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Delayed Inflammatory Response to *Pneumocystis carinii* Infection in Neonatal Mice Is Due to an Inadequate Lung Environment[1]

Beth A. Garvy[2] and Mahboob H. Qureshi

Challenge of neonatal mice with an intranasal inoculation of *Pneumocystis carinii* results in a subclinical infection that takes 6 wk to resolve, whereas adult mice resolve a comparable challenge within 3 wk. This delayed clearance is due to a delayed inflammatory response in neonatal mice; however, the reason for this delay has been unknown. To determine whether the neonatal lung environment is sufficient to attract immunocompetent lymphocytes into the lungs, an adoptive transfer strategy was employed in which splenocytes from adult BALB/c mice were transferred into *P. carinii*-infected neonatal or adult SCID mice. All adults, but no pups, resolved their infections by day 37 postreconstitution. Despite reconstitution with adult splenocytes, pups had a negligible lung inflammatory response until day 24, whereas adult mice had activated $CD4^+$ and $CD8^+$ cells in the lung by day 13. The delay in neonates corresponded to delayed kinetics of expression of lung cytokines TNF-α and IFN-γ mRNA and chemokines lymphotactin, RANTES, and macrophage inflammatory protein-1β mRNA. Phagocytic cells from neonatal mice were significantly less efficient than adult cells at migrating to the draining lymph nodes after phagocytosing fluorescent beads. There were fewer dendritic cells and Ia^+ myeloid cells in the lungs of *P. carinii*-infected neonatal mice compared with adults. These data indicate that the lung environment of neonatal mice is insufficient for migration of T cells, due at least in part to inefficient phagocytosis and migration of APCs to the lymph nodes as well as delayed chemokine and TNF-α mRNA expression. ***The Journal of Immunology,*** **2000, 165: 6480–6486.**

Pneumocystis carinii is an opportunistic, fungal pathogen that is a significant problem in persons with immunodeficiency diseases such as AIDS. Normal, healthy children generally develop Abs specific for *P. carinii* by 2 years of age (1), indicating an early exposure to the organism. It is rare to find clinical cases of *P. carinii* pneumonia (PCP)[3] in children with healthy, intact immune systems. Children <1 year of age who have AIDS have a higher incidence and a more fulminate course of PCP than older children with AIDS (2).

Normal, healthy neonatal rabbits and mice can harbor subclinical *P. carinii* infections until the age of weaning, when an inflammatory response takes place and clears the organisms (3, 4). Additionally, Vargas and colleagues recently reported a high incidence of *P. carinii* infection in autopsy lung specimens obtained from infants who had died of sudden infant death syndrome, confirming that human infants also harbor subclinical *P. carinii* infections (5). We have reported that there is a delay in the inflammatory response in the lungs of mice infected with *P. carinii* as neonates compared with that of infected adult mice (3). Adult mice challenged with an intranasal (i.n.) inoculation of *P. carinii* develop a T cell response within 7 days and resolve the infection within 3 wk. In contrast, neonatal mice challenged with a comparable dose of *P. carinii* do not have detectable T cells in the alveolar spaces until ~3 wk of age and do not resolve the infection until 6 wk (3). This delayed inflammatory response in neonates could be due either to inadequate number or function of specific $CD4^+$ T cells that are required for resolution of the infection, or, alternatively, the lung environment may not be conducive to processing and presenting Ag or attracting effector lymphocytes.

It has been known for some time that resolution of PCP is dependent on the presence of competent $CD4^+$ T cells (6, 7). More recently, it has been determined that B cells are also critical for host defense against primary *P. carinii* infection (8, 9). The function of neonatal lymphocytes has generally been considered to be immature. However, more recent studies have suggested that there is no intrinsic defect in neonatal T cells, but the dose and mode of Ag challenge can affect the data obtained (10–12). Furthermore, stimulating neonatal T cells with costimulatory molecules or adult dendritic cells results in responses similar to those of adults (13, 14). These data suggest that the environment surrounding neonatal T cells is critical to their functional capacity. In this regard it has been shown that infant rats do not express adult level staining patterns for MHC class II-positive airway epithelial and alveolar dendritic cells until the age of weaning (15, 16). This is consistent with the timing of the inflammatory response to *P. carinii* seen in mice infected as neonates, suggesting that the lung environment may not be conducive to mounting an inflammatory response to *P. carinii* until the age of weaning (3).

We have used an adoptive transfer strategy to distinguish between the effects of the lung environment and the function of lymphocytes in the host response to *P. carinii* in neonatal mice. Neonatal or adult SCID mice were given *P. carinii* infections and 5 days later were reconstituted with splenocytes from adult BALB/c donors. We then examined the *P. carinii* lung burden, inflammatory response, and lung cytokine and chemokine mRNA expression and

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[3] Abbreviations used in this paper: PCP, *Pneumocystis carinii* pneumonia; i.n., intranasal; RPA, RNase protection assay; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; TBLN, tracheobronchial lymph nodes.

