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T Cell Costimulatory Molecule Function Determines Susceptibility to Infection with *Pneumocystis carinii* in Mice

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Loss of T cell number and function during HIV infection or secondary to pharmacologic immunosuppression renders individuals susceptible to opportunistic infections, including *Pneumocystis carinii* pneumonia. Because costimulatory receptors are critical for optimal T cell function, we hypothesized that these proteins would regulate susceptibility to opportunistic infections. We found that despite normal T cell numbers, mice deficient in the costimulatory molecules CD2 and CD28 spontaneously developed *P. carinii* pneumonia. In experiments using intratracheal injection of *P. carinii* organisms to induce infection, the loss of CD28 alone was sufficient to render mice susceptible to acute infection; however, the organism was eventually cleared. Examination of inflammatory responses to *P. carinii* revealed that mice deficient in both CD2 and CD28 accumulated CD8+ T cells in their lungs in response to infection and demonstrated markedly reduced specific Ab titers. Analysis of cytokine profiles suggested that regulation of IL-10 and IL-15 may be important elements of the response to this pathogen. Thus, costimulatory molecule function is critical in determining the initial susceptibility to infection with *P. carinii*. Analysis of immunologic responses in these mice may provide important insights into the defects that render individuals susceptible to opportunistic infection, and provide opportunities for novel immunologically based therapies. *The Journal of Immunology*, 2003, 171: 1969–1977.

Infection with *Pneumocystis carinii* remains a significant cause of morbidity and mortality in immunocompromised patients (1). At the onset of the AIDS epidemic, *P. carinii* pneumonia (PCP) 4 was a major cause of death in HIV-infected individuals. Today, the emergence of drug-resistant strains of HIV, the toxicity of antiretroviral agents, and economic factors limit both access to and the effectiveness of antiretroviral therapies, resulting in a large population of HIV-infected patients worldwide who are susceptible to PCP (2). In addition to HIV infection, the administration of immunosuppressive drugs, such as corticosteroids and cyclosporine, can induce susceptibility to opportunistic infection. The increasing population of patients receiving these medications following organ transplantation or for treatment of chronic inflammatory diseases has resulted in greater numbers of individuals at risk for infection with *P. carinii*.

The single most significant determinant of susceptibility to PCP in HIV-infected patients is the absolute number of circulating CD4+ T cells (1, 3). However, before the onset of severe lymphopenia, HIV-infected individuals manifest a number of immunologic defects. In vitro measurements of T cell function demonstrate abnormalities in proliferative responses to mitogen, anti-CD3 Abs, and specific recall Ags (4–8). These deficits precede CD4+ T cell depletion, and the magnitude of the defect is predictive of the development of AIDS.

Optimal T cell activation requires coordinate signaling from the TCR and costimulatory molecules (9). Several proteins have been characterized as costimulatory molecules; among the most important of these is CD28. CD28 is a member of the Ig superfamily that binds both CD80 (B7-1) and CD86 (B7-2) expressed on APCs (10). Coligation of CD28 with the TCR leads to increased T cell proliferation and survival, and enhanced effector function. In HIV infection, CD28 signaling can lead to increased viral replication (11, 12). However, CD28 ligation also results in the down-regulation of the chemokine receptor CCR5, an important coreceptor for M-trophic strains of HIV, thereby rendering these cells less susceptible to viral infection (13). The expression of CD28 has been found to be decreased on T cells from HIV-infected patients, and proliferation following activation through CD3 and CD28 is reduced compared with that on T cells from control subjects (14–17). These abnormalities of T cell function may predispose individuals to opportunistic infections.

We have been interested in studying how costimulatory signals impact host susceptibility to opportunistic infection. Prior studies of T cells from CD28-deficient mice have demonstrated decreased proliferative responses, impaired cytokine secretion, and an overall reduction in cell survival (18, 19). However, the mice are viable, with normal life spans, and have not been reported to be susceptible to opportunistic infections. We previously demonstrated that one of the reasons for the partial preservation of T cell function in CD28-deficient mice is compensation by another receptor, CD2 (20). Similar to CD28, CD2 ligation can augment T cell proliferative responses (21, 22). Mice deficient in both CD2 and CD28 have a profound impairment of T cell function in vitro despite normal numbers of T cells in the thymus and peripheral lymphoid organs.

