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Anti-CD3 Antibody Decreases Inflammation and Improves Outcome in a Murine Model of Pneumocystis Pneumonia

Samir P. Bhagwat,* Terry W. Wright,*† and Francis Gigliotti*,†

The T cell–mediated immune response elicited by Pneumocystis plays a key role in pulmonary damage and dysfunction during Pneumocystis carinii pneumonia (PcP). Mice depleted of CD4* and CD8* T cells prior to infection are markedly protected from PcP-related respiratory deficit and death, despite progressive lung infection. However, the therapeutic effectiveness of Ab-mediated disruption of T cell function in mice already displaying clinical symptoms of disease has not been determined. Therefore, a murine model of PcP-related immune reconstitution inflammatory syndrome was used to assess whether Ab to the pan-T cell molecule CD3 is effective for reducing the severity of PcP when administered after the onset of disease. Mice that received anti-CD3 Ab exhibited a rapid and dramatic halt in the PcP-associated pulmonary function decline within 1 week after treatment, and a striking enhancement of survival rate compared with mice receiving the control Ab. Physiologic improvement in anti-CD3 treated mice was associated with a significant reduction in the number of CD4* and CD8* T cells recovered in lung lavage fluid. This effectiveness of anti-CD3 was noted whether the mice also received antibiotic therapy with trimethoprim-sulfamethoxazole. These data suggest that monoclonal Ab-mediated disruption of T cell function may represent a specific and effective adjunctive therapy to rapidly reverse the ongoing pathologic immune response occurring during active PcP. Thus, the anti-human CD3 monoclonal Ab OKT3, which is already in clinical use, has the potential to be developed as an adjunctive therapy for PcP. The Journal of Immunology, 2010, 184: 497–502.

Pneumocystis carinii pneumonia (PcP) remains a life-threatening disease process prevalent among immunosuppressed populations. Despite many improvements in our ability to care for critically ill patients, the mortality attributable to PcP has changed little and remains unacceptably high (1). Therefore, new treatment modalities that specifically address the pathophysiology of PcP are needed. Mortality is particularly high, up to 80–90%, among those patients who require admission to an intensive care unit. Recent analyses of two large cohorts of patients with AIDS and PcP demonstrated that the need for intensive care or mortality is discernable at or soon after admission (2, 3). Therefore, there is an identifiable group of patients with PcP who would benefit from an improved treatment regimen for PcP, were one available.

It is now becoming increasingly recognized that the host inflammatory response elicited by a microorganism can also produce tissue damage. Studies published by ourselves and others over the past several years have demonstrated that this is a prominent feature of PcP (4–10). Clinical observations in humans also support the link between inflammation and poor outcome in patients with PcP (11, 12), and it has been postulated that the benefit of adjunctive corticosteroid therapy is related to anti-inflammatory effects (12, 13).

The CD4* T lymphocyte is the critical cell type required for normal resistance to P. carinii infection, as well as for immune-mediated clearance of an existing infection (14, 15). A consistent feature of animal model experiments is the finding that in the absence of sufficient CD4* T cells to protect against PcP, the CD8* T cell is a key cell in driving the injurious immune response specific to P. carinii (4, 5, 7, 8, 10, 16). CD4* T cells can also initiate inflammatory injury in response to P. carinii in the context of immune restitution inflammatory syndrome (IRIS), a clinical condition in which a period of immunosuppression and infection is followed by immune recovery and a rapid onset of pulmonary inflammation and respiratory distress (4, 17). Although the immunopathologic role of T cells during PcP is a well-documented concept, effective, specific, and feasible therapeutic regimens to block PcP-related inflammatory processes in clinically relevant models have not been developed. Immunomodulatory therapies should provide a mechanism to improve the outcome of PcP when combined with effective antibiotics to eradicate the infection. Our working hypothesis is that the host’s T cell–mediated immune response to P. carinii infection is a major contributor to the morbidity and mortality of PcP, and that effective control of this response, combined with antibiotic treatment, will improve the outcome of patients presenting with active PcP. In mouse models, we have provided support for this hypothesis by using Ab to specifically deplete T cells before infection (4, 5, 7, 8, 10). However, the effectiveness of Ab-mediated T cell depletion after the onset of PcP has not been determined.

