Resolution of *Pneumocystis carinii* Pneumonia in CD4+ Lymphocyte-depleted Mice Given Aerosols of Heat-treated *Escherichia coli*

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Summary

Mice were thymectomized and depleted of CD4+ lymphocytes by treatment with monoclonal antibody to induce *Pneumocystis carinii* (PC) pneumonia (PCP). These mice were then exposed to aerosols of heat-treated *Escherichia coli* three times a week. Aerosol treatment for 10 d caused a slight reduction in numbers of PC nuclei in the lungs of mice, and treatment for 22 d resulted in nearly complete resolution of PCP. Large numbers of macrophages, polymorphonuclear leukocytes, and lymphocytes accumulated in lungs of aerosol-treated mice. Depletion of either CD8+ lymphocytes or asialo GM1+ cells that remained in the mice after CD4+ cell depletion had no effect on the ability of the mice to resolve PCP after *E. coli* aerosol treatments. However, depletion of Thy-1+ lymphocytes in these mice abrogated their ability to resolve PCP and reduced the numbers of macrophages that accumulated in the lungs. In addition, it was found that resolution of PCP induced by heat-treated *E. coli* aerosol treatments was also abrogated when mice were treated with polyclonal antibodies against tumor necrosis factor α (TNF-α). Thus, resolution of PCP in CD4+ lymphocyte-depleted mice by heat-treated *E. coli* aerosols was not dependent on either CD8+ or asialo GM1+ cells but was dependent on Thy-1+CD4−CD8− lymphocytes and on the participation of TNF. These results indicate that heat-treated *E. coli* aerosols can act as an immune response modifier by inducing resolution of PCP in mice by a mechanism not dependent on the presence of CD4+ lymphocytes.

Certain immunocompromised individuals, such as AIDS patients, are susceptible to *Pneumocystis carinii* (PC) pneumonia (PCP), whereas immunocompetent individuals are resistant. That susceptible individuals usually have deficient CD4+ lymphocyte function suggests that these cells are important in resistance to PCP (1). Indeed, studies in animal models of PCP have confirmed that depletion of host CD4+ lymphocytes increases susceptibility to PCP (2, 3). However, the importance of possible defense mechanisms not dependent on CD4+ lymphocytes has not been established.

One study that has investigated possible CD4+ lymphocyte-independent defense mechanisms against PC is that by Pesanti (4). He found that pulmonary infection by *Pseudomonas aeruginosa* increased resistance of rats to subsequent corticosteroid-induced *Pneumocystis* infection, whereas similar pulmonary infection with *Staphylococcus aureus* did not. Because *Pseudomonas*, but not *Staphylococcus* infection, caused an accumulation of neutrophils in the lung, Pesanti concluded that neutrophil contact with dormant *Pneumocystis* delayed the onset of subsequent corticosteroid-induced *Pneumocystis* infection. Although not discussed by Pesanti, it is also possible that endotoxin associated with the cell walls of the Gram-negative *Pseudomonas* caused the enhanced resistance. Endotoxins from Gram-negative bacteria are known to be potent immune response modifiers (5), and treatment of animals with endotoxin can enhance their resistance to infectious microbes (6) and some tumors (7). It is believed that endotoxin enhances resistance through effects on both specific and nonspecific defense mechanisms (7). However, it is not known whether immune response modifiers such as endotoxin can enhance resistance to *Pneumocystis* in CD4+ lymphocyte-deficient hosts. If such inducible defense mechanisms exist, they would make attractive targets for immunotherapy against PCP in AIDS patients.

The purpose of this study was to determine whether an aerosol of heat-treated *E. coli* could enhance resistance to PCP in mice chronically depleted of CD4+ lymphocytes. An aerosol of heat-treated *E. coli* was used because it has been shown to cause pulmonary inflammation similar to that observed for aerosols of endotoxin (8). In addition, in preliminary experiments we found that mice became tolerant to multiple aerosol exposures of endotoxin but not to heat-treated *E. coli*. Results of the present investigation indicate that heat-treated *E. coli* aerosol treatment can induce resolution of PCP in mice depleted of CD4+ lymphocytes. In additional experiments, it was found that this resolution was not dependent on either CD8+ or asialo GM1+ lymphocytes, but was dependent on Thy-1+CD4−CD8− lymphocytes and TNF.
Materials and Methods

Mice. Female B6D2F1 (C57BL/6J × DBA/2J) mice used in these studies were supplied by the Trudeau Institute Animal Breeding Facility (Saranac Lake, NY). Mice were maintained under standard husbandry conditions in open-topped cages in a conventional animal room.

