

A Serotype 3 Pneumococcal Capsular Polysaccharide-Specific Monoclonal Antibody Requires Fc γ Receptor III and Macrophages To Mediate Protection against Pneumococcal Pneumonia in Mice

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Antibodies to pneumococcal capsular polysaccharide (PPS) are required for PPS-based vaccine-mediated protection against *Streptococcus pneumoniae*. Previous work established that 1E2, a mouse IgG1 to PPS3 that does not induce serotype 3 (ST3) *S. pneumoniae* killing by phagocytes *in vitro*, protects mice from death after intranasal infection with ST3, but its efficacy was abrogated in Fc γ R (F common gamma receptor)-deficient mice. In this study, we determined whether 1E2 efficacy against pulmonary ST3 infection requires Fc γ RIII. 1E2 did not protect Fc γ RIII-deficient (Fc γ RIII^{-/-}) mice. Studies of the mechanism of 1E2-mediated effects showed that it resulted in a marked reduction in lung inflammation in ST3-infected wild-type (Wt [C57BL/6]) mice that was abrogated in Fc γ RIII^{-/-} mice. 1E2 had no effect on early bacterial clearance in the lungs of ST3-infected Wt, Fc γ RIIB^{-/-}, or Fc γ RIII^{-/-} mice, but it reduced levels of bacteremia and serum macrophage inflammatory protein-2 (MIP-2), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF- α) in Wt and Fc γ RIIB^{-/-} mice, strains in which it is protective. As previous work showed that neutrophils were dispensable for 1E2 efficacy, we investigated whether macrophages are required for 1E2 efficacy against intranasal infection with ST3 and found that its efficacy was abrogated in Wt mice depleted of macrophages intranasally. *In vitro* studies revealed that 1E2 promoted ST3 internalization by naïve alveolar macrophages but did not induce early intracellular killing. Macrophages from 1E2-treated ST3-infected mice studied *ex vivo* exhibited more apoptosis than those from Fc γ RIII^{-/-} mice. These findings suggest that 1E2 mediates protection against ST3 in mice by affecting the inflammatory response, perhaps in part via macrophage apoptosis, rather than by inducing early bacterial clearance.

There are ample data that the induction of pneumococcal capsular polysaccharide (PPS) serotype (ST)-specific opsonic antibody (Ab) correlates with vaccine protection against invasive pneumococcal disease (41). However, ST-specific antibodies that mediate protection against pneumococcus in mouse models, but do not promote opsonophagocytic killing *in vitro* (9, 10, 18, 49), have been identified. Such antibodies include PPS3-specific human and mouse monoclonal antibodies (MAbs) that protect mice against lethal pneumonia and sepsis with ST3 (49). Understanding the mechanism(s) by which antibodies to PPS3 mediate protection is important. ST3 pneumonia is associated with a higher risk of death than other STs (29, 55), severe ST3 disease attributed to serotype replacement has been reported in children (4, 11), and to date, although data from the newly introduced 13-valent pneumococcal capsular polysaccharide-protein conjugate vaccine are not yet available, investigational ST3 conjugate vaccines failed to prevent ST3 mucosal disease (36).

The ability of antibodies of the IgG isotype to mediate phagocytosis depends on the availability of Fc γ R (F common gamma receptors). The murine Fc γ R family consists of three activating receptors, Fc γ RI, Fc γ RIII, and Fc γ RIV, which when cross-linked induce cellular activation, and an inhibitory receptor, Fc γ RIIB, which inhibits the activating signal (33). Activating receptors promote and the inhibitory receptor inhibits Fc γ R-dependent antigen internalization and phagocytosis (45). Murine Fc γ RI binds IgG2a; Fc γ RIIB binds IgG1, IgG2a, and IgG2b; Fc γ RIII binds IgG1, IgG2a, and IgG2b; and Fc γ RIV binds IgG2a and IgG2b (33). In a previous study, Tian et al. reported that PPS3-specific mouse IgG1 MAbs that do (7A9 and 5F6) and do not (1E2) promote

phagocyte-mediated opsonophagocytic killing of ST3 *in vitro* were each able to protect wild-type C57BL/6 (Wt) mice against lethal intranasal infection with ST3 (49). The MAbs that promoted *in vitro* killing (7A9 and 5F6) required Fc γ RIIB and neutrophils to mediate protection, whereas the one that did not (1E2) required the FcR common gamma chain (Fc γ R) but not Fc γ RIIB or neutrophils (49). Given that 1E2 requires an activating Fc γ R to mediate protection and Fc γ RIII is the activating Fc γ R to which mouse IgG1 binds (2), we determined whether the efficacy of 1E2 against ST3 pneumonia depends on Fc γ RIII.

MATERIALS AND METHODS

***Streptococcus pneumoniae* and PPS.** The ST3 *S. pneumoniae* WU2 strain was grown in tryptic soy broth (TSB) to mid-log phase as described previously (49). WU2 has been used extensively to study the host response to and survival after infection with ST3 in mice (6, 26, 30, 37, 48, 49, 54). Purified PPS3, isolated from strain 6303 and obtained from the American Type Culture Collection, was used for enzyme-linked immunosorbent assay (ELISA)-based analyses of MAb binding.

Received 14 October 2011 Returned for modification 3 November 2011

Accepted 19 January 2012

Published ahead of print 30 January 2012

Editor: J. N. Weiser

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doi:10.1128/IAI.06081-11

Mice. Wt C57BL/6 male mice (National Cancer Institute) (6 to 8 weeks old) were used. Fc γ RIII-deficient (Fc γ RIII^{-/-}) male and female mice (22) obtained from Jackson Laboratories and Fc γ RIIB-deficient (Fc γ RIIB^{-/-}) male and female mice (46) obtained from Taconic Laboratories and bred in the Animal Institute of the Albert Einstein College of Medicine (AECOM) were used. All mouse experiments were conducted according to the rules, regulations, and ethical standards for animal use of the Animal Care and Use Committee of AECOM.

MABs and F(ab')₂ Fragments of MABs. Mouse IgG1 MABs to PPS3, 1E2, 7A9, and 5F6 were used in this study. Their production, PPS specificity, and efficacy were described previously (49). Each MAB has a different PPS3 specificity and protects Wt mice against intranasal (49) and intraperitoneal (19) infection with ST3 (strain WU2) in Wt mice. None of the MABs required complement to mediate protection in Wt mice (49). An IgG1 MAB that binds PPS8, 31B12 (56), was used as an isotype control.

