2-treated mice than in 7A9-treated mice or control MAb-treated mice (Fig. 4). Thus, 1E2-treated, ST3-colonized mice exhibited a minimal proinflammatory cytokine response that was significantly weaker than the response seen in 7A9- or control MAb-treated mice.

Effect of PPS MAbs on ST3 agglutination. PPS antibodies that agglutinate the homologous pneumococcal ST in the NP have been shown to reduce pneumococcal colonization in mice (28). Given that 1E2 can agglutinate ST3 *in vitro* (25), we performed several experiments to determine if ST3 agglutination contributed to the ability of 1E2 to reduce NP colonization. NP tissue sections from MAb-treated mice collected 2 h (data not shown) or 1 day (Fig. 2, right) postinfection did not reveal ST3 agglutination in 1E2-treated mice. Since 1E2 only agglutinates ST3 at certain concentrations *in vitro*, we determined the concentrations of the MAbs in NP lavage fluid from uninfected mice 1 day after i.p. MAb treatment (Table 1) and tested whether the measured concentration of 1E2 agglutinated ST3 *in vitro*. Some small aggregates were observed with 0.1 and 1 μg/ml 1E2, but obvious agglutination was only observed with 10 and 100 μg/ml (Fig. 5). 7A9 and the control MAb did not induce agglutination at any concentration (Fig. 5). We also found that NP lavage fluid from uninfected mice 1 day after MAb treatment did not agglutinate ST3 *ex vivo* (data not shown). Thus, 1E2 induced minimal aggregates at lower concentrations and marked agglutination of ST3 at a concentration ~250 times higher than what we detected in lavage fluid.

Effects of i.n. administered MAbs and F(ab′)$_2$ fragments on ST3 colonization, NP inflammation, and dissemination. Since antibody Fc regions are responsible for effector function, but F(ab′)$_2$ fragments can agglutinate *S. pneumoniae* (28, 29), we produced F(ab′)$_2$ fragments of each MAb and examined their effects on colonization, inflammation, and dissemination. Mice were treated with 10 μg of either whole MAbs or the corresponding F(ab′)$_2$ fragments intranasally (i.n.) prior to infection to ensure NP delivery of the latter. Concentrations of MAbs in NP lavage fluid after i.n. administration were ~50-fold higher than those measured after i.p. treatment (Table 1). In this i.n. model, at 1 day postinfection, mice given whole 1E2 or 7A9 had significantly fewer NP CFU than control MAb-treated mice, with 1E2-treated mice having the fewest CFU overall (Fig. 6A). Mice treated with the 1E2 F(ab′)$_2$ fragment had significantly fewer CFU than mice treated with F(ab′)$_2$ fragments of 7A9 and the control MAb (Fig. 6A). Comparing the whole MAbs to their corresponding F(ab′)$_2$ frag-

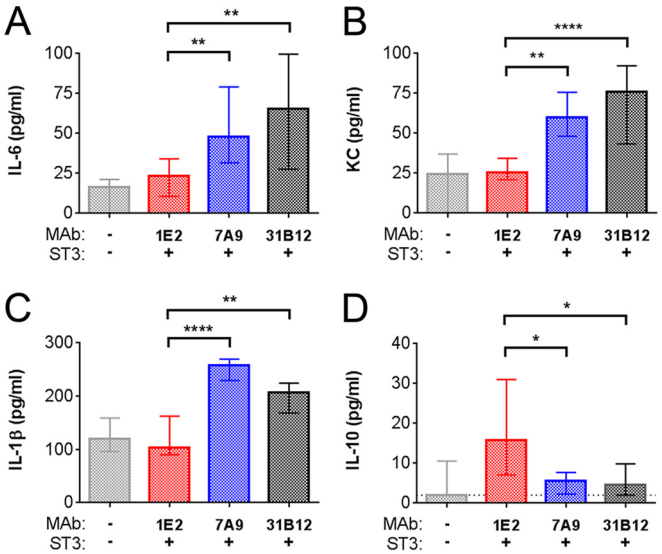

FIG 4 NP cytokines in ST3 bacterium-colonized mice 1 day postinfection. Mice were treated i.p. with 1E2, 7A9, or 31B12 2 h before i.n. infection with 10^5 CFU of ST3 bacteria. IL-6 (A), KC (B), IL-1β (C), and IL-10 (D) levels in NP lavage fluid 1 day postinfection are shown for the MAbs indicated. Data from untreated, uninfected mice are shown in gray. Bars represent median values ± interquartile ranges from two independent experiments. There were eight mice per condition. The overall P value is <0.05 by one-way analysis of variance. ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$ by Dunn's multiple comparison test.