Although anti-CD8 and anti-CD4 Abs for use in humans are not available, the pan T cell Ab OKT3 (muromonab-CD3; Mur-omomab; Ortho Biotech, Bridgewater, NJ) is currently approved for use in patients. Importantly, OKT3 exerts its effects on both CD4* and CD8* T cells. Because some PcP patients may have residual CD4* T cell function, administration of an OKT3-like Ab would have the combined benefit of interfering with the function of both CD8* and CD4* T cells that may be contributing to the pathologic inflammatory response. This result would make such

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Abbreviations used in this paper: BAL, bronchoalveolar lavage; IRIS, immune restitution inflammatory syndrome; PcP, Pneumocystis carinii pneumonia; TMP-SMX, trimethoprim-sulfamethoxazolozole.

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an approach especially useful for the treatment of PCP in the setting of IRIS, in which CD4+ T cells are known to play a role in immunopathogenesis. A final benefit of using an OKT3 or OKT3-like Ab is that there is extensive clinical experience using these molecules, which would facilitate using them as adjunctive therapy for PCP should such an approach be validated. We therefore undertook a series of experiments to provide experimental animal data for the effect of anti-CD3 Ab on the outcome of PCP. We used an immune reconstitution model that mimics PcP-induced IRIS. Our findings suggest that this approach may have a clinical use in the management of moderate to severe PCP.

Materials and Methods
Preparation of F(ab')2 fragments
Hybridoma cell line 145 2C11 that produces hamster anti-mouse CD3 Ab was obtained from the American Type Culture Collection (18). Ab was purified by saturated ammonium sulfate precipitation and protein A purification of ascites fluid obtained from SCID mice. F(ab')2 fragments were prepared from intact Ab by a previously published protocol (19) with following modifications: the Ab was digested with pepsin at 37°C for four hours. The Fc fragments were removed by passing the Ab over a protein A column that allowed the F(ab')2 to pass through. Whole hamster IgG was used to produce control F(ab')2 using the same method. F(ab')2 fragments were used for these experiments to avoid the potentially confounding effects of the generalized cytokine release sometimes seen when CD3 is cross-linked by intact Ig (19).

Preparation of mouse Pneumocystis organisms
The CB.17 scid/scid mice with heavy P. carinii infections were euthanized and their lungs removed aseptically. P. carinii organisms were isolated from the lung tissue as previously described (10, 20). The final preparation was stained with ammoniacal silver to enumerate cysts, and Diff-Quick (Dade AG, Dudingen, Switzerland) to screen for bacterial contamination. In addition, the preparations were routinely plated on commercially available chocolate blood agar plates to test for the presence of contaminating microorganisms.

Treatment of mice with F(ab')2 fragments in the mouse model of PCP-related IRIS
Mice were infected by intranasal inoculation with 1 × 10⁸ P. carinii cysts. Four weeks later, the infected SCID mice were immune reconstituted with 5 × 10⁸ spleenocytes from syngeneic donor strains to mimic IRIS as previously described (4, 8). Anti-CD3 F(ab')2 treatment was begun when the mice were clearly symptomatic, which we defined as either the average body weight loss of >10% or average respiratory rate of >400 respirations per min. Thus, the experimental mice were monitored with body weight and respiratory rate determination, and F(ab')2 treatment was begun ~1 wk after immune reconstitution (days 7–9). Experimental mice were given either 150 µg 145 2C11 F(ab')2 or control F(ab')2 i.p. every other day. In one experiment, a group of mice received anti-CD3 F(ab')2 only on treatment days 1 and 3 in addition to the trimethoprim-sulfamethoxazole (TMP-SMX) treatment. TMP-SMX (Sicor Pharmaceuticals, Irvine, CA) treatment was initiated 1 d after anti-CD3 treatment as a single daily dose until the end of the experiment. TMP-SMX was diluted in saline and given at 20 mg TMP per kilogram body weight as described (21).