MAB F(ab')₂ fragments were produced and purified with a mouse IgG1 F(ab')₂ preparation kit as described by the manufacturer (Pierce). The purity of the F(ab')₂s was analyzed by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining; neither an Fc fragment nor an IgG1 protein band was detected for either MAB (data not shown). Protein concentrations of F(ab')₂ fragments were determined by protein assay as described by the manufacturer (Bio-Rad). F(ab')₂ fragments were sterile filtered, and aliquots were stored at -80°C. The antigen binding capability of F(ab')₂ fragments to PPS3 was determined by ELISA as previously described (49). F(ab')₂ fragments of both MABs bound to PPS3 (data not shown).

Pneumococcal infection. Mice were infected intranasally with ST3 (WU2) as previously described (49). For MAB protection experiments, 10 μ g of purified MAB was diluted in phosphate-buffered saline (PBS) and 100 μ l given intraperitoneally to mice 2 h before intranasal infection with 10⁸ CFU ST3 as previously described (49). Inocula were confirmed by CFU on Trypticase soy agar with 5% sheep's blood plates (BD), plated before and after infection. For all studies that did not evaluate survival, groups of 4 to 14 mice were infected intranasally with 2 \times 10⁷ CFU of ST3 2 h after intraperitoneal administration of 10 μ g of the PPS3 and control MABs. Mice were killed 24 h after infection.

Lung and blood bacterial burden. Mice were anesthetized and bled from the retro-orbital plexus by the use of heparinized hematocrit capillary tubes (Fisher Scientific) and killed by cervical dislocation, and the lungs were aseptically removed and homogenized in Hanks balanced salt solution. Samples were serially diluted in TSB and plated onto blood agar plates. The plates were incubated for 18 h at 37°C in 5% CO₂, and then the number of CFU was counted.

Measurement of cytokine levels in the lungs and blood. Cytokine levels were measured as described previously (10, 40). Sera were separated from clotted blood by centrifugation at 3,000 \times g for 30 min. ELISA DuoSet kits for macrophage inflammatory protein-2 (MIP-2), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) (R&D Systems) were used for cytokine determinations according to the manufacturer's protocols. These mediators were chosen because they are increased by Fc γ RIII-mediated phagocytosis (21), in models of pneumococcal pneumonia and sepsis (13, 14, 24, 27, 54) and pneumococcal phagocytosis in *S. pneumoniae*-infected inhibitory Fc γ R (Fc γ RIIB)-deficient mice (12).

Histopathology. Mice were infected as described above and killed 24 h after infection. Lungs were inflated with buffered formalin for 48 h, removed, and paraffin embedded. Thin sections were stained with hematoxylin and eosin (H&E) and evaluated by a veterinary pathologist.

Alveolar macrophage depletion. To deplete alveolar macrophages, Wt mice were given a dose of 1 mg liposome-encapsulated clodronate intranasally as described for other studies (25, 42, 53) 2 days before infection. Liposomes without clodronate were used as a control. Clodronate was a gift of Roche Diagnostics GmbH and encapsulated in liposomes as previously described (51). Macrophage depletion was confirmed by performing bronchial alveolar lavage (see details below) on several "test" mice, staining the samples with F4/80-fluorescein isothiocyanate and de-

termining percent expression of F4/80 on a FACScan gating on F4/80⁺ cells. This analysis revealed <20% macrophages in clodronate-liposome-treated compared to control mice.

Bacterial uptake and killing assay. These experiments were performed as described previously (58) with some modifications. ST3 was incubated with 1 μ g/ml of the PPS3 or control MABs for 30 min at 37°C in veronal buffer (VB) (Lonza). Alveolar macrophages were isolated from mice by bronchial lavage using 10 ml per mouse through an intratracheal catheter with PBS supplemented with 0.6 mM EDTA. Lavage fluids were pooled and centrifuged at 300 \times g for 7 min; fluids contained 97% monocytes/macrophages by morphology on trypan blue-stained cells counted in a hemocytometer. PPS3/MAB mixtures were then incubated for 1 h at 37°C with 10% mouse serum and naïve Wt or Fc γ RIII^{-/-} alveolar macrophages with a macrophage-to-ST3 (E:T) ratio of 1:10, after which the mixtures were washed with VB to remove unbound bacteria. Then, cold water at pH 10.5 was added to part of the mixture (aliquot A), processed with a vortexer for 1 min, and incubated for 30 min at room temperature to lyse the macrophages. The nonlysed mixture was then treated with 100 μ g/ml of gentamicin for 15 min at 37°C to kill extracellular bacteria and divided in half (aliquots B and C). After washing, aliquot B was lysed with water and aliquot C was incubated for an additional hour and then lysed with water. Experiments were also performed with Wt macrophages in which aliquot C was incubated for 2 h and with Fc γ RIII^{-/-} macrophages with 1-h incubations as described above. Each lysed aliquot was diluted in VB, plated on sheep blood agar plates, and incubated at 37°C in 5% CO₂ overnight. The total number of bound (extra- and intracellular) bacteria was defined as aliquot A. The total number of bacteria internalized was defined as aliquot B, and the number of bacteria that were killed by the macrophages was determined by subtracting CFU of aliquot C from aliquot B.

Cellular apoptosis. Apoptosis of alveolar macrophages obtained from MAB-treated, infected Wt and Fc γ RIII^{-/-} mice was determined using annexin V and 7-aminoactinomycin D (7-AAD) staining and an annexin V kit (BD) as described previously (27) according to the manufacturer's instructions. F4/80 fluorescein isothiocyanate was added to the samples with annexin V allophycocyanin and 7-AAD according to the manufacturer's instructions. Samples were run on a FACScan and analyzed with FlowJo software by gating on cell size using forward/side scatter and then F4/80 positivity. F4/80-positive (F4/80⁺) cells that stained as positive for annexin V and negative for 7-AAD were considered apoptotic, and 7-AAD⁺ cells were considered necrotic.