Unexpectedly, we observed premature death due to spontaneous infection with *P. carinii* in our colony of CD2, CD28-double...
deficient mice. This observation formed the basis for studies specifically addressing the roles of these costimulatory molecules in susceptibility to PCP. We hypothesized that defects in costimulatory molecule function might be one mechanism by which hosts become susceptible to *P. carinii* despite normal numbers of CD4⁺ T cells. This investigation provides us with the opportunity to define which aspects of T cell function determine susceptibility to PCP, perhaps suggesting novel therapies to enhance immunity in susceptible individuals. Furthermore, these observations may provide a model for understanding the immunologic defects present in the prelymphoplasatic stages of HIV infection.

**Materials and Methods**

**Mice**

C57BL/6j and B6.CB17-Prkdc<sup>-/-</sup>SjL mice (B6.SCID) were purchased from The Jackson Laboratory (Bar Harbor, ME). CD2-deficient, CD28-deficient, and CD2/CD28 double-deficient mice have been previously described (24). In brief, lungs were harvested from infected B6.SCID mice. Those with evidence of *P. carinii* cysts and foamy exudate present throughout alveoli in most regions. Histologic scoring for intensity of inflammation present in histologic sections stained with the Gomori methenamine silver stain. This scale ranges from 0 (no inflammation) to 5⁺ (severe perivascular and peribronchial inflammation with effacement of alveolar parenchyma and small airways by sheets of inflammatory cells). All slides were graded in a blinded fashion.

**Histologic scoring**

To determine the intensity of *P. carinii* infection, we performed histologic grading of lung sections using a method previously described and validated (25). Lungs were inflated with formalin, fixed overnight, paraffin-embedded, and sectioned. Scoring for intensity of infection was performed on sections stained with the Gomori methenamine silver stain. This scale ranges from 0 (no *P. carinii* organisms detected in any field) to 4⁺ (4⁺ *P. carinii* cysts and foamy exudate present throughout alveoli in most regions). Scoring for intensity of inflammation was performed on sections stained with H&E. This scale ranges from 0 (no inflammation) to 5⁺ (severe perivascular and peribronchial inflammation with effacement of alveolar parenchyma and small airways by sheets of inflammatory cells). All slides were graded in a blinded fashion.

**Preparation and injection of *P. carinii* inoculum**

As *P. carinii* cannot be maintained in long-term culture, organisms were serially passaged in the lungs of B6.SCID mice. Fresh inocula of *P. carinii* were prepared for each experiment as previously described (24). In brief, lungs were harvested from infected B6.SCID mice. Those with evidence of bacterial pneumonia as determined by examination of a Gram-stained touch preparation were excluded. The lungs were frozen for 2 h at −20 °C, homogenized in sterile PBS, and centrifuged at 500 × g for 10 min. The pellet was resuspended in PBS and filtered through nylon mesh, and the number of organisms was determined by counting a representative aliquot stained with Diff-Quick (Baxter Scientific Products, Miami, FL). The concentration of organisms was adjusted to 2 × 10<sup>6</sup>/mL. Pentobarbital anesthesia, 100 μL of the *P. carinii* preparation was injected directly into the trachea following tracheal ligation, followed by injection of air to clear the central airways. To assure the virulence of the inoculum, B6.SCID mice were housed under specific pathogen-free conditions. Sentinel mice were routinely examined to exclude the presence of unintended pathogens. All animal experimentation was approved by the Washington University animal studies committee.

**Immunohistochemistry and flow cytometry**

All Abs were purchased from BD PharMingen (San Diego, CA). Cells isolated from bronchoalveolar lavage (BAL) and spleen were stained with FITC- or PE-conjugated mAb and analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences, Mountain View, CA). For immunohistochemistry, the lungs were inflated with 50% OCT (Tissue-Tek, Torrance, CA) in PBS and frozen on dry ice. Ten-micrometer sections were cut, fixed in acetone, and stained with control or specific Abs. Detection was performed with the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) using HRP-conjugated avidin and developed with diaminobenzidine substrate. Images were captured using a BX51 microscope coupled to a Magnafire digital camera and software (Olympus, Melville, NY).