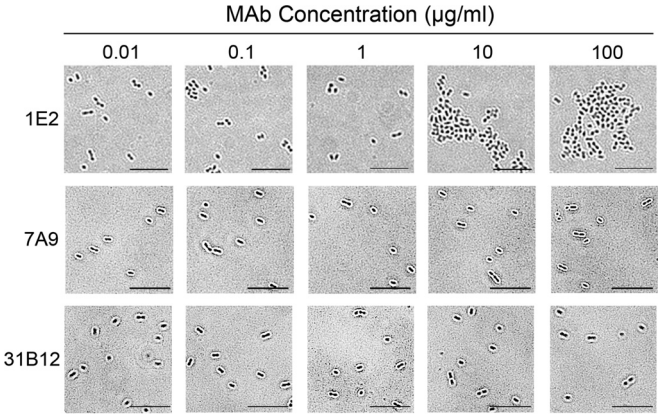

FIG 5 *In vitro* agglutination of ST3 bacteria. A total of 10^5 CFU of ST3 bacteria were incubated with the indicated amounts of purified MAbs in PBS for 1 h. Images were captured at ×630 magnification and are representative of three independent experiments performed in duplicate for each MAb concentration. Scale bars, 10 μm.

ments, CFU were comparable for 1E2 and its F(ab′)$_2$ fragment, but for 7A9, CFU counts were significantly lower for the whole MAb (Fig. 6A). Quantitative PCR (qPCR) analysis of NP lavage fluid confirmed the CFU results (see Fig. S3 in the supplemental material). Thus, in the i.n. model, treatments with whole 1E2 and its F(ab′)$_2$ fragment each reduced colonization, but only whole 7A9 was able to reduce colonization.

To examine the effects of MAb Fc regions on the NP cytokine response, we determined levels of IL-6, IL-1β, KC, and IL-10 in NP lavage fluid from mice treated with whole MAbs or F(ab′)$_2$ fragments at 1 day postinfection. Similar to i.p. MAb treatment, i.n. treatment with whole 1E2 resulted in lower levels of IL-6, IL-1β, and KC than 7A9 or the control MAb (Fig. 6B). In contrast, F(ab′)$_2$ fragments had no effect on cytokine levels (Fig. 6B). Comparing the whole MAbs to their corresponding F(ab′)$_2$ fragments, IL-6 and KC levels were significantly higher for the F(ab′)$_2$ fragment of 1E2 than for the whole MAb (Fig. 6B). There were no differences for 7A9. We did not detect IL-10 in mice treated with any of the MAbs or F(ab′)$_2$ fragments in the i.n. model (data not shown).

Finally, we examined the effects of i.n. delivered MAbs and F(ab′)$_2$ fragments on ST3 dissemination to the lungs and blood at 3 days postinfection. Similar to the i.p. model, whole 1E2 prevented dissemination to the lungs, but 7A9 did not (Fig. 6C). Although there were no significant differences in blood CFU counts at 3 days postinfection for mice treated with whole MAbs, no 1E2-treated mice exhibited any detectable blood CFU (see Fig. S4 in the supplemental material). Similar to whole MAbs, F(ab′)$_2$ fragments of 1E2, but not 7A9, prevented dissemination to the lungs 3 days postinfection (Fig. 6C). Thus, the Fc region was required for the ability of 1E2 to reduce inflammation but not for its ability to reduce colonization or dissemination, whereas the ability of 7A9 to reduce colonization was Fc-dependent.

TABLE 1 PPS3 concentrations in NP lavage fluid

	Mean concn (μg/ml) ± SEM	
Antibody	Immunized i.p.	Immunized i.n.
1E2	0.0039 ± 0.0028	0.21 ± 0.12
7A9	0.0071 ± 0.0040	0.43 ± 0.15
31B12	ND[a]	0.20 ± 0.08

[a] ND, not determined.