Statistical analysis. Mouse survival was evaluated statistically by Kaplan-Meier plotting and the log rank test as described previously (49). The levels of blood and lung CFU and cytokines were analyzed using one-way analysis of variance (ANOVA) with Dunn's multiple-comparison test. Comparisons of the effects of the MABs in the macrophage uptake and apoptosis assays were performed using one-way ANOVA with Bonferroni's multiple-comparison test. All statistical analyses were performed using Prism (GraphPad Software). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Effect of MAB on survival in mice. A previous study established that MABs 1E2 and 7A9 (and 5F6) protect Wt (C57BL/6) mice against lethal intranasal infection with ST3 and that 1E2 efficacy required the common Fc γ chain (49). Hence, this study was undertaken to determine whether the efficacy of 1E2 requires the presence of Fc γ RIII. As shown in Fig. 1A, ST3-infected Fc γ RIII^{-/-} mice were protected from death by 7A9 but not 1E2 (*P* < 0.01, comparing 7A9 to control MAB or 1E2). F(ab')₂ fragments of 7A9 and 1E2 did not protect ST3-infected Wt (Fig. 1B), Fc γ RIIB^{-/-}, or Fc γ RIII^{-/-} (data not shown) mice. Hence, 1E2 and 7A9 require an Fc region to mediate protection in the infection model used in this study.

Bacterial burden in blood and lungs. (i) Lung CFU. At 24 h

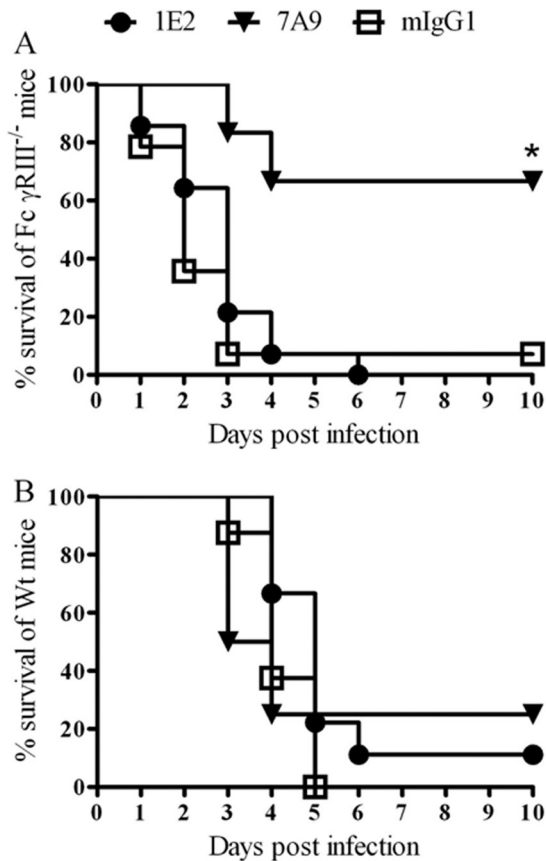


FIG 1 The dependence of PPS3-specific MAbs on Fc γ RIII and their Fc regions to protect mice against intranasal infection with 10^8 CFU ST3. (A) Survival of ST3-infected Fc γ RIII^{-/-} mice after intraperitoneal immunization with PPS3 MAbs, 1E2 and 7A9, or a control MAb (mIgG1) 2 h prior to infection. *, $P < 0.01$, comparing 7A9- to 1E2- and mIgG1-treated mice. Kaplan-Meier log-rank test, $n = 12$ to 14 mice per group. (B) Survival of ST3-infected Wt mice after passive immunization with F(ab')₂ fragments of 1E2 and 7A9 2 h prior to infection. F(ab')₂ fragments did not mediate protection; there was no significant difference in survival between the groups. Kaplan-Meier log-rank test, $n = 8$ mice per group.

after infection, 7A9-treated Wt and Fc γ RIII^{-/-} mice had, respectively, fewer lung CFU than control mIgG1-treated and 1E2-Fc γ RIII^{-/-} mice ($P < 0.01$ and $P < 0.01$, respectively) (Fig. 2A). Among ST3-infected Fc γ RIIB^{-/-} mice, 7A9-treated mice had more lung CFU than control mIgG1-treated mice ($P < 0.01$) (Fig. 2A). For 1E2-treated mice, lung CFU in Wt mice and each Fc γ R-deficient strain were not statistically different from CFU in mIgG1-treated mice. Hence, lung CFU correlated with protection in 7A9- but not 1E2-treated mice 24 h after infection.

(ii) Blood CFU. At 24 h after infection, 7A9-treated Wt, Fc γ RIIB^{-/-}, and Fc γ RIII^{-/-} mice had fewer blood CFU than mIgG1-treated mice ($P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively) (Fig. 2B). For 1E2-treated mice, Wt and Fc γ RIIB^{-/-} mice had fewer blood CFU than mIgG1-treated mice ($P < 0.01$ and $P < 0.01$, respectively), but 1E2-treated Fc γ RIII^{-/-} mice had more CFU than 7A9-treated Fc γ RIII^{-/-} mice ($P < 0.01$). Hence, blood CFU correlated with protection for 1E2 and 7A9, except for 7A9-treated Fc γ RIIB^{-/-} mice 24 h after infection.

Cytokine levels in the lungs and sera. (i) **Lung cytokines.** At 24 h after infection, 7A9-treated Wt mice had lower levels of lung