**Measurement of *P. carinii*-specific Ig**

*P. carinii* Ag was prepared by sonicating freshly isolated *P. carinii* in PBS and centrifuging at 15,000 × g for 30 min. Immulon 2 microtiter plates (Dynatech, Chantilly, VA) were coated with a 1/10 dilution of *P. carinii* Ag in coating buffer (0.1 M NaHCO₃, pH 8.2) at 4°C overnight. The following day the wells were washed with PBS/0.5% Tween 20 and blocked with PBS/0.5% Tween 20 containing 2% BSA for 1 h at room temperature. Serial dilutions of serum were added to triplicate wells and incubated at room temperature for 2 h. The wells were then washed extensively and incubated with alkaline phosphatase-conjugated goat anti-mouse Ig specific for total IgG, IgG1, IgG2a, or IgM for 1 h. Detection was performed by incubation with p-nitrophenyl phosphate, and OD was determined at 405 nm using a Spectramax plate reader and SoftMax Pro software (Molecular Dynamics, Sunnyvale CA).

**RNase protection assay (RPA)**

Total RNA was isolated from lung tissue of mice using TRIzol reagent. Cytokine mRNA was determined by RPA using the RiboQuant multiprobe kit with the mCK-1 probe set (BD PharMingen) according to the manufacturer’s protocol. The following cytokines were analyzed: IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, and IFN-γ.

**Statistics**

Statistical analysis for comparison of intensity of inflammation and organism burden was performed using the Kruskal-Wallis test using InStat3 software (GraphPad, San Diego, CA). Comparison of the numbers of cells recovered in the BAL and Ig titers was performed with a two-tailed independent *t* test using Excel software (Microsoft, Seattle, WA). Statistical significance was accepted for *p* < 0.05.

**Results**

**Deficiency of CD28 confers susceptibility to *P. carinii* pneumonia**

Autopsy of several mice that died prematurely in our colony of CD2/CD28 double-deficient mice revealed the cause of death to be spontaneous infection with *P. carinii*. Although we had not observed similar mortality in mice deficient in CD2 or CD28, it was possible that infection occurred, but went clinically undetected.

To test whether the singly deficient mice were susceptible to PCP, we directly inoculated mice of each genotype with *P. carinii*. Consistent with previous work, wild-type mice clear *P. carinii* inoculum of this virulence by 4 wk after intratracheal inoculation, with no residual organisms or inflammation present in histologic sections. Periodic inclusion of wild-type controls confirmed no evidence of infection histologically or as determined by real-time PCR analysis of organism burden (data not shown and Fig. 1D) (24). Examination of histologic sections obtained from mice deficient in only CD2 or CD28 revealed that only those deficient in CD28 developed PCP 4 wk after inoculation (Fig. 1A). In CD2-deficient mice a brisk peribronchial and peribronchial inflammatory process was evident as well as the classic foamy alveolar exudates and interstitial alveolar thickening. A similar pattern was observed in the CD2/CD28 double-deficient mice, although their tendency to be more extensive alveolar involvement. Interestingly, the CD2-deficient mice also developed prominent inflammatory changes; however, in this strain the inflammation had the appearance of...
lymphoid nodules, and there was no alveolar exudate seen. Inflammation scores obtained by blinded grading of the specimens confirmed that lungs from the CD2/CD28 double-deficient mice had significantly greater inflammation than lungs from the CD2-deficient mice (Fig. 1B). Silver staining demonstrated the presence of P. carinii cysts in both strains of mice as well as in SCID mice (Fig. 1A). No organisms were observed in Gomori Methenamine Silver (GMS)-stained specimens obtained from CD2-deficient mice. Grading of multiple specimens confirmed that P. carinii was only detected in the SCID, CD28-deficient, and CD2/CD28 double-deficient mice (Fig. 1C). Quantification of organism burden by real-time PCR, performed on lungs from a randomly selected subset of mice in each experimental group, confirmed infection in the CD28-deficient and CD2/CD28 double-deficient mice, but not in wild-type or CD2-deficient mice (Fig. 1D). The organism burden at this time point was higher in the samples from CD28-deficient mice than in those from the CD2/CD28 double-deficient mice (p<0.05); however, the biologic significance of this magnitude of difference remains to be determined.