Physiologic assessment of dynamic lung compliance and resistance in mice
Dynamic lung compliance and resistance was measured in live mice using a previously described method with modifications (8, 10, 22). Mice were anesthetized by i.p. injection of 0.13 mg sodium pentobarbital per gram body weight. A tracheostomy was performed and a 20-gauge cannula was inserted 3 mm into an anterior nick in the exposed trachea. The thorax was then opened to equalize airway and transpulmonary pressure. To assure that the mice tolerated the procedure, they were examined for spontaneous respirations before proceeding further. Mice were immediately placed into a plethysmograph designed for anesthetized mice (Buexco Electronics, Wilmington, NC), and connected to a Harvard rodent ventilator (Harvard Apparatus, Southnack, MA). Mice were ventilated with a tidal volume of 0.01 ml per gram body weight at a rate of 150 breaths per minute. Respiratory flow and pressure were measured using transducers attached to the plethysmograph chamber. Data were collected and analyzed using the Biosystems XA software package (Buxco Electronics). Dynamic lung compliance was calculated in ml · cm⁻¹ · H₂O from the flow and pressure signals using the method by Amdur and Mead (23), and then normalized for peak body weight. Lung resistance values were calculated in cm H₂O · ml⁻¹ · s⁻¹ from the same input signals.

Bronchoalveolar lavage and lung tissue preparation
Bronchoalveolar lavage (BAL) and lung tissue samples were obtained following dynamic compliance measurements. The chest cavity was surgically opened to expose the lungs and trachea, and the left lung lobe was tied off securely at the bronchus with surgical silk and removed with sterile scissors. The remaining lung lobes were gently lavaged with four, 1-ml aliquots of 1 × HBSS via the tracheal cannula. Recovered lavage fluid (~3.5 ml per mouse) was centrifuged at 250 × g for 5 min to obtain the cellular fraction. The cells were resuspended in fresh HBSS, enumerated, centrifuged onto glass slides, and stained with Diff-Quick for differential counting. In addition, multiparameter flow cytometric analysis was performed on BAL cells following staining with fluorochrome-conjugated Ab. Anti-CD4-Fluorescein (clone RM4-4), and anti-CD8a-peridinin chlorophyll-a-protein (clone 53-6.7) were purchased from BD Biosciences (San Diego, CA). At least 5000 events per BAL sample were routinely analyzed on a FACSCalibur cell sorter (BD Biosciences).

Summary of bronchoalveolar lavage
The BAL cells were counted using a hemocytometer. The number of alveolar macrophages (AM) was calculated as the percentage of AM in the BAL cell population. The percentage of CD4+ T cells was determined by flow cytometry using a FACSCalibur (BD Biosciences) and the following Abs: anti-CD4-FITC, anti-CD8a-Pacific Blue, and anti-CD11c-APC. Results
The experiments were designed to test the hypothesis that anti-CD3 F(ab')2 fragments could be used therapeutically to improve the morbidity and mortality associated with PCP. We chose to use an immune reconstitution mouse model of PCP, because it allows for a vigorous inflammatory response to P. carinii that resembles the IRIS seen in patients with PCP. SCID mice infected with P. carinii were immune-reconstituted with congenic splenocytes to induce IRIS. After 9 d, when the mice exhibited obvious signs of PCP, treatment was begun with anti-CD3 or control F(ab')2 fragments. Our results demonstrated a clear cut advantage, both in terms of mortality and improved compliance among survivors, in those mice receiving anti-CD3 F(ab')2 fragments when compared with mice receiving control F(ab')2 fragments. Overall survival

Statistical analyses
All values reported for each experimental group are mean ± 1 SE of measurement. Analysis was performed using Sigma-Stat version 3.5 (SyStat Software, Point Richmond, CA). Different groups were compared by performing a one-way ANOVA. In cases in which the ANOVA was significant, individual groups were tested using pairwise t test. Survival analysis was done using Kaplan-Maier Log-rank test. In addition, individual groups were compared in pairs using Fisher’s exact chi-square test. A test was considered significant at p < 0.05.
was increased from 50 to 92% in mice receiving anti-CD3 ($p = 0.003$; Table I; group I versus III). Three replicate experiments were performed, and there was improved survival in all three trials. Only the second experiment was large enough to demonstrate a statistically significant ($p = 0.017$) improved survival when individually analyzed. For these experiments, no other intervention was performed other than the use of Ab.