Aerosol Exposures of Mice to Heat-treated E. coli. Lyophilized cells of strain W (9637; American Type Culture Collection [ATCC], Rockville, MD) E. coli were purchased from Sigma Chemical Co. (St. Louis, MO). 1 g of the E. coli cells was suspended in 100 ml water and boiled for 45 min to remove the capsular antigens exposing the O antigens (8). The cells were then washed several times and resuspended in 20 ml of sterile water, aliquoted, and kept frozen at −70°C. For aerosol exposures, 1 ml of the E. coli suspension was thawed, added to 40 ml of sterile water, and placed in the reservoir of a T updraft nebulizer (Hudson, Temecula, CA). The solution was then nebulized under ~20 lb/in² air pressure and an airflow rate of ~6 liters/min. The nebulized solution then flowed into a 20 × 20 × 40-cm plexiglass chamber that contained the mice.

Antibodies and FACS® Analysis. The hybridoma GK 1.5 (Dr. Frank Fitch, University of Chicago, Chicago, IL) secreting rat IgG2b anti-CD4 mAb, hybridoma TIB-210 (ATCC) producing rat IgG2b anti-Thy-1.2, and hybridoma HB151 (ATCC) producing rat IgG2b anti-HLA-DR5 were raised as ascites and purified by ammonium sulfate precipitation. Rabbit polyclonal antibodies against TNF-α and control rabbit Ig were produced and purified as described elsewhere (9), and were the gift of Dr. Edward Havell (Trudeau Institute). Rabbit anti-asialo GM1 was purchased from Wako Chemicals (Richmond, VA).

FITC-conjugated F(ab')2 fragments of anti-Thy-1.2, anti-CD4, and anti-CD8 were prepared as described elsewhere (10) and used to stain lung lavage cells. Asialo GM1+ cells were enumerated by utilizing anti-asialo GM1 as a first antibody and F(ab')2 fragments of FITC-conjugated goat anti-rabbit Ig (Organon Teknika, Durham, NC) as a secondary antibody. Fluorescence was measured using a FACScan® cytofluorometer (Becton Dickinson & Co., Sunnyvale, CA). Cells from lung lavage fluids were collected by lung lavage and processed as previously described (11).

Depletion of CD4+ , CD8+ , Thy-1.2+ , and Asialo GM1+ Cells. B6D2 mice were made susceptible to PCP by chronic depletion of CD4+ cells as previously described (3). Briefly, 4-wk-old mice were thymectomized, rested for 2 wk, and then given 0.5 mg of GK1.5 once a week as an intraperitoneal injection. After 5 wk, the mice were inoculated intratracheally (11) with 0.1 ml of SCID mouse lung homogenate containing 5 × 10⁸ PC nuclei/ml. Some of the mice were used in experiments after 3 wk of additional GK1.5 injections. Other mice received a second inoculation of PC 2 wk after the first and were used in experiments after an additional 5 wk. All mice remained on anti-CD4 treatment the entire time of the experiments.

Some mice were treated with additional mAbs (anti-CD8 or anti-Thy-1.2; 0.5 mg) or anti-asialo GM1 serum (40 μl) by intraperitoneal injections twice weekly beginning on the first day of E. coli aerosol exposure and extending to the end of the experiment. As controls, one group of mice not exposed to E. coli aerosols and one group exposed to E. coli aerosols both received intraperitoneal injections of HB151 (anti-HLA-DR5; 0.5 mg) twice a week also beginning on the first day of aerosol exposures.

Enumeration of PC in Lungs of Mice. The number of PC nuclei in the lungs of mice was determined as described elsewhere (12), but with some modifications, as described previously (3). This method entails pushing the lungs through a stainless steel screen into HBSS, diluting the homogenate, and making a cytospin smear. The smear was stained with Diff-Quick (American Scientific Products, McGaw Park, IL) and the number of PC nuclei per 20-40 oil emersion fields determined. This number was then used to calculate total PC nuclei per lung. With this method, one nuclei counted in 40 fields equates to slightly more than 10⁴ PC nuclei per lung. Therefore, 10⁴-1 PC nuclei was considered the limit of detectability.