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found that the delay in inflammatory response and clearance of *P. carinii* in mice is due to deficiencies in the lung environment and can be attributed to, at least in part, delayed TNF-α and chemokine production. This may be due to ineffective phagocytosis of *P. carinii* in the lungs and Ag presentation in the draining lymph nodes.

Materials and Methods

Mice

Pregnant BALB/c or BALB/c SCID mice in midgestation and adult female BALB/c or BALB/c SCID mice (8 wk old) were purchased from the National Cancer Institute (Bethesda, MD). Donor 6- to 8-wk-old female BALB/c mice were purchased from the National Cancer Institute or from Harlan Sprague Dawley (Indianapolis, IN). *P. carinii* were obtained from Dr. Allen Harmsen at Trudeau Institute (Saranac Lake, NY) and maintained in C.B17 SCID mice originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in the Lexington Veterans Affairs Medical Center veterinary medical unit.

Inoculation of P. carinii

Lungs were excised from *P. carinii*-infected mice and pushed through stainless steel mesh in HBSS. Cell debris was removed by centrifugation at $100 \times g$ for 2 min. Aliquots of lung homogenates were spun onto glass slides, fixed in methanol, and stained with Diff-Quik (Dade International, Miami, FL). *P. carinii* nuclei were enumerated by microscopy as previously described (3). Mice were anesthetized lightly with halothane gas to inhibit the diver's reflex. The *P. carinii* inoculum was placed over both nares. The entire volume of 10 μl for neonates and 50 μl for adults was aspirated because mice are obligate nose breathers. Neonatal mice (<72 h old) were routinely inoculated with 2×10^6 *P. carinii* organisms, and adults (8 wk old) were injected with 10^7 organisms.

Adoptive transfer of immunocompetent splenocytes

Spleens were obtained from adult (8-wk-old) female BALB/c mice and pushed through mesh screens in HBSS to form single cell suspensions. Erythrocytes were removed using a hypotonic lysing buffer, and cells were washed and resuspended at 10^7/ml in PBS. Four days after infection with *P. carinii*, adult and neonatal mice were given i.p. injections of 5×10^7 and 10^7 cells, respectively.

Isolation of cells from alveolar spaces, lungs, and lymph nodes

Mice were killed by exsanguination under deep halothane anesthesia. The lungs were lavaged with HBSS containing 3 mM EDTA. Right lung lobes were excised, minced, and digested in RPMI supplemented with 3% FCS, 1 mg/ml collagenase A, and 50 U/ml DNase for 1 h at 37°C. Digested lung fragments were pushed through mesh screens, and aliquots were removed for enumeration of *P. carinii*. Erythrocytes were removed from digests using a hypotonic lysing buffer, cells were washed, and single-cell suspensions were enumerated. Tracheobronchial lymph nodes (TBLN) were pushed through mesh screens in HBSS and enumerated.

Enumeration of P. carinii *in the lungs of neonatal and adult mice*

Aliquots of lung digests were diluted, and 100-μl aliquots were spun onto glass slides, fixed in methanol, and stained with Diff-Quik (Dade International). *P. carinii* nuclei were enumerated microscopically as previously described (3). Lung burden is expressed as $\log_{10}$ *P. carinii* nuclei per right lung lobes. The limit of detection ranged from 2.93–3.23 in pups and was 3.23 in adults.

Flow cytometric analysis of lung and lymph node lymphocytes

Lung lavage, lung digest, and TBLN cells were washed in PBS with 0.1% BSA and 0.02% NaN_3 (PBA) and stained with the appropriate concentrations of fluorochrome-conjugated Abs specific for murine I^d, CD4, CD8, CD11c, CD44, CD62 ligand, CD19, CD80, and CD86. Abs were purchased from PharMingen (San Diego, CA). Expression of these molecules on the surface of lymphocytes was determined by multiparameter flow cytometry using a FACScalibur cytofluorometer (Becton Dickinson, Mountain View, CA).

For intracellular cytokine staining, lung digest cells were stimulated for 4 h with 50 ng/ml PMA and 1 μg/ml ionomycin at 37°C under an atmosphere of 5% CO_2. Brefeldin A (10 μg/ml) was added for the final 2 h of incubation to inhibit secretion of cytokines. Cells were surface stained with anti-CD4 and anti-CD8 as described above, fixed in 5% formalin, and permeabilized with PBA containing 0.5% saponin. Nonspecific binding sites were blocked with anti-CD16/CD32 (Fc block, PharMingen). For detection of cytokines, cells were incubated with fluorochrome-conjugated Abs specific for IFN-γ and IL-10. Quantitation of the number of $CD4^+$ or $CD8^+$ cells expressing IFN-γ or IL-10 was performed using a FACScalibur cytofluorometer.