Because patients with PcP would receive TMP-SMX in addition to any adjunctive therapies, we included mice in each experiment that received TMP-SMX in addition to anti-CD3 or control F(ab\textsuperscript{2}'). As was observed in mice not receiving antibiotics, mice treated with TMP-SMX had an improved survival if they received anti-CD3 F(ab\textsuperscript{2}) (96 versus 38%; $p < 0.001$; compared with the group that received control F(ab\textsuperscript{2}); Table I; group IV versus II). Again, three replicate experiments were done and the improvement in survival was seen in all three trials.

To delineate the pattern of mortality over the ~2-wk course of the experiment, the results from Table I were plotted as a Kaplan-Meier graph. As seen in Fig. 1, mortality did not differ between anti-CD3 F(ab\textsuperscript{2}) treated and control mice over the first several days of treatment. However, by the end of the first week after start of the treatment, the increased mortality in the control mice became apparent.

**Postonset anti-CD3 treatment causes a rapid halt in pulmonary function decline during PcP**

To determine the direct effect of the anti-CD3 therapeutic regimen on the progression of PcP, body weight loss was tracked non-invasively on all mice, and random mice were selected for physiologic measurement of dynamic lung compliance and resistance. By 2 d after the initiation of treatment, the PcP-related weight loss of the anti-CD3 F(ab\textsuperscript{2}) treated groups had already begun to separate from the control F(ab\textsuperscript{2}) treated groups (Fig. 2). By 4 d after treatment, the control F(ab\textsuperscript{2}) treated groups with and without TMP-SMX had both lost nearly 10% more weight than the anti-CD3 treated groups. This relative difference was maintained out to 1 wk after the onset of treatment, indicating that anti-CD3 treatment was able to rapidly halt the progression of PcP-related IRIS whether TMP-SMX was administered. Direct physiologic assessment of pulmonary function demonstrated that after 1 wk of treatment, pulmonary dynamic compliance was significantly improved in mice treated with anti-CD3 F(ab\textsuperscript{2}) plus TMP-SMX ($p = 0.004$; compared with mice treated with control F(ab\textsuperscript{2}) plus TMP-SMX; Fig. 3A). Likewise, lung resistance was also significantly improved at this time in the anti-CD3 F(ab\textsuperscript{2}) treated group ($p = 0.002$; compared with mice treated with control F(ab\textsuperscript{2}); Fig. 3B). In the case of Ab only treated groups (in the absence of any TMP-SMX treatment), compliance of Anti-CD3 F(ab\textsuperscript{2}) treated mice was also improved compared with control F(ab\textsuperscript{2}) ($p = 0.055$). However, it is important to note that the differences between treated and control mice are likely underestimated because so many of the control mice died an obvious respiratory death, effectively removing those mice with poorest lung function from the analysis. Overall, these results demonstrate that administration of anti-CD3 to mice already presenting with PcP produces a dramatic improvement in survival and preserves lung function.

**Anti-CD3 treatment reduces PcP-related pulmonary T cell responses**

Improved pulmonary function in mice receiving anti-CD3 F(ab\textsuperscript{2}) was associated with decreased numbers of inflammatory cells in the lung (Table II). As would be expected, anti-CD3 F(ab\textsuperscript{2}) produced a marked drop in both CD8\textsuperscript{+} and CD4\textsuperscript{+} T lymphocytes. In addition, both macrophages and neutrophils were also reduced, indicative of an overall downregulation of the pathologic inflammatory response.

**Anti-CD3 treatment interferes with host-mediated clearance of *P. carinii* in immune reconstituted SCID mice**

An important feature of these experiments was the finding that TMP-SMX did not improve survival in the absence of anti-CD3. The SCID mice used in these experiments were allowed to develop significant *P. carinii* infections before they were immune reconstituted, and the consequently developed severe PcP-related IRIS. Whereas the TMP-SMX did reduce *P. carinii* numbers compared with mice not treated with antibiotic, the exuberant inflammatory response produced a pulmonary insult that was not optimally responsive to antibiotics alone. Similarly, it is likely that anti-CD3 treatment alone would not be sufficient. Although mice treated with anti-CD3 in the absence or presence of TMP-SMX were healthier than mice treated with control F(ab\textsuperscript{2}) or control F(ab\textsuperscript{2})

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Kaplan-Maier plot of survival analysis. Survival data of mice pooled from three experiments summarized in Table I. Proportion of survivors versus time (days after treatment start) are plotted. To make the analysis more conservative, the mice that were sacrificed for lung function and inflammatory measurements were assumed to be “survived” until the last day of the experiment. Kaplan-Maier Log-rank test was performed using Sigma-Stat version 3.5, which was found to be significant ($p < 0.001$).