Statistics. The significance of differences in means of cell numbers in the lungs of groups of mice was evaluated utilizing the Student's t test (two tailed), whereas that for PC numbers was calculated utilizing the Mann-Whitney U test (two tailed).

Results

Effect of E. coli Aerosol Treatment on Numbers of PC in Lungs of Mice. B6D2 mice that received only one inoculation of PC were divided into two groups. One group was exposed to heat-treated E. coli aerosols and the other group was left as

### Table 1. Numbers of PC Nuclei in the Lungs of CD4+ Lymphocyte-depleted Mice after Treatment with Heat-killed E. coli Aerosols

| Treatment          | Duration | No. of PC nuclei | No. of mice with undetectable* no. of PC/total mice |
|--------------------|----------|------------------|--------------------------------------------------|
| Control            | d        | log₁₀ per lung   | 0/5                                              |
| 10                 | 6.37 ± 0.66† |                  | 0/5                                              |
| E. coli exposed    | 10       | 5.32 ± 0.49‡     | 0/5                                              |
| Control            | 22       | 6.73 ± 0.49      | 0/5                                              |
| E. coli exposed    | 22       | 4.37 ± 0.60†     | 4/5                                              |

* The limit of detectability for PC in the lungs was 4.1 (log₁₀).
† Numbers are means ± 1 SD; n = 5 mice.
‡ p = 0.0947 vs. 10-d control group (Mann-Whitney U test, two tails).
† p = 0.0122 vs. 22-d control group (Mann-Whitney U test, two tails).
controls. After 10 or 22 d, five mice each from control and from the exposed group were killed. Their lungs were lavaged, excised, and processed for PC counts. The numbers of PC nuclei in the lungs of these mice are shown in Table 1. After 10 d of aerosol treatment, PC numbers were reduced in the lungs of mice, but not significantly. However, after 22 d, mice treated with the E. coli aerosols had >100-fold fewer PC in their lungs than did the controls not given the aerosols (p = 0.0122). In fact, only one of five mice in the aerosol-exposed group contained detectable numbers of PC in their lungs after 22 d of aerosol treatment, whereas all mice in the other groups contained detectable numbers of PC.

The numbers of various leukocytes in lung lavage fluids of CD4⁺ lymphocyte-depleted mice after treatment with heat-killed E. coli aerosols are shown in Table 2. After 10 d, mice treated with the aerosols had significantly more macrophages, lymphocytes, and PMN in their lungs that did controls (p < 0.05). This increase in all three types of cells was still observed at 22 d of aerosol exposure (p < 0.05).

Effect of Depletion of Various Lymphocytes on Resolution of PCP. B6D2 mice that had been thymectomized, depleted of CD4⁺ lymphocytes, and inoculated with PC two times were split into five groups. These mice had greater numbers of PC in their lungs at the start of E. coli aerosol treatment than did mice in the previous experiment as a result of the additional inoculation and longer anti-CD4 treatment. As control, one group of mice did not receive aerosol treatment but was given intraperitoneal injections of anti-HLA-DR5. The other four groups all received E. coli aerosol treatments three times a week. Each of these four groups of mice also received twice weekly intraperitoneal injections of antibodies that included anti-Thy-1.2, anti-CD8, anti-asialo GM1, and anti-HLA-DR5 as control Ig. At 21 d after the start of E. coli aerosol and antibody injections, the mice were killed and the numbers of PC and different cell types in their lungs determined. The results representing one of three experiments are shown in Table 3. Again, E. coli aerosol treatment caused nearly complete resolution of PCP in CD4⁺ lymphocyte-depleted mice. Treatment of mice with either anti-CD8 or anti-asialo had no significant effect on the aerosol-induced resolution of PCP. However, treatment with anti-Thy-1.2 eliminated the ability of mice to resolve PCP in response to E. coli aerosol treatment.