DISCUSSION

Our data show that the opsonic and nonopsonic PPS3 MAbs, 7A9 and 1E2, respectively, mediated entirely different effects on ST3 bacterial NP colonization, dissemination, and the NP cytokine response. In both i.p. and i.n. MAb administration models, whole 1E2 reduced early NP colonization with ST3 bacteria, prevented bacterial dissemination to the lungs and blood, and induced an antiinflammatory NP cytokine profile. In contrast, whole 7A9 reduced colonization when administered i.n., but not when administered i.p. Furthermore, despite reducing NP CFU counts when administered whole, 7A9 did not prevent dissemination to the lungs and blood and induced a proinflammatory cytokine profile.

The difference in the efficacy of 7A9 in the i.n. and i.p. models was likely a function of the ~60-fold larger amount of MAb present in the NP in the i.n. model. In fact, the amount of whole 7A9 in the NP in the i.n. model was ~100 times the amount of whole 1E2 in the NP in the i.p. model. Although the PPS3 affinity of 7A9 is approximately 2.5-fold lower than that of 1E2 (19), NP lavage fluid from mice that received each MAb exhibited equal binding to PPS3, and there was almost twice as much 7A9 as 1E2 in the NP in both models. Nonetheless, 1E2 was still more effective and prevented dissemination. Therefore, while its somewhat higher affinity could contribute to the superior activity of 1E2 in the NP, the functional activities of the MAbs differ in several ways. First, the ability of 7A9 to reduce NP CFU was Fc-dependent, but that of 1E2 was Fc-independent. Thus, the activity of 7A9 depends on the quantity of 7A9-ST3 complexes and the availability of Fc receptor

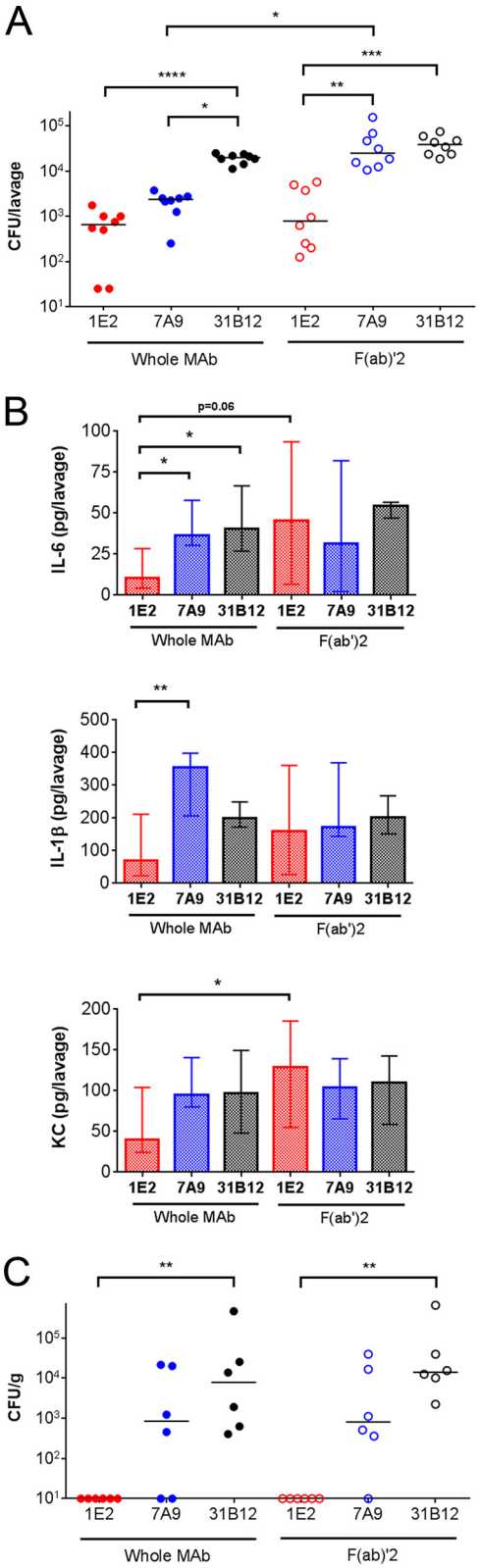

FIG 6 Differential effects of whole 1E2 and 7A9 and F(ab′)$_2$ fragments on colonization, inflammation, and dissemination. (A) NP CFU counts 1 day postinfection are shown for mice treated i.n. with whole MAbs (left side) or the corresponding F(ab′)$_2$ fragments (right side) 2 h before i.n. infection with 10^5 CFU of ST3 bacteria. Data represent median values from two independent experiments, with data for individual mice shown as circles. There were eight

(Continued)

ligands. Although it did not need its Fc to reduce CFU counts, whole 1E2 was more effective in the i.n. model, when there was ~50 times more NP antibody than in the i.p. model.