### Table I. **Overall survival in mice with PcP treated with anti-CD3 F(ab\textsuperscript{2}) fragments with or without additional TMP-SMX treatment**

<table>
<thead>
<tr>
<th>Group</th>
<th>Ab Treatment</th>
<th>TMP-SMX</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control F(ab\textsuperscript{2})</td>
<td>No</td>
<td>86% (6/7)</td>
<td>31% (4/13)</td>
<td>50% (3/6)</td>
<td>50% (13/26)</td>
</tr>
<tr>
<td>II</td>
<td>Control F(ab\textsuperscript{2})</td>
<td>Yes</td>
<td>50% (3/6)</td>
<td>36% (5/14)</td>
<td>33% (2/6)</td>
<td>38% (10/26)</td>
</tr>
<tr>
<td>III</td>
<td>Anti-CD3 F(ab\textsuperscript{2})</td>
<td>No</td>
<td>100% (7/7)</td>
<td>85% (11/13)</td>
<td>100% (5/5)</td>
<td>92% (25/25)</td>
</tr>
<tr>
<td>IV</td>
<td>Anti-CD3 F(ab\textsuperscript{2})</td>
<td>Yes</td>
<td>100% (7/7)</td>
<td>93% (13/14)</td>
<td>100% (6/6)</td>
<td>96% (26/27)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are the number of survivors/total number of mice in the group.
with TMP-SMX, the anti-CD3 treatment given without TMP-SMX was found to interfere with *P. carinii* clearance. Mice treated for 2 wk with anti-CD3 F(ab\''\')2 alone had a *P. carinii* burden of 1.5 ± 0.6 × 10^5 kexin copies, compared with 3.2 ± 1.2 × 10^5 kexin copies in mice receiving both TMP-SMX and anti-CD3 F(ab\''\')2 (p = 0.02). This observation suggests that although anti-CD3 F(ab\''\')2 reduces inflammatory injury and improves lung function and survival of mice with PCP, it also prevents host-mediated clearance of *P. carinii* from the lungs. Thus, the combined effects of anti-CD3 and TMP-SMX appeared optimal for outcome of PCP.

### Discussion

PCP is an infectious disease with significant morbidity and mortality attributable to the host inflammatory response, as demonstrated in both patients (11, 12) and animal models (4, 5, 8). The presence of T cells has been shown to be necessary to initiate the inflammatory immune response to *P. carinii*, which in turn produces so-called bystander injury to the lung. In most clinical scenarios, patients lack CD4+ T cells, thereby leaving a predominantly CD8+ T cell–driven response that is ineffective in killing *P. carinii* but produces critical lung injury (4, 5, 8). In other clinical situations, such as IRIS, CD4+ T cells also participate in the inflammatory response. In contrast to CD8+ T cells, the CD4+ T cell–driven immune response contributes to the eradication of *P. carinii*, although these responses may result in even greater lung damage (4).

We have shown previously that the inflammatory injury associated with PCP-related IRIS can be significantly prevented by depleting CD4+ and CD8+ T cells prior to immune reconstitution (4, 8). Although this is easily accomplished in mouse models, therapeutic agents to accomplish this in humans are not presently available. A pan T cell Ab, OKT3 (Muromonab), is available for clinical use as an immunosuppressive agent especially in the control of transplant rejection. OKT3 and similar Abs work by causing partial depletion of T cells and more importantly by disrupting the interaction of CD3 with the T cell receptor necessary for T cell Ag recognition and activation (25). Because both CD4+ and CD8+ T cells express CD3, we reasoned that using an Ab directed against CD3 would result in T cell inactivation similar to that observed with specific monoclonal Abs in mice.