The numbers of various leukocytes in lung lavage fluids of CD4⁺ lymphocyte-depleted mice after treatments with heat-killed E. coli aerosols and antibodies to various cells are shown in Table 4. The numbers of macrophages, lymphocytes, and PMN in lungs of the control mice are higher than those shown for control mice in Table 2. The values shown in Table 4 are from mice with much heavier burdens of PC, and we have consistently found that inflammatory cells accumulate in the lungs of mice as their PC burdens increase. In this regard, as shown in Table 4, E. coli aerosol treatment had little additional effect on macrophage, lymphocyte, and
Table 4. Numbers of Various Leukocytes in Lung Lavage Fluids of CD4⁺ Lymphocyte-depleted Mice after Treatments with Heat-killed E. coli Aerosols and Antibodies to Various Cells

| Treatment                       | Macrophages (x 10⁶) | Lymphocytes (x 10⁶) | PMN (x 10⁶) |
|--------------------------------|---------------------|---------------------|-------------|
| Control + control Ig           | 0.80 ± 0.11         | 0.43 ± 0.20         | 2.52 ± 0.45 |
| E. coli + control Ig           | 1.03 ± 0.59         | 1.14 ± 0.29         | 2.92 ± 1.16 |
| E. coli + α-CD8                | 0.89 ± 0.42         | 1.18 ± 0.26         | 1.92 ± 0.18 |
| E. coli + α-asialo GM1         | 1.35 ± 0.37         | 2.66 ± 1.11         | 3.39 ± 0.97 |
| E. coli + α-Thy-1.2            | 0.39 ± 0.07         | 0.34 ± 0.11         | 1.24 ± 0.54 |

* Numbers are means ± 1 SD; n = 3 mice.
† p < 0.05 vs. E. coli + control Ig (Student's t test, two-tailed).

PMN numbers. In addition, neither anti-CD8 nor anti-asialo GM1 affected the accumulation of these inflammatory cells, whereas anti-Thy-1.2 treatment did reduce macrophage, PMN, and lymphocyte accumulation, with the latter cell being significantly (p < 0.05) reduced in number.

The numbers of lymphocytes staining positively for CD4, CD8, Thy-1, or asialo GM1 present in lung lavage fluids of CD4⁺ lymphocyte-depleted mice that were treated with heat-killed E. coli aerosols and antibodies to these surface markers were determined cytofluorometrically. The results in Table 5 show that CD4⁺ lymphocytes were undetectable in all groups of mice, thus confirming the efficacy of the CD4⁺ lymphocyte depletion protocol. In addition, CD8⁺, Thy-1.2⁺ or asialo GM1⁺ cells were each depleted by respective treatments with mAbs or antibodies. It is also interesting to note that in mice depleted of CD8⁺ lymphocytes, a large number of Thy-1.2⁺ lymphocytes accumulated in their lungs and that these cells did not stain with either anti-CD4 or anti-CD8 reagents.

Effect of Anti-TNF-α Treatment on E. coli Aerosol-induced Resolution of PCP. The ability of CD4⁺ lymphocyte-depleted mice to respond to heat-treated E. coli aerosols by the production of TNF was determined. Mice were divided into three groups of four mice each, one of which received eight E. coli aerosol exposures (three times per week), another received one aerosol exposure, and the last group was not exposed to E. coli aerosols and served as control. 6 h after the last exposure, the mice were killed, and TNF activity in homogenates of their lungs was determined as previously described (13). All control mice not exposed to the E. coli aerosols contained TNF activity of <20 U/lung. Mice exposed once to E. coli aerosols contained 160 (range, 160-320) U/lung, and all mice exposed eight times contained 320 U/lung. The limit of detectability for TNF activity is 20 U/lung. Thus, TNF activity was not detected in lungs of mice not exposed to E. coli aerosols, whereas substantial activity was detected after a single aerosol exposure and this activity persisted through eight aerosol exposures.

Whether TNF plays a role in resolution of PCP induced by E. coli aerosols was investigated. Mice depleted of CD4⁺

Table 5. Numbers of Various Lymphocytes in Lung Lavage Fluids of CD4⁺ Lymphocyte-depleted Mice after Treatments with Heat-killed E. coli Aerosols and Antibodies to Various Cells

| Treatment                       | CD4⁺ (x 10⁶) | CD8⁺ (x 10⁶) | Thy-1⁺ (x 10⁶) | Asialo GM1 (x 10⁶) |
|--------------------------------|--------------|--------------|----------------|-------------------|
| Control + control Ig           | <0.01        | 1.31 ± 0.55  | 1.40 ± 0.57    | 0.91 ± 0.25       |
| E. coli + control Ig           | <0.01        | 1.34 ± 0.66  | 1.59 ± 0.73    | 0.70 ± 0.16       |
| E. coli + α-CD8                | <0.01        | <0.01        | 0.91 ± 0.19    | 0.47 ± 0.17       |
| E. coli + α-asialo GM1         | <0.01        | 1.77 ± 0.39  | 2.02 ± 0.30    | 0.05 ± 0.06       |
| E. coli + α-Thy-1.2            | <0.01        | 0.13 ± 0.06  | 0.07 ± 0.05    | 0.10 ± 0.07       |