We developed the i.n. model to ensure the delivery of F(ab′)$_2$ fragments to the NP, which would likely be hindered in the i.p. model, as antibody translocation across epithelial surfaces relies heavily on Fc-mediated transport (30). However, we note that the i.p. model most closely recapitulates how vaccine-elicited serum antibodies might work in the NP. In this regard, given that PPS3 induces a lower level of ST-specific opsonic antibody than other PCV13-included STs (31, 32), our data support the assertion that insufficient opsonic antibody at the site of infection could contribute to the failure of PPS3-included PCVs to prevent ST3 disease (14, 33), and the finding that for some STs, more antibody than previously thought might be needed for protection (15). At the same time, our data also show that a nonopsonic MAb was more effective than an opsonic MAb in reducing NP colonization, raising the possibility that the presence of such antibodies could augment vaccine efficacy. There are currently no assays to identify nonopsonic antibodies in sera, but our group is working to develop ways of detecting such antibodies.

We did not observe marked differences in inflammation in NP tissue sections of MAb-treated mice at 1 day postinfection, but there were markedly fewer ST3 bacteria in the submucosal areas of 1E2-treated mice than in 7A9- or control MAb-treated mice. Consistent with this finding, 1E2-treated mice exhibited no dissemination to the lungs or blood following NP colonization, whereas mice treated with whole and F(ab′)$_2$ fragments of 7A9 and the control MAb each exhibited dissemination. Numerous studies have linked pneumococcal invasiveness to a proinflammatory milieu, such as that induced by influenza virus (34–36). NP IL-6, KC, and IL-1β levels were higher in 7A9- and control MAb-treated mice than in 1E2-treated mice, in which dissemination did not occur. Thus, these mediators could have enhanced ST3 invasiveness in 7A9- and control MAb-treated mice. Notably, 7A9 and 1E2 had opposite effects on IL-1β in both the i.n. and i.p. models, whereby 7A9 increased and 1E2 decreased IL-1β levels in the NP. Pneumococcus stimulates the production of IL-1β via pneumolysin, which activates the NLRP3 inflammasome (37, 38). Interestingly, immune complexes can inhibit inflammasome activation and IL-1β secretion by ligation of activating FcγRs, such as FcγRIII, but not the inhibitory receptor, FcγRIIb (39). Since 1E2 efficacy in the lungs required FcγRIII, but that of 7A9 required FcγRIIb (19, 24), it is logical to posit that 1E2-ST3 immune complexes might have inhibited inflammasome activation. On the other hand, 7A9-ST3 immune complexes might have indirectly

Figure Legend Continued

mice per group. (B) Levels of the indicated cytokines in NP lavage fluid from panel A were determined by ELISA. Bars represent median values ± interquartile ranges from two independent experiments. There were six mice per group. (C) CFU counts per gram of lung tissue 3 days postinfection are shown for mice treated with the MAbs indicated. Data represent median values from two independent experiments, with data for individual mice shown as circles. Limit of detection, 10 CFU/g. There were six mice per group. For panels where intergroup comparisons between whole MAbs or F(ab′)$_2$ fragments are shown, the overall P value determined by one-way analysis of variance is <0.05. ****, $P < 0.0001$; ***, 0.001; **, $P < 0.01$; *, $P < 0.05$ by Dunn's multiple comparison posttest. For comparisons of whole MAbs with the corresponding F(ab′)$_2$ fragments, the P value is <0.05 by the Mann-Whitney test (*).

enhanced inflammasome activation via pneumolysin released during FcR-mediated phagocytosis and intracellular killing of ST3 bacteria. Further work is needed to validate these hypotheses. Nonetheless, our findings suggest that, as in protection against pneumonia (24), optimal prevention of ST3 bacterial dissemination from the NP could require antibody-mediated immunomodulation. In this regard, nonopsonic antibodies that contribute to protection against pneumonia could explain why opsonic antibody titers are unreliable correlates of PCV protection. However, inflammatory cytokine levels alone were not a reliable indicator of dissemination in our model, as 1E2 $F(ab')_2$-treated mice had the same levels as 7A9- and control MAb-treated mice yet exhibited no ST3 dissemination, albeit with fewer NP CFU. On the other hand, NP CFU counts alone were also not a reliable indicator of dissemination, as the CFU counts in mice treated i.n. with whole 7A9 were lower than those in mice given 1E2 i.p., yet bacteria disseminated in the 7A9-treated mice. These data highlight the complex relationships among ST3 colonization, inflammation, and dissemination and suggest that more work is needed to understand the relationship between the immune milieu and ST3 invasiveness.