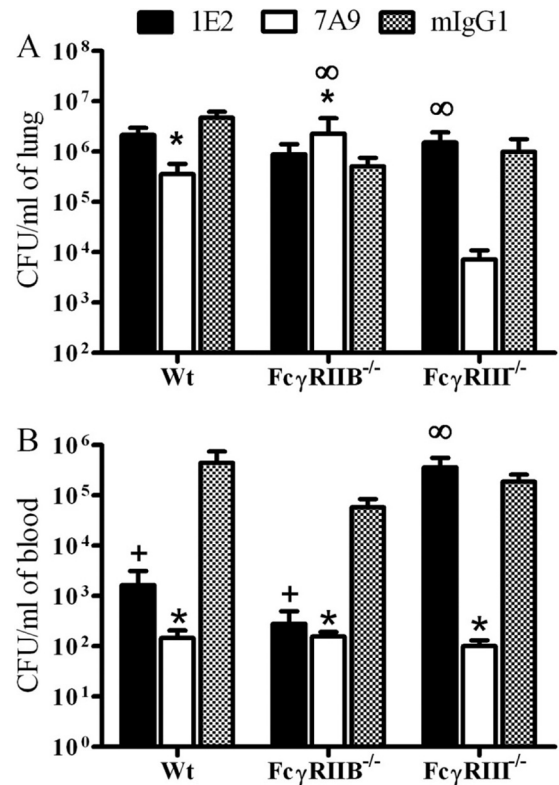


FIG 2 Lung (A) and blood (B) of 1E2-, 7A9-, and mIgG1-treated Wt, Fc γ RIIB^{-/-}, and Fc γ RIII^{-/-} mice 24 h after intranasal infection with 2×10^7 CFU ST3. Lung CFU were lower among 7A9- than mIgG1-treated Wt and Fc γ RIII^{-/-} mice and in 7A9- than mIgG1-treated Wt, Fc γ RIIB^{-/-}, and Fc γ RIII^{-/-} mice. Blood CFU were lower in 1E2- than mIgG1-treated Wt and Fc γ RIIB^{-/-} mice and in 7A9- than mIgG1-treated Wt, Fc γ RIIB^{-/-}, and Fc γ RIII^{-/-} mice. $P < 0.05$, comparing (+) 1E2- to mIgG1-treated mice, (*) 7A9- to mIgG1-treated mice, and (∞) 1E2- to 7A9-treated mice. One-way ANOVA with Dunn's multiple-comparison test. $n = 8$ to 14 mice per group. Error bars represent standard errors of the means.

MIP-2 and 7A9-treated Fc γ RIII^{-/-} mice had lower levels of TNF- α ($P < 0.03$ and $P < 0.01$, respectively) (Fig. 3A and C) than mIgG1-treated mice. Hence, for 7A9, lower levels of these mediators in the lungs 24 h after infection correlated with lung CFU and protection. At 24 h after infection, 1E2-treated Fc γ RIII^{-/-} mice had lower lung levels of MIP-2 and IL-6 than mIgG1-treated mice ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 3A and B).

(ii) Serum cytokines. At 24 h after infection, 7A9-treated Wt and Fc γ RIII^{-/-} mice had lower levels of MIP-2 ($P < 0.01$ and $P < 0.01$, respectively), IL-6 ($P < 0.01$ and $P < 0.01$, respectively), and TNF- α ($P < 0.01$ and $P < 0.01$, respectively) than mIgG1-treated mice (Fig. 3D, E, and F). At 24 h after infection, 1E2-treated Wt and Fc γ RIIB^{-/-} mice had lower levels of MIP-2 ($P < 0.01$ and $P < 0.01$, respectively), IL-6 ($P < 0.01$ and $P < 0.01$, respectively), and TNF- α ($P < 0.01$ and $P < 0.01$, respectively) than mIgG1-treated Wt and 7A9-treated Fc γ RIIB^{-/-} mice (Fig. 3D, E, and F), but 1E2-treated Fc γ RIII^{-/-} mice had higher levels of MIP-2 and TNF- α ($P < 0.01$ and $P < 0.01$, respectively) than control mIgG1- and 7A9-treated Fc γ RIII^{-/-} mice. Hence, for 7A9 and 1E2, lower levels of serum cytokines 24 h after infection correlated with protection, except for 7A9-treated Fc γ RIIB^{-/-} mice.

Inflammatory response in the lungs. Histopathological studies were performed to evaluate inflammation in lung sections

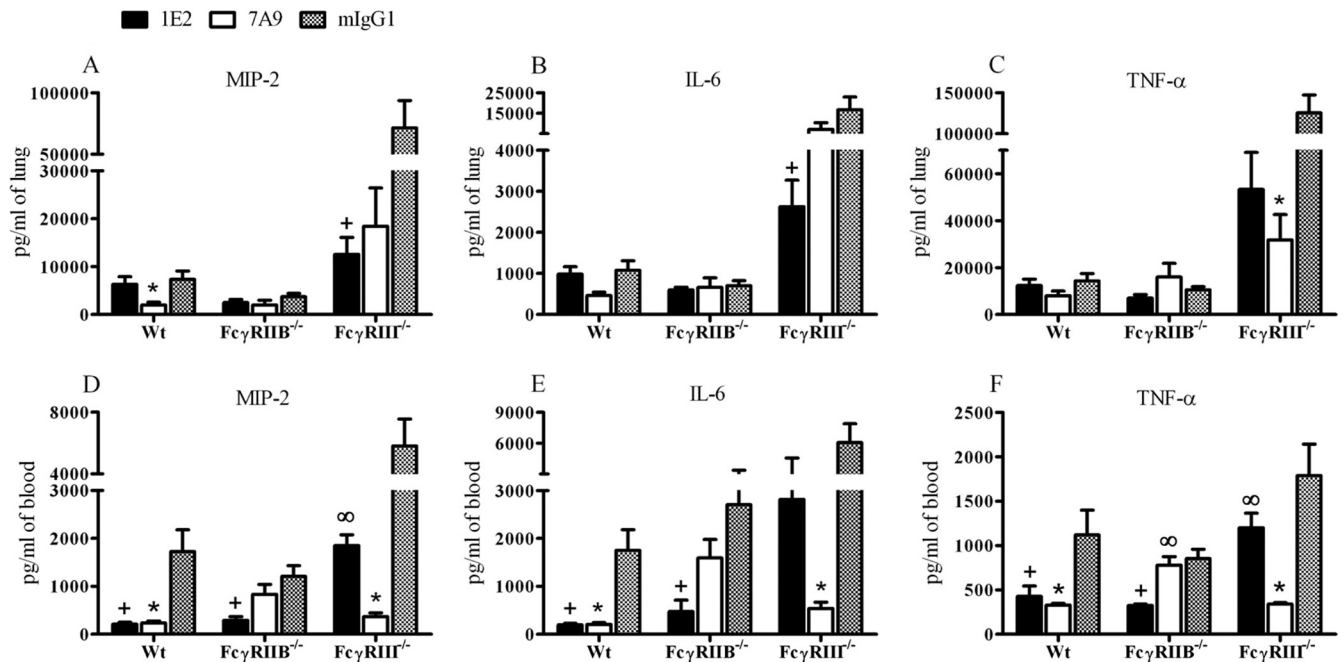


FIG 3 Cytokines in lung and serum from 1E2-, 7A9-, and mIgG1-treated Wt, FcγRIIB^{-/-}, and FcγRIII^{-/-} mice 24 h after intranasal infection with 2×10^7 CFU ST3. (A) MIP-2 in lung, (B) IL-6 in lung, (C) TNF-α in lung, (D) MIP-2 in serum, (E) IL-6 in serum, (F) TNF-α in serum. $P < 0.05$ comparing (+) 1E2- to mIgG1-treated mice, (*) 7A9- to mIgG1-treated mice, and (∞) 1E2- to 7A9-treated mice. One-way ANOVA with Dunn's multiple-comparison test. $n = 8$ to 14 mice per group. Error bars represent standard errors of the means.