All animals were killed 21 d after the first exposure to E. coli aerosol. Antibodies were given on the day of first exposure and every 3-4 d thereafter.

* Numbers are means ± 1 SD; n = 3 mice.
lymphocytes were divided into three groups. One group was not exposed to E. coli aerosols and served as a control. Two other groups of mice were exposed to E. coli aerosols three times a week. Mice in one of these groups were given anti-TNF IgG (10^4 NU) intraperitoneally 2 h before each aerosol exposure, and mice in the other group received an equivalent amount of control IgG before each aerosol exposure. At 19 d after the first aerosol exposure, the mice were killed and numbers of PC nuclei in their lungs determined. Results are shown in Table 6. As found in the above experiments, the E. coli aerosol treatment significantly reduced numbers of PC nuclei in the lungs of mice. However, treatment of the aerosol-exposed mice with anti-TNF IgG significantly reduced the ability of the mice to resolve PCP as judged by mean numbers of PC nuclei.

Discussion

Our results show that aerosols of heat-treated E. coli induce resolution of PCP in mice depleted of CD4+ lymphocytes. Pesanti (4) found that bacterial pneumonia resulting in acute pulmonary inflammation rendered rats more resistant to PCP. In this regard, the heat-treated E. coli aerosol used in the present investigation also induced an acute inflammatory response characterized by an accumulation of macrophages, lymphocytes, and PMN (Table 2). The ability of the E. coli aerosol to induce such an inflammatory response may be the result of the presence of endotoxins in these organisms. Indeed, aerosols of heat-treated E. coli do cause an inflammatory response in the lungs similar to that caused by aerosols of endotoxin (8). It is believed that boiling the E. coli removes capsular antigens thus exposing O antigens (8), which are associated with the lipopolysaccharides of the organism. It is also possible that the ability of the E. coli aerosol to cause resolution of PCP could reside in a component of the E. coli cell wall that is not endotoxin related. Such components have been described and some have biological activities similar to endotoxin (14). Future studies will attempt to identify the cell wall component responsible for inducing resolution of PCP.

Macrophages and neutrophils did accumulate in the lungs of mice treated with heat-treated E. coli aerosols but whether these cells play a central role in the resolution of PCP caused by the aerosol treatment is not known. We found that CD4+ lymphocyte-depleted mice with few PC in their lungs (Table 2) also had few macrophages and neutrophils in their alveoli, whereas mice with larger numbers of PC also had larger numbers of alveolar macrophages and neutrophils in their lungs (Table 4) regardless of whether they had been exposed to E. coli aerosols. These results suggest that accumulation of inflammatory phagocytes in the lungs alone is not sufficient for resolution of PCP. However, depletion of Thy-1+ cells resulted in reduction of macrophage and neutrophil accumulation in the lungs, and it is possible that this effect on phagocytic accumulation caused a decrease in resistance to PC. If indeed phagocytes are capable of killing PC as others have suggested (15, 16), it is possible that this process is dependent on soluble factors (cytokines) that could have been induced by the heat-treated E. coli aerosols.

One factor that is known to mediate phagocytic cell function is TNF. For this reason and for the reason that endotoxin is known to be a potent inducer of TNF (17, 18), we investigated the role of TNF in E. coli aerosol-induced resolution of PCP. We found that aerosol treatment did indeed cause production of TNF in lungs of mice depleted of CD4+ lymphocytes. Furthermore, treatment of mice exposed to E. coli aerosols with anti-TNF IgG ablated the ability of the mice to resolve PCP. Thus, TNF appears to play a role in the resistance to PC induced by E. coli aerosols. This is consistent with our previous finding that TNF is required in host resistance to PC induced by immunological reconstitution of SCID mice (13). It is not known how TNF enhances host resistance to PC, but that TNF is important in both CD4+ lymphocyte-dependent and independent resolution of PCP implies that this cytokine plays a pivotal role.