Although previous work with 1E2 and 7A9 showed that each MAb needed its Fc region to protect mice against lethal ST3 pneumonia (19), another group found that PPS-specific $F(ab')_2$ fragments enhanced opsonophagocytosis (29) and reduced NP colonization via ST-specific agglutination (28). The Fc-dependent activity of 7A9 in the i.n. model is not surprising because it is an opsonic MAb that enhances effector cell phagocytosis and killing of ST3 *in vitro* (19). However, the activity of 1E2 is more complex. As noted above, and consistent with its efficacy in the pneumonia model (24), its immunomodulatory activity is Fc-dependent. However, in contrast to the pneumonia model, in which it had no effect on the early CFU count in the lungs (24), 1E2 induced an early reduction of the CFU count in the NP. The latter activity, which was Fc-independent, could be a function of the ability of 1E2 to agglutinate ST3. This is a plausible hypothesis, because 1E2 agglutinates ST3 bacteria *in vitro* (25 and herein), and another group has shown that whole and $F(ab')_2$ fragments of immune serum-derived IgG that agglutinated bacteria *in vivo* reduced ST-specific colonization (28). Although we did not observe ST3 agglutination in tissue sections of 1E2-treated mice, compared to the aforementioned study, our model used a different ST and inoculum and used MAbs rather than polyclonal antibody preparations. Nonetheless, 1E2 might agglutinate ST3 in tissue when present in higher concentrations and/or in the presence of other factors in the NP, which might, in turn, result in more ST3 clearance. However, 1E2 prevented dissemination despite being present in the NP at low concentrations in both models.

Another hypothesis for how 1E2 might mediate ST3 clearance is that it may exert direct effects that render bacteria more susceptible to innate host responses or to harsh environmental conditions in the NP, where growth conditions are far from optimal (40). Although many classical mechanisms of antibody action require effector cells (41), direct effects of antibody on bacteria and fungi have been identified. In fact, this was described for 1E2, which altered ST3 gene expression and enhanced bacterial fratricide in the presence of competence-stimulating peptide 2 (CSP2) *in vitro* (25). Notably, 1E2 altered the expression of bacteriocin and competence genes even in the absence of CSP2, although it did not affect the expression of genes known to be associated with pneumococcal invasion (25). While it is tempting to hypothesize that 1E2 might enhance pneumococcal fratricide *in vivo*, this has not been addressed experimentally. Nonetheless, antibodies have been shown to alter the biological state of other microbes. Gene expression of another encapsulated pathogen, *Cryptococcus neoformans*, was altered by a capsule-specific MAb (42, 43), bovine immune colostrum induced metabolic changes in *Streptococcus mutans* and *S. sobrinus* (44), and lipopolysaccharide-specific MAbs had direct, wide-ranging effects on *Vibrio cholerae* and *Shigella flexneri* (45, 46). 1E2 $F(ab')_2$ fragments reduced NP colonization and prevented dissemination without reducing inflammatory cytokine levels, giving credence to the hypothesis that it might reduce ST3 viability *in vivo* and limit its invasive potential. While more work needs to be done to validate this hypothesis, a MAb that could reduce colonization and prevent dissemination in an Fc-independent manner would be a very useful therapeutic adjunct for neutropenic and/or other immunocompromised patients.