Costimulation-deficient mice recruit increased numbers of CD8+ T cells to the alveolar space

We examined the cellular constituents of the inflammatory infiltrate in costimulation-deficient mice inoculated with P. carinii. Total cell recovery in the BAL was not significantly different among the strains examined (Fig. 2A). All except SCID mice recruited a mixed population of cells, including macrophages, neutrophils, and lymphocytes (Fig. 2B). The overall recruitment of lymphocytes was significantly greater in the CD2/CD28 double-deficient mice than in either the CD2-/- or CD28-/- mice (p<0.01). Analysis of the lymphocyte subsets present in the lung revealed primarily CD4+ T cells in the CD2-deficient mice (Fig. 2C). Interestingly, accumulation of CD8+ T cells was greater in the CD28-deficient and CD2/CD28 double-deficient mice compared with the CD2-deficient mice (Fig. 2D). Comparison of the ratio of CD4+ to CD8+ T cells recovered in the BAL revealed an inversion of the ratio, with the CD28-/- and CD2/CD28-deficient mice having a ratio of <1.0 in the BAL, but a normal ratio of >1 in the spleen (Fig. 2E). In the absence of costimulation, T cell activation might be sufficiently impaired to functionally render the mice as if they were CD4 cell depleted. To assess this possibility, we measured the expression of the activation marker CD25 on CD4 cells recovered in the BAL. CD25 expression on cells isolated from all genotypes was low (5.13 ± 1.25% for CD2-/-, 4.77 ± 1.69% for CD28-/-, and 1.49 ± 1.06% for CD2/CD28-/-). The low expression of CD25 may represent impaired T cell activation or indicate that the cells have returned to a quiescent state.

To complement the flow cytometric analysis of the BAL we examined the cell types present in the lungs of infected mice by immunohistochemistry (Fig. 3). Macrophages and neutrophils...
were evident in the tissue of all mice examined. The nodular lymphoid aggregates seen in the CD2-deficient mice consisted primarily of T and B cells. Both CD4^+ and CD8^+ T cells were evident in the lungs of the CD28-deficient and CD2/CD28 double-deficient mice, but few CD8^+ T cells were evident in specimens from CD2-deficient mice. The difference in CD4^+ and CD8^+ cell recruitment to the lung was less apparent on immunohistochemical staining than in the BAL. However, these techniques are complementary and examine different tissue compartments. This may, in fact, represent real differences in the ability of the cells to traffic from the tissue to the alveoli. All mice, with the exception of SCID mice, had significant accumulations of B cells as determined by B220 expression. Staining for macrophages and neutrophils with anti-CD11b and anti-Gr1 Abs, respectively, demonstrated the presence of these cell types in the lungs of all strains. Thus, there were no obvious defects in the recruitment of inflammatory cells to the lung in susceptible mice, implying that alterations in the function of these cells must account for susceptibility to PCP.

Costimulation-deficient mice demonstrate decreased specific Ab production

As CD28-deficient mice have been shown to have impaired Ab responses when immunized with protein Ags (27), we assayed the relative amounts of *P. carinii*-specific Ig present in the serum of mice 4 wk after inoculation. Mice deficient in only CD2 had significantly higher levels of *P. carinii*-specific IgG than the susceptible strains. As expected, no *P. carinii*-specific Ig was detected in mice of any genotype that had not been inoculated with *P. carinii*.
Interestingly, CD28-deficient mice had slightly reduced levels of specific IgG than CD2-deficient mice, but this difference was not statistically significant \((p < 0.08)\). However, the combined loss of CD2 and CD28 led to a greater reduction in Ab titer that was highly significant \((p < 0.01)\). Determination of the specific IgG isotypes revealed a marked defect in IgG1 production by both CD28-deficient and CD2/28 double-deficient mice, but a relative preservation of IgG2a production in the CD28-deficient strain. Thus, despite the recruitment of B cells to the lung, there is impaired development of humoral immunity that may contribute to the susceptibility to infection with \(P. carinii\).

**Analysis of cytokine gene expression in the lungs of \(P. carinii\)-infected mice**

To determine whether there were specific differences in the profile of cytokines expressed in the lungs of resistant compared with susceptible mice, we isolated total RNA from the lungs of mice infected with \(P. carinii\). Cytokine gene expression was analyzed by RPA using a probe set that hybridizes to multiple cytokine genes (Fig. 5A). Quantification was performed using a densitometer, and the ratio of each gene to GAPDH is presented in Fig. 5B. Only IL-15 was detected in samples obtained from inoculated wild-type mice 4 wk after inoculation, after organisms had been cleared. Mice lacking CD28 displayed increases in IL-10, IFN-\(\gamma\), and IL-6 expression. The cytokine profile of CD2/CD28 double-deficient mice was distinct from the profile observed in samples from CD28-deficient mice and more closely resembled that obtained from CD2-deficient mice. Other cytokines examined, including IL-4, IL-5, IL-9, and IL-2, did not show detectable signals in lungs from any group of mice (data not shown). The expression of TNF-\(\alpha\) mRNA was also assayed by RT-PCR analysis. All samples demonstrated similar levels of TNF-\(\alpha\) mRNA (data not shown). We attempted to confirm the data obtained by RPA by directly assaying IL-10 and IFN-\(\gamma\) protein levels in the BAL by ELISA. However, all samples were below the limit of detection, perhaps due to sample dilution during the BAL.