Results of the present investigation also indicate that resolution of PCP in CD4+ lymphocyte-depleted mice is dependent on Thy-1+ CD4+CD8- lymphocytes. The exact identity of this cell is not known, but results of the present investigation indicate that the Thy-1+ CD4+CD8- cells probably are not NK cells since depletion of asialo GM1+ cells had no effect on the ability of mice to resolve PCP. Therefore, these cells are probably either Thy-1+ CD4+CD8- α/β T cells or Thy-1+ CD4+CD8- γ/δ T cells. Dunn and North (10) described a similar cell type in mice depleted of both CD4+ and CD8+ cells and showed these cells to be important in resistance to Listeria monocytogenes. Regardless of the identity of the cell involved, results presented indicate that resistance to PCP can be induced in CD4+ lymphocyte-depleted mice and that such resistance is dependent on Thy-1+ CD4+CD8- lymphocytes. These results do not contradict previously published results that show that CD4+ lymphocytes are required for resistance to PCP in mice (2, 3). It is possible that in the present experiments E. coli aerosols

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Table 6. Number of PC Nuclei in Lungs of CD4+ Lymphocyte-depleted Mice after Exposure to Heat-killed E. coli Aerosols and Treatment with Anti-TNF-α

| Treatment | No. of PC nuclei | Log_{10} per lung | PC/total mice |
|-----------|-----------------|------------------|--------------|
| Control   | 6.41 ± 0.37 (4)*| 0/4              |              |
| E. coli + control IgG | 4.97 ± 0.94 (7) | 3/7              |
| E. coli + α-TNF IgG | 6.21 ± 1.09 (7)†| 1/75             |

All mice were killed 19 d after first exposure to E. coli aerosol. Control IgG or anti-TNF-α IgG (10^4 NU) was given intraperitoneally 2 h before each aerosol exposure.

* Numbers are means ± 1 SD (numbers of mice) in one of two experiments with similar results.

† p = 0.001 vs. control IgG group (Mann-Whitney U test, two tails).

The exact identity of this cell is not known, but results of the present investigation indicate that the Thy-1+ CD4+CD8- cells probably are not NK cells since depletion of asialo GM1+ cells had no effect on the ability of mice to resolve PCP. Therefore, these cells are probably either Thy-1+ CD4+CD8- α/β T cells or Thy-1+ CD4+CD8- γ/δ T cells. Dunn and North (10) described a similar cell type in mice depleted of both CD4+ and CD8+ cells and showed these cells to be important in resistance to Listeria monocytogenes. Regardless of the identity of the cell involved, results presented indicate that resistance to PCP can be induced in CD4+ lymphocyte-depleted mice and that such resistance is dependent on Thy-1+ CD4+CD8- lymphocytes. These results do not contradict previously published results that show that CD4+ lymphocytes are required for resistance to PCP in mice (2, 3). It is possible that in the present experiments E. coli aerosols
"replaced" some function normally accomplished by CD4+ lymphocytes. Thus, resistance to PCP in immunocompetent hosts could require both CD4+ and Thy-1+ CD4−CD8− lymphocytes. Alternatively, the mechanisms involved in Thy-1+ CD4−CD8− lymphocyte-dependent resistance to PCP might be independent of CD4+ lymphocytes. It is possible for example, that chronic depletion of CD4+ lymphocytes results in the depletion of an additional cell type that normally functions together with Thy-1+ CD4−CD8− lymphocytes in resistance to PCP. Thus, CD4+ lymphocytes and Thy-1+ CD4−CD8− lymphocytes may be parts of the same pathway that leads to resistance to PCP, or each cell may be part of separate pathways.

In conclusion, results of the present investigation indicate that heat-treated E. coli aerosols act as an immune response modifier by enhancing resistance to PCP in mice depleted of CD4+ lymphocytes. This induced resistance was dependent on the presence of Thy-1+ CD4−CD8− lymphocytes. Whether AIDS patients possess equivalent cells that could also be stimulated by immune response modifiers resulting in enhanced resistance to PCP is not known. However, if such potential resistance exists in humans, it could represent a possible target for activation by immunologic therapy.

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