Although this report is limited to two PPS3 MAbs, our group generated a protective, nonopsonic human MAb to PPS3 (47) and protective, nonopsonic human and mouse MAbs to PPS8 (18). These MAbs were elicited by PPS-based vaccines. Thus, the phenomenon of protective nonopsonic antibodies is not limited to ST3, which is a unique ST with unique capsular synthesis machinery (48). Nonetheless, we do not know the frequency of nonopsonic antibodies in natural or PPS-based vaccine responses to different pneumococcal STs and are devising ways to detect such antibodies in vaccine recipients. Notably, a feature of the nonopsonic MAbs we have identified to date is that they also agglutinate their homologous ST (25). However, another group identified a human IgA MAb that enhanced ST2 opsonophagocytosis and agglutination in its polymeric form, although its monomeric form was not agglutinating (49). Additionally, agglutinating IgG antibodies that enhance opsonophagocytosis of ST4 have been reported, although the antibodies were purified from sera and were not monoclonal (29). Thus, although studies of agglutination and opsonic activity need to be expanded to include more STs, antibodies, and isotypes, we posit that these activities stem from the function of individual antibodies that are both present in polyclonal serum. Available data suggest that 1E2 and 7A9 have different PPS3 specificities (19), and we are now seeking to identify their PPS3 epitopes. If this effort is successful, it will allow us to probe sera for each antibody type. However, we might be able to cast an even wider net by screening sera for agglutination, as this could be a correlate of protection against colonization (28). Thus, incorporation of assays for agglutination and/or nonopsonic antibodies with known PPS specificity into future studies of new PPS-based vaccines could provide additional correlates of protection and new insights into vaccine efficacy, particularly against pneumonia.

A central hypothesis for how conjugate vaccines work against encapsulated pathogens is that they induce capsule-specific antibodies that mediate bacterial clearance in the NP (26, 27). While our findings support this concept in principle, they extend it to include nonopsonic, in addition to opsonic, antibodies with distinct mechanisms of action. The opsonic MAb used here induced Fc-dependent bacterial clearance, whereas the nonopsonic MAb did so with and without its Fc region. Importantly, whole 1E2 dampened the inflammatory cytokine response, as it did in an ST3 pneumonia model (24). Thus, our data raise the intriguing possibility that certain nonopsonic MAbs may actually be better than

opsonic MAbs for controlling colonization, in part because they can reduce inflammation that could have the unintended consequence of enhancing dissemination. On the other hand, opsonic MAbs might be more effective than nonopsonic antibodies in the periphery, where their effects on inflammation could be beneficial in accelerating and enhancing bacterial clearance. This hypothesis remains to be tested directly, but is reinforced by previous work in the pneumonia model, in which 7A9 reduced lung and blood CFU counts more effectively than 1E2 did (24), and the data herein showing that i.p. delivered 7A9 eventually reduced bacteremia. Ultimately, it is likely that both antibody types work in concert to mediate protection, but the potential importance of nonopsonic antibodies illustrated by our data challenges current thinking about how PPS antibodies prevent pneumococcal disease and opens new avenues to advance our understanding of protection against pneumococcal disease.

MATERIALS AND METHODS

Bacteria. *S. pneumoniae* ST3 strain A66.1 (originally a gift from D. E. Briles) was grown at 37°C in 5% CO_2 on Trypticase soy agar with 5% sheep blood (BD Biosciences) or in Todd-Hewitt broth (BD Biosciences) supplemented with 0.5% yeast extract without shaking. For mouse infections, ST3 was grown to early log phase (optical density at 600 nm of 0.1 to 0.2), washed once with PBS, and frozen in growth medium with 15% glycerol at −80°C until use. Frozen aliquots were thawed and washed twice with PBS immediately prior to use.

Mouse MAbs and F(ab′)$_2$ generation. The derivation and biological activities of PPS3 mouse IgG1 MAbs, 1E2 and 7A9, and the PPS8 mouse IgG1 MAb, 31B12, which was used as a specificity and isotype control, were previously described (19, 50). MAb F(ab′)$_2$ fragments were generated with the Mouse IgG1 Fab and F(ab′)$_2$ Micro Preparation kit (Thermo) according to the manufacturer's instructions. Briefly, 250 μg of each MAb was digested for 28 h with immobilized ficin in the presence of 4 mM cysteine. Undigested MAbs and digested Fc fragments were bound by protein A agarose beads, and the F(ab′)$_2$-containing unbound fractions were collected. Proper F(ab′)$_2$ generation was verified by Coomassie blue staining, and F(ab′)$_2$ binding to PPS3 (for 1E2 and 7A9) or PPS8 (for 31B12) was verified via enzyme-linked immunosorbent assay (ELISA) as previously described (data not shown) (51).