from Wt and FcγRIII^{-/-} mice 24 h after infection. ST3-infected mIgG1-treated Wt mice exhibited neutrophilic and necrotizing pneumonia, with areas of fragmented, degenerating neutrophils and diffuse cellular infiltration radiating from blood vessels (Fig. 4A and B). 1E2-treated Wt mice had no evidence of pneumonia and very few infiltrating cells (Fig. 4C and D). In contrast, 1E2-treated (Fig. 4G and H) and mIgG1-treated (Fig. 4E and F) FcγRIII^{-/-} mice each had pneumonia with similar patterns of wide-spread inflammation with cellular infiltration in alveolar spaces that resembled the inflammation in mIgG1-treated Wt mice. Hence, 1E2 mediated a reduction in inflammation in Wt mice that was abrogated in FcγRIII^{-/-} mice.

1E2 requires alveolar macrophages to mediate protection. The survival of ST3-infected control PBS-liposome-treated Wt mice that received 1E2 was significantly longer than that of clodronate-liposome-treated mice ($P < 0.01$) (Fig. 5). There was no statistical difference in the survival rates of PBS-liposome- and clodronate-liposome-treated mice that received 7A9. Hence, in our model, alveolar macrophages were required for 1E2- but not 7A9-mediated protection.

1E2 Promotes internalization but not intracellular killing of ST3 by macrophages. To investigate whether 7A9 and 1E2 promote macrophage interaction with ST3, we assessed three processes: binding, internalization, and killing. Results are shown in Table 1. Compared to control mIgG1, 1E2 and 7A9 each induced more binding of ST3 to (naïve) alveolar macrophages ($P < 0.03$ and $P < 0.01$, respectively) and more ST3 internalization ($P < 0.01$ and $P < 0.01$, respectively). In the killing experiments there were more viable bacteria in 1E2- than 7A9- or mIgG1-treated cells ($P < 0.01$). We would have liked to perform additional studies with 7A9, but unfortunately, the cell line was lost. Instead, we

used another protective IgG1 MAb, 5F6, that like 7A9 mediated killing in the opsonophagocytosis assay (OPA) and required FcγRIIB and neutrophils to mediate protection against ST3 (49). Results with 5F6 in Wt cells were similar to those with 7A9: compared to mIgG1-treated control cells, 1E2 and 5F6 each induced more ST3 binding ($P < 0.03$ and $P < 0.05$, respectively) and internalization ($P < 0.05$ and $P < 0.03$, respectively), and there were more viable ST3 in 1E2- than in 5F6- or mIgG1-treated cells ($P < 0.01$) (data not shown). To determine if there was a delay in 1E2-mediated killing, we extended the final incubation from 1 to 2 h and found there were no viable CFU inside 1E2-, 5F6-, or mIgG1-treated cells (data not shown). We also performed the binding, internalization, and killing experiments with naïve alveolar macrophages from FcγRIII^{-/-} mice (Table 1). ST3 binding to FcγRIII^{-/-} macrophages was markedly less than to Wt macrophages, and the amounts of binding to FcγRIII^{-/-} cells did not differ between the MAb-treated groups. 7A9 and 1E2 each induced less ST3 internalization by FcγRIII^{-/-} than Wt macrophages, with the numbers of live intracellular CFU being similar. Although 7A9 internalized more ST3, this was not statistically significant. Hence, the ability of 7A9 and 1E2 to bind to and induce ST3 uptake in FcγRIII^{-/-} macrophages was impaired and the increased viability of ST3 in 1E2-ST3-treated Wt macrophages was abrogated.

1E2 promotes alveolar macrophage apoptosis. Annexin V staining was performed to examine alveolar macrophage apoptosis 24 h after ST3 infection. The results are shown in Table 2. Among Wt mice, 1E2-treated mice had significantly more apoptotic (annexin V⁺/7-AAD⁻ F4/80⁺) alveolar macrophages than 7A9-treated ($P < 0.01$)- and mIgG1 ($P < 0.01$)-treated mice; mIgG1-treated mice had more apoptotic alveolar macrophages

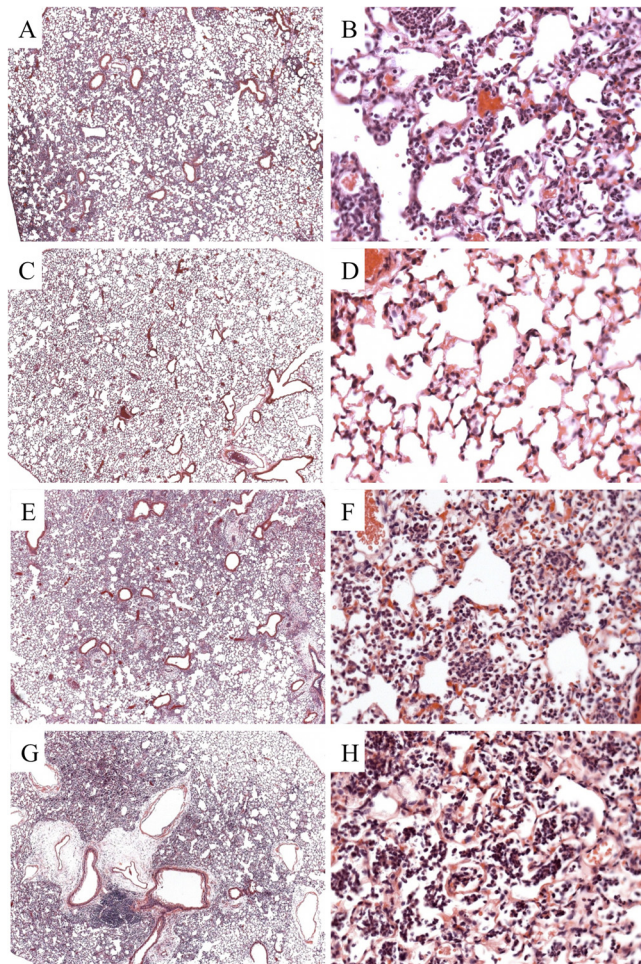


FIG 4 Histological sections of Wt and $Fc\gamma RIII^{-/-}$ mice treated with MAbs 24 h after intranasal infection with 2×10^7 CFU ST3. H&E sections of control mIgG1-treated Wt mice (A and B) had increased cellular infiltration compared to 1E2-treated Wt mice (C and D). In contrast, mIgG1-treated (E and F) and 1E2-treated (G and H) $Fc\gamma RIII^{-/-}$ mice had similar patterns of cellular infiltration. Panels A, C, E, and G are $\times 4$ magnification; panels B, D, F, and H are $\times 40$ magnification.