SCID mice with PCP that were immune reconstituted and treated with anti-CD3 F(ab\''\')2 fragments showed a marked physiologic benefit. These improvements are statistically significant and clinically relevant. Treated mice demonstrated reduced pulmonary inflammation, were able to clear *P. carinii* from their lungs, and were able to live significantly longer than untreated or control-treated mice. The regimen of anti-CD3 F(ab\''\')2 treatment in this model was designed to be performed every other day, but this reduction was not statistically significant (0.81 and 0.99, respectively; p = 0.231). This experiment suggests that the effect of limited anti-CD3 F(ab\''\')2 treatment is temporary, but still potentially useful in improving the morbidity and mortality in this model.

Limitet postonset Anti-CD3 treatment improves survival of mice with PCP-related IRIS

To test whether a shorter treatment with anti-CD3 F(ab\''\')2 was also effective in improving morbidity and mortality of mice with PCP-related IRIS, we compared two doses of Ab: given on days 1 and 3 versus every other day as used in our other experiments. TMP-SMX was continued until the end of the experiment in all groups. The results are summarized in Fig. 4. At the conclusion of the experiment, all the mice in both the Anti-CD3 F(ab\''\')2 treatment groups survived, whereas only three and two of six mice in control F(ab\''\')2 treatment groups survived with or without TMP-SMX treatment, respectively. The mice that received Anti-CD3 F(ab\''\')2 every other day showed weight gain until the end of the experiment. The mice that received only two doses of Anti-CD3 F(ab\''\')2 gained weight until day 12 and then slowly began losing weight, probably because of restoration of lymphocyte-mediated inflammation as the Anti-CD3 F(ab\''\')2 was halted. A number of mice that received control F(ab\''\')2 either with or without TMP-SMX died during the course of the experiment, as denoted by black arrows in Fig. 4. The surviving mice in the control F(ab\''\')2+TMP-SMX group had improved average body weight at the end of the experiment, but that was mainly due to a single mouse of six that had substantial improvement in body weight. The compliance of mice with only two Anti-CD3 F(ab\''\')2 doses was reduced, compared with the mice that received Anti-CD3 F(ab\''\')2 every other day, but this reduction was not statistically significant (0.81 and 0.99, respectively; p = 0.231). This experiment suggests that the effect of limited Anti-CD3 F(ab\''\')2 treatment is temporary, but still potentially useful in improving the morbidity and mortality in this model.

**FIGURE 2.** Changes in the body weight of experimental mice. Percent loss of body weights (compared with the peak weight) for different groups of experimental mice is plotted against the days post reconstitution. Each data point represents arithmetic mean ± SEM. *p < 0.001, when day 7 weights of all the mice was compared with their day 3/4 weights (n = 102). **p < 0.001, when Anti-CD3 F(ab\''\')2 + TMP-SMX group was compared with the control F(ab\''\')2 + TMP-SMX group (n = 13). Data are combined from a pool of three independent experiments. ⊗, control IgG F(ab\''\')2; ⊙, control IgG F(ab\''\')2 + TMP-SMX; ▲, anti-CD3 F(ab\''\')2; △, anti-CD3 F(ab\''\')2 + TMP-SMX.

**FIGURE 3.** Effect of anti-CD3 treatment on lung function during PCP-associated IRIS in mice. Pulmonary function testing was performed on the mice at either day 6 (in experiment two) or 8 (in experiment one) after the anti-CD3 treatment was initiated. The results are pooled data from two independent experiments, and each data point is the arithmetic mean ± 1 SEM (n = 7–11 mice for all groups). A, Dynamic lung compliance measurements (*p = 0.004). B, Airway resistance measurements (**p = 0.002).
inflammation, improved dynamic compliance, improved lung resistance, and improved survival. Increased neutrophil numbers in pulmonary lavage fluid have been correlated with increased severity of PcP in patients and mouse models (12, 26). Using anti-CD3 F(ab’)2 in our model resulted in a significant reduction in neutrophil numbers in lavage fluid that was associated with less severe disease. Interestingly, macrophage numbers were also decreased, although the significance of this observation in regard to the injury resulting from the P. carinii–driven inflammatory response is unknown.

The experimental approach described in this report differs from that described in our earlier publications (4, 8) in one important respect. In the earlier experiments, CD4+ or CD8+ T cell depletion that described in our earlier publications (4, 8) in one important response is unknown.

Disclosures
The authors have no financial conflicts of interest.
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