Mice, mouse infections, and organ burden assays. NP colonization with ST3 bacteria was performed as previously described (52). Briefly, 6- to 8-week-old female wild-type C57BL/6 mice obtained from Jackson Laboratories (Bar Harbor, ME) were anesthetized with isoflurane and i.n. infected with 1×10^5 to 2×10^5 CFU of ST3 bacteria resuspended in 10 μl of PBS. The actual number of ST3 bacteria administered was determined by plating the inoculum given to each mouse. In pilot experiments, lung CFU counts and NP colonization were determined 4 h postinfection as described below. These experiments confirmed that, in this model, ST3 bacteria were confined to the NP without detectable CFU in the lungs at this time (data not shown).

CFU counts in the NP, lungs, and blood were determined 1, 2, 3, 4, or 6 days postinfection in MAb-treated and untreated mice. In some experiments, mice were passively immunized 2 h before infection by i.p. injection of 10 μg of PPS3 MAbs (1E2 and 7A9) or a PPS8 MAb (31B12) as a control in a total volume of 100 μl of PBS. For experiments with F(ab′)$_2$ fragments, mice were passively immunized 2 h before infection by i.n. administration of 10 μg of each MAb or F(ab′)$_2$ fragment in a total volume of 20 μl of PBS and then infected as described above. In pilot colonization experiments, we saw no differences in NP CFU counts or cytokine levels between 31B12-immunized mice and mock (PBS)-immunized mice and therefore omitted mock-immunized groups from the experiments described here (see Fig. S2 in the supplemental material).

To determine NP CFU counts, mice were humanely killed, the trachea was cannulated and lavaged with 500 μl of PBS, and lavage fluid was collected from the nares. Lavage fluid was vigorously vortexed to disrupt bacterial aggregates and serially diluted in PBS, and dilutions were plated in duplicate. To determine lung CFU counts, lungs were removed, weighed, and homogenized in 2 ml of PBS with a Brinkmann Polytron Homogenizer. Homogenates were vortexed and serially diluted in PBS, and dilutions were plated in duplicate. Blood was collected from mice via cardiac puncture and centrifuged for 10 min at 250 × *g*. Supernatants were then serially diluted in PBS and plated in duplicate. Undiluted lung homogenate and blood were plated to yield limits of detection of 10 CFU/g and 10 CFU/ml, respectively. Bacterial burdens in the NP, lungs, and blood were calculated from colony counts and are expressed in CFU/ml, CFU/g of tissue, and CFU/ml, respectively. All experiments were performed two or three times with groups of three to five mice per experiment, as indicated in the figure legends. All mouse experiments were done in accordance with the guidelines and with the approval of the Animal Institute of the Albert Einstein College of Medicine.

Real-time qPCR. To confirm that NP CFU counts were representative of bacterial burdens, the pneumococcal DNA gyrase gene (*gyrA*) was quantified by qPCR as previously described (28). Briefly, 2 μl of lavage fluid was added directly to 10 μl of SYBR green PCR master mix (Life Technologies) and 8 μl of water containing the following primers at 50 nM: F, CCCTTTGGCAGTCCGACCA; R, ACGTGGGGGTCGTGGTGTCC. Amplification was performed on a StepOne Plus Real Time PCR System (Life Technologies) under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Genome equivalence was determined on the basis of a standard curve of *gyrA* amplification from known quantities of ST3 bacteria. Data analysis was performed with StepOne Software (Life Technologies). Experiments were performed in duplicate with samples from three mice per MAb, and all reactions were run in triplicate.

Immunofluorescence microscopy. For visualization of bacteria in the NP, mice were immunized i.p. and infected as described above. At 2 or 24 h postinfection, the mice were humanely killed and their heads were collected. To prepare tissues for sectioning, we used the protocol described by Nelson et al. (53). Tissues were fixed for 48 h at room temperature (RT) in PBS with 4% paraformaldehyde. Following fixation, tissues were decalcified by sequential 7-day incubations in 0.12 M EDTA, pH 7.2, over a period of 28 days. Decalcified tissues were embedded in Tissue-Tek OCT embedding medium in a Tissue-Tek cryomold (Miles), flash frozen in liquid nitrogen-cooled isopentane, and stored at −80°C. Frozen samples were cut into 6-μm-thick sections and stored at −80°C until staining.