than 7A9-treated Wt mice ($P < 0.01$). 1E2- and 7A9-treated mice each had fewer 7-ADD⁺ alveolar macrophages than mIgG1-treated mice ($P < 0.01$ and $P < 0.01$, respectively). The level of 7-AAD⁺ macrophages among mIgG1-treated Wt mice could represent either necrosis or late apoptosis (52, 57). Similar studies were performed in $Fc\gamma RIII^{-/-}$ mice 24 h after ST3 infection. These mice had fewer F4/80⁺ cells than Wt mice. There were no differences in the numbers of apoptotic alveolar macrophages among 1E2-, 7A9-, or mIgG1-treated $Fc\gamma RIII^{-/-}$ mice (Table 2), but 1E2- and 7A9-treated mice each had fewer 7-ADD⁺ macrophages than mIgG1-treated mice ($P < 0.01$ and $P < 0.01$, respectively). Hence, the 1E2-mediated increase in apoptotic macrophages observed in Wt mice was abrogated in $Fc\gamma RIII^{-/-}$ mice.

DISCUSSION

There is ample evidence that PPS-specific antibodies that promote phagocyte killing of pneumococcus *in vitro* (referred to as “opsonophagocytic” or “opsonic” antibodies) are critical mediators of protection against invasive pneumococcal disease (17, 23, 39).

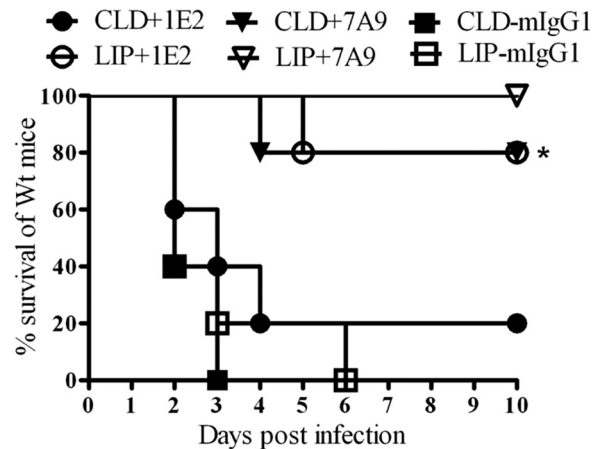


FIG 5 Survival of ST3-infected alveolar macrophage-depleted Wt mice after intraperitoneal administration of MAbs and intranasal infection with 10^8 CFU ST3. Alveolar macrophage depletion with chlodronate liposomes had no significant effect on protection by 7A9, but it abrogated protection by 1E2. *, $P < 0.001$, Kaplan-Meier log rank test. $n = 5$ mice per group. CLD, chlodronate liposomes; LIP, liposome control.

However, there is less information on protective PPS-specific antibodies that do not enhance pneumococcal killing in the opsonophagocytic killing assay that has been used to evaluate pneumococcal conjugate vaccine responses (41). In this article, we show that the efficacy of MAb 1E2, a PPS3-specific IgG1 that does not promote ST3 killing *in vitro* and protects $Fc\gamma RIIB^{-/-}$ but not $Fc\gamma R^{-/-}$ mice against lethal intranasal infection with ST3 WU2 (49), depends on $Fc\gamma RIII$ and alveolar macrophages. We also show that the efficacy of MAb 7A9, a PPS3-specific IgG1 that promotes ST3 killing *in vitro* and requires $Fc\gamma RIIB^{-/-}$ and neutrophils but not $Fc\gamma R^{-/-}$ to mediate protection (49), does not depend on $Fc\gamma RIII$ or macrophages. Given that $F(ab)'_2$ s of 7A9 and 1E2 were not protective, the efficacy of these IgG1 MAbs requires an Fc region and $Fc\gamma Rs$. Hence, the different requirements for neutrophils (7A9), macrophages (1E2), and an activating (1E2) or the inhibitory (7A9) $Fc\gamma R$ exhibited by these MAbs parallel their difference in PPS3 specificity (49).

Analogous to its inability to mediate phagocyte killing *in vitro* (49), 1E2 did not mediate early bacterial clearance in ST3-infected Wt or $Fc\gamma RIIB^{-/-}$ mice, even though it is protective in these strains (49). In contrast, 7A9 was able to mediate a reduction in lung CFU in the strains in which it is protective, Wt and $Fc\gamma RIII^{-/-}$ mice. Given that $Fc\gamma RIII$ is not expected to induce efficient neutrophil-mediated phagocytosis or bacterial killing (2, 20), the ability of 7A9 to mediate bacterial clearance in $Fc\gamma RIII^{-/-}$ mice corresponds to its ability to induce neutrophil-mediated killing *in vitro* and its requirement for neutrophils to mediate protection against intranasal infection (49). On the other hand, 1E2 and 7A9 each mediated a marked (>2 log) reduction in blood CFU in the mouse strains in which they are protective. However, blood CFU were also reduced in 7A9-treated $Fc\gamma RIIB^{-/-}$ mice, a strain in which 7A9 is not protective (49). The latter is likely to stem from the ability of 7A9 to induce phagocyte-mediated killing (49), as described by Clatworthy and Smith for $Fc\gamma RIIB^{-/-}$ mice that were able to phagocytose and control bacteremia but died of cytokine storm in a ST1 intraperitoneal infection model (12). Given that serum MIP-2, IL-6, and TNF- α were not reduced in 7A9-

treated Fc γ RIIB^{-/-} mice, the reduction in blood CFU in these mice is most consistent with phagocytosis-induced cytokine activation via Fc γ RIII in the absence of the anti-inflammatory effect of the inhibitory Fc γ R, Fc γ RIIB. Hence, in the setting of phagocytosis, neither bacterial killing *in vitro* nor bacterial clearance *in vivo* is predictive of protection against disease/death *in vivo* in our model.