Migration of fluorescent beads from the lungs to the TBLN

Neonatal and adult mice were given i.n. inoculations of 1.2×10^7 1-μm fluorescent (NYOcarboxylate, Polysciences, Warrington, PA) microspheres/g of body weight. Two days later lungs were lavaged with HBSS/EDTA, and TBLN were excised and pushed through mesh screens in HBSS. Cells associated with fluorescent microspheres were enumerated using a Nikon Eclipse microscope (Nikon, Melville, NY) with a mercury arc lamp. Using this method it was not possible to determine definitively that the particles were phagocytosed and not bound to the surface of the cells. However, experiments performed by Harmsen et al. (17) in which beads were placed into different lung lobes of dogs indicate that it is unlikely that unphagocytosed beads migrated to the draining lymph nodes.

RNase protection assays (RPAs)

Total RNA was prepared from frozen lung tissue using TRIzol (Life Technologies, Gaithersburg, MD) according to manufacturer's instructions. Briefly, 50–100 mg of lung tissue was homogenized in TRIzol reagents followed by chloroform extraction and ethanol precipitation. Air-dried RNA pellets were dissolved in diethylpyrocarbonate-treated water. Multiprobe DNA templates for cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-9, IL-13, and IL-15) and chemokines (lymphotactin, RANTES, eotaxin, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, monocyte chemoattractant protein (MCP)-1, and TCA-3) were purchased from PharMingen along with the RiboQuant in vitro transcription kit and RiboQuant RPA kit. RPA was performed according to manufacturer's instructions. DNA templates were used to synthesize antisense riboprobes labeled with [α-^{32}P]UTP (Amersham Pharmacia Biotech, Piscataway, NJ) using T7 RNA polymerase. Labeled probes were hybridized with 10 μg of total RNA and samples were digested with RNase A and T1 and treated with proteinase K. Labeled, protected RNA was extracted with phenol/chloroform/isoamyl alcohol and resolved on 5% denaturing polyacrylamide gels. Dried gels were exposed to storage PhosphorImager screens, and images were developed using a Storm 860 imaging system (Molecular Dynamics, Sunnyvale, CA). The intensity of each specific band was measured using ImageQuant software (Molecular Dynamics) and was standardized for mRNA levels by dividing by the intensity signal of the housekeeping gene L32 for each sample.

Statistical analysis

Differences between experimental groups were determined using Student's *t* tests or ANOVA, followed by Student-Newman-Keuls post-hoc test where appropriate. Differences were considered statistically significant when $p < 0.05$. SigmaStat statistical software (SPSS, Chicago, IL) was used for all analyses.

Results

Resolution of P. carinii *infection is delayed in pups reconstituted with adult cells*

To examine the possibility that delayed resolution of *P. carinii* in mice challenged as neonates is due to the lung environment, SCID pups or adults were infected with *P. carinii* and reconstituted 4 days later with splenocytes from adult immunocompetent BALB/c mice. Because neonatal mice (pups) weighed 5- to 10-fold less than adult female mice, pups were routinely inoculated with 2×10^6 organisms and 10^7 cells, and adults were given 10^7 organisms and 5×10^7 cells. The normal kinetics of clearance of *P. carinii* from BALB/c mice infected as neonates is delayed compared with that from adults as shown in Fig. 1*A*. SCID pups reconstituted with adult splenocytes also had delayed clearance of *P. carinii* compared with reconstituted SCID adults (Fig. 1*B*). Adult SCID mice had resolved PCP by about 5 wk postreconstitution, whereas only two of five SCID pups had cleared *P. carinii* from the lungs by almost 7 wk postreconstitution (Fig. 1*B*). These data suggest that the delayed clearance of *P. carinii* in neonatal mice is due to the