Tissue sections were prepared for immunofluorescence microscopy as described above and fixed for 15 min in a 1:1 acetone-methanol mixture at −20°C. Sections were washed three times in double-distilled H_2O, blocked in PBS with 1% bovine serum albumin (BSA) for 2 h at RT, and incubated overnight at 4°C with a rabbit *S. pneumoniae* polyclonal antibody (AdB Serotec catalog number 0300-0218) diluted 1:200 in PBT buffer (1× PBS, 0.5% BSA, 0.1% Triton X-100). Sections were then washed three times with PBT and incubated for 2 h at RT with a tetramethyl rhodamine isocyanate-conjugated goat anti-rabbit secondary antibody (Life Technologies catalog number t-2769) diluted 1:400 in PBT. Labeled tissue sections were visualized with an AxioImager Z1 microscope (Zeiss), and representative images were obtained and processed with an AxioCam MR camera and AxioVision LE software (Zeiss). For quantitative analysis of pneumococcal localization, images from 15 fields per MAb were examined and scored on a scale of 0 to 3 according to the amount of bacteria in the NP lumen, epithelial cell surface, or deep tissue. Analysis was done in a blind fashion, and scores from two independent scorers were averaged for each image. All experiments were performed in duplicate with two mice per MAb per experiment and two sections per sample.

Quantitation of PPS antibodies and cytokines in NP lavage fluid. To determine if i.p. delivered PPS MAbs entered the NP, we conducted a pilot experiment and detected PPS3- or PPS8-specific antibodies in NP lavage fluid by ELISA as previously described (19). Briefly, mice were passively immunized as described above and humanely killed 1, 2, or 3 days postim-

munization. NP lavage fluid was collected as described above and concentrated to a final volume of 200 μl/mouse with 3-kDa centrifugal filter units (Amicon). High-binding 96-well enzyme immunoassay/radioimmunoassay plates (Costar) were coated with 5 μg/ml purified ST3 PPS3 (ATCC catalog number 169-X) or ST8 PPS8 (ATCC catalog number 185-X) overnight at 4°C in 100 μl of PBS. PPS-coated plates were then blocked with PBS containing 0.5% BSA for 2 h, incubated with 25 μl of lavage fluid concentrate for 2 h, washed, and then incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG1 antibody (Southern Biotech) for 1 h. Wells were washed and incubated with pNPP substrate (Sigma), and absorbance at 405 nm was read. In separate experiments, the amounts of 1E2, 7A9, and 31B12 in NP lavage fluid were determined by ELISA with a standard curve of purified MAbs (1 to 0.001 μg/ml) as a reference. All samples were assayed in duplicate, and the experiments were performed two times with two mice per MAb.

For analysis of NP cytokine levels, mice were immunized i.p. (for studies with whole MAbs alone) or i.n. [for studies with MAbs and $F(ab')_2$ fragments] and infected as described above. At 1 day postinfection, NP lavage fluid was collected, clarified via centrifugation (12,000 $\times$ *g*, 5 min), and stored at −20°C until use. Duoset ELISA kits were used to detect IL-1β, IL-6, IL-10, and KC (R&D Systems) according to the manufacturer's instructions. Absorbance readings for each sample were compared to a standard curve for each cytokine, and concentrations were determined and are expressed in pg/ml of lavage fluid. All samples were assayed in duplicate, and the experiments were performed two times with three or four mice per group, as indicated in the figure legends.

ST3 agglutination assays. To examine the ability of NP lavage fluid to agglutinate bacteria *ex vivo*, 10^5 CFU of ST3 bacteria were washed with PBS and resuspended in 50 μl of lavage fluid from MAb-treated mice. To examine the ability of purified MAbs to agglutinate bacteria *in vitro*, 10^5 CFU of ST3 bacteria were resuspended in 50 μl of PBS containing 0.01 to 100 μg/ml 1E2, 7A9, or 31B12. For each experiment, bacteria were incubated in NP lavage fluid or with purified MAbs for 2 h at 37°C, spotted onto 1% agarose pads, and visualized with an AxioImager Z1 microscope (Zeiss). Studies with NP lavage fluid were performed two times with pooled fluid from two mice per MAb; studies with purified MAbs were performed three times for each MAb concentration.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02260-15/-/DCSupplemental.

Figure S1, JPG file, 0.2 MB.
Figure S2, JPG file, 0.2 MB.
Figure S3, JPG file, 0.3 MB.
Figure S4, JPG file, 0.1 MB.

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