The data herein reveal complex associations between the lung bacterial burden, lung cytokines, and survival. The absence of a decrease in cytokines in the lungs of 1E2-treated ST3-infected Wt or Fc γ RIIB^{-/-} mice (mouse strains in which 1E2 is protective [49]) corresponds to the bacterial burden at the time these studies were performed, which was not reduced. In contrast, the reduction in lung CFU in 7A9-treated Wt and Fc γ RIII^{-/-} mice, mouse strains in which 7A9 is protective, was accompanied by a decrease in lung cytokines, albeit only the reduction in TNF- α was statistically significant. The latter could reflect Fc γ RIIB-mediated inhibition of phagocytosis-induced cytokine release, as described by Clatworthy and Smith (12), but it could also correspond to the reduction in the lung bacterial burden in 7A9-treated Wt and Fc γ RIII^{-/-} mice. Nonetheless, 1E2-treated Fc γ RIII^{-/-} mice also exhibited a reduction in lung MIP-2 and IL-6 without a corresponding decrease in lung CFU, perhaps reflecting a lack of Fc γ RIII-mediated cytokine production and/or Fc γ RIIB-mediated inhibition of cytokine production in the setting of Fc γ RIII deficiency. Consistent with the latter, successful host defense requires both Fc γ R-mediated activation and inhibition to achieve a balance between detrimental and beneficial phagocytosis-mediated inflammation and bacterial clearance (12, 38). Fc γ RIII-mediated phagocytosis can induce IL-6 and TNF- α (21) and Fc γ RIIB mediates anti-inflammatory activity via inhibition of Fc γ RI/III (32) and clearance of immune complexes (50), but unbalanced/overexpression increases pneumococcal lethality in mice, despite phagocytosis (7). We are cognizant that cytokine levels in our model could have differed at other times and other cytokines might have exhibited relationships. In addition, we realize that more work is needed to understand the many factors influencing cytokine levels in our model.

Lung sections from 1E2-treated ST3-infected mice exhibited little to no lung inflammation, whereas 1E2-treated Fc γ RIII^{-/-} mice had marked neutrophilic inflammation resembling the inflammatory response of mIgG1-treated Wt mice. Hence, the anti-inflammatory effect of 1E2 depends on Fc γ RIII, although it does not depend on early bacterial clearance, as the bacterial burdens in 1E2- and mIgG1-treated mice were comparable 24 h after infection, the time at which the histopathological analysis was performed. The precise mechanism by which 1E2 reduces lung inflammation remains under investigation. However, given that 1E2-mediated protection required alveolar macrophages in our model, our findings are analogous to those of Knapp et al. that alveolar macrophages enhance mouse resistance to ST3 (6303) by reducing lung inflammation, rather than mediating bacterial clearance (25). In the latter study, the anti-inflammatory effect of macrophages was attributed to clearance of apoptotic neutrophils. Although we did not examine neutrophils directly, the neutrophilic infiltrates observed in lung sections from control (mIgG1-treated) Wt and 1E2-treated Fc γ RIII^{-/-} mice were not observed in 1E2-treated Wt mice. We also found that 1E2-treated mice had more apoptotic alveolar macrophages than either 7A9- or mIgG1-treated ST3-infected mice 24 h after infection, an effect that was abrogated in Fc γ RIII^{-/-} mice, and that 1E2-, 5F6-, and mIgG1-

TABLE 1 PPS3-specific MAb-mediated binding, uptake, and intracellular killing of ST3 by naive alveolar macrophages from Wt and Fc γ RIII^{-/-} mice^a

Strain	No. of ST3 bound to macrophages			No. of ST3 internalized(% of bound ST3 \pm SD)			No. of ST3 alive inside(% of internalized ST3 \pm SD)		
	1E2	7A9 or 5F6 ^b	mIgG1	1E2	7A9 or 5F6 ^b	mIgG1	1E2	7A9 or 5F6 ^b	mIgG1
Wt	60,000 \pm 3,536 ⁺	77,000 \pm 4,950*	4,000 \pm 1,414	5,400 \pm 502 ⁺ (9.0 \pm 0.3) ⁺	7,700 \pm 206* (9.0 \pm 0.3)*	80 \pm 58 (2.0 \pm 0.8)	7,074 \pm 960 ^{+∞} (131.0 \pm 5.7) ^{+∞}	154 \pm 39 (2.3 \pm 0.5)	35 \pm 11 (42.5 \pm 17.1)
Fc γ RIII ^{-/-}	18,000 \pm 3,857	33,000 \pm 9,232	15,000 \pm 8,550	1,188 \pm 437 (6.6 \pm 3.7)	2,607 \pm 3,893 (7.9 \pm 6.7)	210 \pm 34 (1.4 \pm 0.6)	520 \pm 426 (43.8 \pm 36.2)	521 \pm 698 (20.0 \pm 26.5)	10 \pm 11 (4.3 \pm 5.1)

^a Numbers shown are means with standard deviations; $P < 0.05$, comparing ⁺ 1E2- to mIgG1-treated cells, ^{*} 7A9- or 5F6- to mIgG1-treated cells, and [∞] 1E2- to 7A9- or 5F6-treated cells. Data represent results of one-way ANOVA with Bonferroni's multiple-comparison test. $n = 4$ separate experiments with data combined.

^b 5F6, an IgG1 that, like 7A9, promotes *in vitro* killing (49) was used in experiments with Fc γ RIII^{-/-} mice.