**Long term infection of costimulation-deficient mice**

To determine the long term outcome of PCP in the costimulation-deficient mice, we inoculated mice of each genotype and followed them longitudinally for evidence of disease. Although both CD28-deficient and CD2/CD28 double-deficient mice reproducibly developed PCP 4 wk following direct intratracheal inoculation, the
mice did not appear clinically ill. This observation suggested that the mice tolerated the infection without significant morbidity or that the mice were eventually able to clear the infection. Histologic examination of lungs at 14 wk postinoculation revealed no significant histologic evidence of disease, although there was a slight increase in CD4+ T cells at this time point, although there was a slight increase in CD4+ T cell number recovered from the CD2/CD28 double-deficient mice (Fig. 6B). Differential analysis demonstrated a decrease in the number of lymphocytes and neutrophils recovered in the BAL from the susceptible genotypes compared with wild-type mice, although this did not reach statistical significance (Fig. 6C). In contrast to the results obtained at 4 wk, neither CD28-deficient nor CD2/CD28 double-deficient mice had increased numbers of CD8+ T cells at this time point, although there was a slight increase in CD8+ T cell number recovered from the CD2/CD28 double-deficient mice (Fig. 6B).

Analysis of cytokine gene expression by RPA demonstrated minimal expression of any cytokines, and there was no statistical difference in expression between samples (Fig. 6D). IL-10 and IFN-γ protein levels were not detectable in BAL by ELISA (data not shown). Serum Ab responses demonstrated similar levels of P. carinii-specific IgM in all genotypes, with the exception of the SCID mice. While total P. carinii-specific IgG was higher in the CD28-deficient mice at the time point, by 4 wk it was decreased, making the early elevation of uncertain clinical significance. Thus, little difference was detected in the response of resistant vs susceptible mice at an early time point in the course of infection.

**Discussion**

Impaired T cell function has been recognized as a risk factor for susceptibility to infectious diseases in individuals treated with immunosuppressive drugs or as a result of diseases that lead to the depletion of T cells. While the functions of costimulatory molecules are well described in regulating T cell activation, specific defects in these pathways have not been previously identified as leading to susceptibility to opportunistic infections. We have demonstrated that the loss of T cell costimulatory molecule function is one mechanism by which mice become susceptible to acute infection with P. carinii. Mice deficient in both CD2 and CD28 were initially found to be susceptible to environmental acquisition of the disease, while mice deficient in only CD2 or CD28 did not acquire the infection spontaneously. Following direct inoculation with P. carinii both CD28-deficient and CD2/CD28-double deficient mice were included as controls. Organism burden, as determined by real-time PCR analysis, was similar in all groups at this time point (Fig. 6A). No animals, including the SCID mice, had histologic evidence of disease at this time point, although SCID mice inoculated simultaneously did progress to develop PCP when examined at 4 wk (data not shown). Total BAL cell recoveries were not significantly different between groups (Fig. 6B). Differential analysis demonstrated an increase in the number of lymphocytes and neutrophils recovered in the BAL from the susceptible genotypes compared with wild-type mice, although this did not reach statistical significance (Fig. 6C). In contrast to the results obtained at 4 wk, neither CD28-deficient nor CD2/CD28 double-deficient mice had increased numbers of CD8+ T cells at this time point, although there was a slight increase in CD8+ T cell number recovered from the CD2/CD28 double-deficient mice (Fig. 6B). Analysis of cytokine gene expression by RPA demonstrated minimal expression of any cytokines, and there was no statistical difference in expression between samples (Fig. 6D). IL-10 and IFN-γ protein levels were not detectable in BAL by ELISA (data not shown). Serum Ab responses demonstrated similar levels of P. carinii-specific IgM in all genotypes, with the exception of the SCID mice. While total P. carinii-specific IgG was higher in the CD28-deficient mice at this time point, by 4 wk it was decreased, making the early elevation of uncertain clinical significance. Thus, little difference was detected in the response of resistant vs susceptible mice at an early time point in the course of infection.
developed PCP when examined at 4 wk. However, both CD28-deficient and CD2/CD28 double deficient mice retain sufficient immune function to clear the infection, albeit with delayed kinetics. Thus, deficiency of costimulation mediated by CD28 confers susceptibility to acute infection with P. carinii, despite the presence of normal numbers of CD4+ T cells.

P. carinii pneumonia is a fungal infection that occurs virtually exclusively in immunocompromised hosts. Since its initial description as plasma cell interstitial pneumonia in malnourished children, it has heralded in the post-transplant era of pharmacologic immunosuppression and, most recently, the AIDS epidemic (28). In HIV-infected patients, a reduction in the number of CD4+ T cells below 200 cells/μl identifies individuals at increased risk for pneumonia (3, 29). However, even before the development of lymphopenia, HIV-infected patients are at increased risk of infection with a variety of pathogens. This increased susceptibility might be caused by specific defects in CD4+ T cell function that occur before cellular depletion (8).

CD4+ T cells influence the outcome of immune responses both directly and by regulating the effector responses of other cell types. The secretion of cytokines such as TNF-α exert direct cytotoxic effects as well as activating phagocytes, while other cytokines promote the development of humoral responses. The host response to P. carinii is particularly complex, involving granulocytes, mononuclear phagocytes, as well as T cell and Ab responses (30). Experimental and observational data have confirmed the critical role of the CD4+ T cell in the response to P. carinii; however, the elements of CD4+ T cell function that render hosts immune or susceptible to pneumonia have not been clearly defined (31–33).

Although we initially observed spontaneous, environmentally acquired infection as the cause of death in the CD2/CD28 double-deficient mice, that finding was not reproduced in formal experiments testing the outcome of established P. carinii infection. The initial observations were made during backbreeding of mice in a mixed genetic background to the C57BL/6J background; thus, it remains a possibility that genetic background accounts for these differences. Additionally, there may be differences in the virulence of the organism that initially infected the mice compared with that used for intratracheal inoculation. Alternatively, these mice may have succumbed due to a coexistent infection or disease that was not detected upon postmortem examination.

After intratracheal inoculation with P. carinii, mice from all strains developed mixed inflammatory responses to the organism. Despite similar infectious burden, the CD2/CD28 double-deficient mice had an overall increase in lymphocyte recruitment to the lung. The observed difference in lymphocyte recruitment at 4 wk may, in fact, reflect kinetic differences in the host response to infection. Mice of the susceptible genotypes had increases in the numbers of CD8+ T cells recovered from their lungs and inversions of the CD4/CD8 cell ratio in the BAL fluids. A similar accumulation of CD8+ T cells has been reported in the BAL of CD4-depleted mice infected with P. carinii (24, 31). Selective depletion of CD8+ T cells alone has not been shown to render mice susceptible to infection (31, 34). However, mice simultaneously depleted of CD4 and CD8 subsets develop more intense infections with P. carinii than mice depleted of CD4+ T cells alone, indicating that CD8+ T cells can contribute to defense (34). Our observation of increased numbers of CD8+ T cells in the lungs of CD2/CD28 double-deficient mice confirms that these cells alone are ineffective at bestowing immunity acutely. However, the fact that the mice cleared the infection at later times suggests that the CD8 cell response may aid in a delayed response to the organism.

Ab responses are a prominent part of the host response to P. carinii. Mice deficient in B cells are susceptible to infection, and passive transfer of specific Ig can aid in clearance of the infection (35–38). CD28 is important not only in direct activation of the T cell, but also in the cellular communication between T and B cells. Mice deficient in CD28 have been shown to have decreased Ig secretion, impaired germinal center formation, and defective somatic hypermutation in response to protein immunization (27). Surprisingly, the CD28-deficient mice mounted significant IgG responses to P. carinii infection, perhaps due to the longer time of