TABLE 2 Analysis of apoptosis of alveolar macrophages from MAb-treated Wt and FcγRIII^{-/-} mice 24 h after intranasal infection with 2 × 10⁷ CFU ST3^a

Strain	No. of F4/80 ⁺ macrophages (× 10 ⁵)			No. of annexin V ⁺ /7-ADD ⁻ /F4/80 ⁺ macrophages (× 10 ⁵)(% ± SD)			No. of 7-AAD ⁺ /F4/80 ⁺ macrophages (× 10 ⁵)(% ± SD)		
	1E2	7A9 or 5F6 ^b	mIgG1	1E2	7A9 or 5F6 ^b	mIgG1	1E2	7A9 or 5F6 ^b	mIgG1
Wt	96 ± 64	111 ± 36	120 ± 20	16 ± 2 ⁺⁺ (13 ± 1) ⁺⁺	3 ± 1* (3 ± 1)*	6 ± 2 (6 ± 2)	5 ± 2 ⁺ (4 ± 1) ⁺	3 ± 1* (3 ± 1)*	13 ± 2 (14 ± 2)
FcγRIII ^{-/-}	28 ± 7	24 ± 16	30 ± 20	6 ± 2 (24 ± 3)	2 ± 1 (18 ± 6)	7 ± 2 (19 ± 2)	3 ± 1 ⁺ (12 ± 2) ⁺	2 ± 1* (11 ± 1)*	4 ± 2 (16 ± 1)

^a Numbers shown are means with standard deviations of total F4/80⁺ macrophages. The percentages with standard deviations of apoptotic (annexin V⁺/7-ADD⁻) and necrotic (7-ADD⁺) F4/80⁺ macrophages are shown in parentheses. *P* < 0.05, comparing ⁺ 1E2- to mIgG1-treated mice, * 7A9- or 5F6- to mIgG1-treated mice, and ⁺⁺ 1E2- to 7A9-treated mice. Data represent results of one-way ANOVA with Dunn's multiple-comparison test. *n* = 4 to 5 mice/group.

^b 5F6, an IgG1 that, like 7A9, promotes *in vitro* killing (49) was used in experiments with FcγRIII^{-/-} mice.

treated FcγRIII^{-/-} mice each had a similar amount of apoptotic macrophages. In view of the anti-inflammatory effect of 1E2 in Wt mice, our findings are consistent with data from Marriott et al. showing that apoptotic alveolar macrophages reduced lung inflammation and bacterial bloodstream invasion in a ST2 pulmonary infection model (28).

7A9 and 5F6, but not 1E2, were previously shown to promote ST3 killing by neutrophils and J774-like macrophages *in vitro* (49). Based on these findings, 7A9 and 5F6 were characterized as opsonic and 1E2 as nonopsonic. However, data presented herein challenge these characterizations, as 1E2 mediated an amount of ST3 uptake by naïve alveolar macrophages similar to that of 7A9 or 5F6 *in vitro*. Nonetheless, and corresponding to the lack of early bacterial clearance in the lungs of 1E2-treated mice, 1E2-opsonized ST3 induced significantly less intracellular bacterial killing *in vitro* after 1 h of incubation. Although ample data establish that mouse survival in pneumococcal infection requires bacterial clearance and control of bloodstream invasion (8, 13, 27, 44, 54), this study did not directly demonstrate how 1E2 mediates bacterial clearance. Given that pneumococcal pathogenesis stems from bacterial ligands binding to host receptors (3, 34) 1E2-induced macrophage internalization of ST3 could temporarily limit ST3 access to host receptors. However, 1E2-mediated protection is associated with alveolar macrophage apoptosis *in vivo* and our *in vitro* results suggest that macrophage internalization of 1E2 could result in delayed macrophage killing. 1E2-opsonized ST3 was viable in macrophages for 1 h *in vitro*, but there were no viable intracellular bacteria in 1E2-, 5F6-, or control mIgG1-treated cells 2 h after incubation. Consistent with our finding that 1E2- and 7A9-induced ST3 uptake was reduced but not abrogated in FcγRIII^{-/-} macrophages, FcγRIIB and FcγRIII each promote (human) macrophage-associated apoptotic killing of *S. pneumoniae* (1) and opsonized *S. pneumoniae* induced delayed macrophage apoptosis-associated bacterial killing via FcγRIII and FcγRII (1). Hence, the kinetics of mouse and human macrophage apoptosis could differ. Nonetheless, Dockrell et al. showed that ST1 uptake resulted in alveolar macrophage apoptosis-mediated bacterial clearance in mice (15, 16) and killing in human macrophages (15). Further, Ali et al. described a delay in apoptosis-associated killing of *S. pneumoniae* by human macrophages (1), possibly reflecting recently described relationships between phagolysosomal events, apoptosis, and late-stage pneumococcal killing (5). Interestingly, Bewley et al. found that inhibition of macrophage apoptosis-associated killing of ST1 was associated with increased neutrophil recruitment to the lungs (5), providing a mechanistic hypothesis for our data that 1E2 efficacy requires

macrophages and that its anti-inflammatory effect requires FcγRIII and is associated with alveolar macrophage apoptosis.

In summary, our data show that 1E2-mediated protection is a function of macrophage FcγRIII availability and reveal a critical role for FcγRIII in 1E2-mediated macrophage uptake and internalization of ST3 *in vitro* and alveolar macrophage apoptosis and immunomodulation *in vivo*. Fc-independent antibody-mediated immunomodulation was described in a *Streptococcus mutans* model (40). However, to our knowledge, despite several studies linking FcγRIII to inhibition of the inflammatory response (31, 35, 47), current thought holds that Fc-dependent immunomodulation depends on FcγRIIB (12, 43, 45, 50). Our data support but do not prove that 1E2 induces ST3 killing via late macrophage apoptosis, and the precise mechanism by which 1E2 mediates bacterial clearance *in vivo* remains to be determined. Nonetheless, our model provides a novel platform to dissect mechanisms of antibody-dependent macrophage apoptosis-associated pneumococcal killing and antibody-mediated regulation of the inflammatory response to pneumococcal infection. Finally, our data suggest that antibodies which do not promote phagocyte-mediated bacterial killing *in vitro* (41), but promote bacterial uptake by macrophages, warrant further study as potential modulators of the inflammatory response to pneumococcal pneumonia.

ACKNOWLEDGMENTS

We thank Rani Sellers of the Histopathology Core Facility for expert histopathological analysis. We also thank Lydia Tesfa of the Flow Cytometry Core Facility for assistance with the FACScan. The Flow Cytometry Core Facility is a shared facility supported by the Einstein Cancer Center (P30CA013330).

This work was supported by the National Institutes of Health (R01-AI045459 and R01-AI044374 to L.P.) and an HIV, AIDS and Opportunistic Infection Institutional AIDS Training Grant (T32-AI007501 to S.W.).

No author has a conflict of interest with the data presented in this article.

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