**FIGURE 6.** Analysis of mice 2 wk after inoculation. Mice (n = 3–5 for each genotype) were inoculated with P. carinii intratracheally, and 2 wk later samples were collected for analysis. A, Real-time PCR analysis demonstrated similar levels of P. carinii rRNA between genotypes. B, Cell counts and flow cytometric analysis of BAL fluid. C, Manual differential analysis of cell recovered in the BAL. D, Cytokine mRNA levels were determined by RPA using the same probe set as that described for Fig. 5. E, P. carinii-specific IgG and IgM in the serum.
exposure to the organism compared with Ab responses studied in response to acute immunization. However, there was a significant impairment in the production of P. carinii-specific IgG1 by CD28-deficient mice, with a relative preservation of IgG2a. The presence of specific IgG in CD28-deficient mice may have contributed to the eventual resolution of the infection; however given the important role of CD28 in providing B cell help, it is possible that the Ab is of lower affinity or is otherwise ineffective at promoting clearance of the pathogen in vivo. Mice deficient in both CD2 and CD28 had markedly decreased titers of all IgG isotypes. These observations confirm the cooperative nature of CD2 and CD28 in promoting T-dependent B cell responses.

Disruption of the CD40/CD154 pathway has been shown to render mice susceptible to P. carinii infection (39). CD40 was initially described as being expressed on B cells, but has been identified on many cell types. Ligation of CD40 on B cells promotes their activation, survival, and Ab production (40). This pathway is intricately linked with the CD28/B7 pathway of costimulation. Ag and CD28 stimulation of T cells up-regulates the expression of CD154. Conversely, engagement of CD40 leads to increased expression of CD80 and CD86 (41, 42). Our finding of susceptibility in CD28-deficient mice suggests the possibility of a common mechanism.

Analysis of cytokine gene expression did not reveal a specific profile unique to the susceptible strains of mice. All samples had detectable expression of IL-15. This cytokine has been shown to be important in NK cell function and is induced in response to infection with another fungus, Cryptococcus neoformans (43–45). Of note, we did observe an induction of IL-10 and IL-6 in the CD28-deficient mice. IL-10 functions to limit systemic inflammation by inhibiting the production of TNF-α and IL-12 as well as the expression of class II MHC and costimulatory molecules by phagocytes and APC (46). IL-10 has been shown to impair the host response to other infectious agents, including Leishmania major and Mycobacterium tuberculosis (47–49). Thus, the expression of IL-10 following infection of the CD28-deficient mice might result in an environment that is permissive for the survival of the organism. Recent data demonstrated that IL-10 does, in fact, alter the kinetics of clearance of P. carinii, as IL-10-deficient mice had enhanced resolution of the infection (50). Previous work has highlighted the role of TNF-α in the response to P. carinii (51, 52). Even though mRNA levels of TNF-α did not appear to be decreased in CD28-deficient mice, it is possible that the expression of IL-10 in CD28-deficient mice might inhibit the secretion of TNF-α, thereby impairing the response to the organism; however, this remains to be formally tested.

The CD2/CD8 double-deficient mice expressed less IL-10 than the CD28-deficient mice. This raises the possibility that the mechanism of susceptibility of these two strains differ. One model is that the T cells from the CD28-deficient mice become activated, but that the cytokine milieu is inhibitory, whereas in the absence of both CD2 and CD28, the susceptibility is based on a failure to even activate the CD2/CD8 double-deficient T cells. A more detailed kinetic analysis of T cell function during infection may clarify this point. Our examination of mice at an earlier time point in infection did not reveal any specific differences between susceptible and resistant strains of mice. However, at 4 wk, the susceptible genotypes had significant decreases in serum-specific Ab as well as increased numbers of CD8+ T cells. Thus, it may be that the failure to mount an Ab response coupled with the changes in T cell recruitment and cytokine milieu render CD28-deficient and CD2/CD8 double-deficient mice susceptible to the development of pneumonia.

The unexpected observation of PCP in mice deficient in CD2 and CD28 has provided us with important new insights into the host factors that determine susceptibility to P. carinii infection. Despite normal peripheral T cell numbers, mice deficient in T cell costimulation are susceptible to acute infection with PCP. Sufficient immunity is retained to mediate a delayed clearance of the infection, providing us with a novel model to examine the elements of immune function that lead to resolution of PCP. This demonstrates that cellular functions regulated by CD28 are critical in bestowing resistance to acute P. carinii infection and suggests that examination of this aspect of T cell function in immunosuppressed people might reveal similar defects. Development of strategies directed at specifically augmenting this aspect of T cell function may be beneficial in the development of immunologically based therapies to treat this important human disease.

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