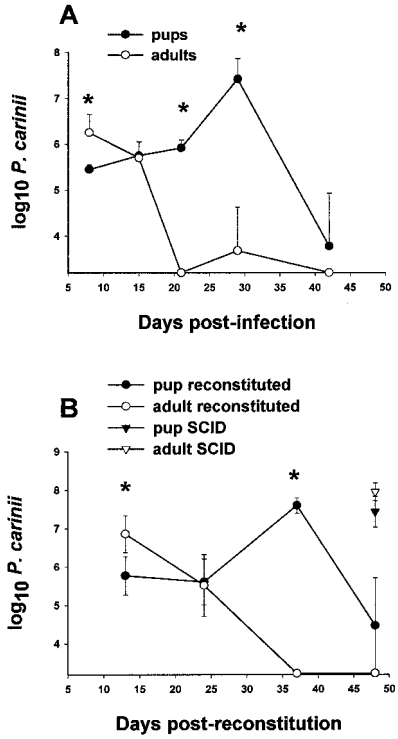


FIGURE 1. Resolution of lung *P. carinii* burden is delayed in mice infected as neonates compared with that in adult mice. *A*, BALB/c mice were given i.n. inoculations of *P. carinii* as neonates (pups) or as adults, and lung burden was determined over time. Lungs were excised, and *P. carinii* organisms were enumerated microscopically after fixation and staining with Diff-Quik. *B*, Adult or neonatal SCID mice were given i.n. inoculations of *P. carinii* and reconstituted 4 days later with splenocytes from adult BALB/c donors. For comparison, lung *P. carinii* burden is shown for unreconstituted SCID pups and adults on day 42 postreconstitution. Data are expressed as $\log_{10}$ *P. carinii* per right lung lobes, with a limit of detection of $\log_{10}$ 3.23 organisms/lungs. Data are the mean ± SD of three to five mice per group and are representative of three separate experiments. ∗, $p < 0.05$ compared with adults at the same time point.

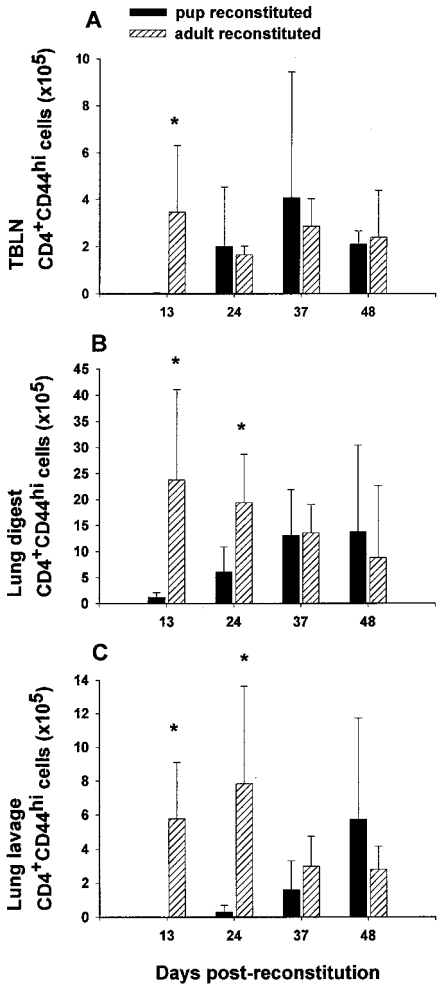


FIGURE 2. The $CD4^+$ T cell response in the TBLN and lungs of SCID neonates reconstituted with immunocompetent splenocytes is delayed compared with that in reconstituted adult SCID mice. SCID neonates and adults were given i.n. inoculations of *P. carinii* and 4 days later reconstituted with splenocytes from BALB/c donors. At the times indicated postreconstitution, lungs were lavaged, and lungs and TBLN were excised. Single-cell suspensions were analyzed for the presence of activated $CD4^+CD44^{high}$ cells by flow cytometry. *A*, TBLN; *B*, lung digest; *C*, lung lavage cells. Data are the mean ± SD of three to five mice per group and are representative of three separate experiments. ∗, $p < 0.05$ compared with pups at the same time point.

inability of the lung environment to support effector function of lymphocytes.

Migration of lymphocytes is delayed in neonatal mice

We have previously reported that there is a delay in the lung inflammatory response to *P. carinii* in immunocompetent mice infected as neonates compared with that in adults (3). Consistent with these data, there was a delayed inflammatory response in *P. carinii*-infected SCID pups reconstituted with adult lymphocytes compared with that in reconstituted adult SCID mice. *P. carinii*-infected adult SCID mice had a significant number of $CD4^+$ cells in the draining lymph nodes, lung digest, and lung lavages when examined on day 13 postreconstitution using flow cytometry (Fig. 2). In contrast, $CD4^+$ cells were nearly undetectable in SCID pups until day 24 postreconstitution. $CD4^+$ T cells appeared in the TBLN and lung digest of the pups on day 24; however, very few cells were found in the alveolar spaces until day 37 postreconstitution (Fig. 2*C*). The appearance of B and $CD8^+$ T cells in neonatal tissues was also delayed compared with that in adults (data not shown). Notably, increasing the inoculation dose of *P. carinii* in the neonatal mice to equal that in adult mice did not result in an earlier inflammatory response or clearance of PCP (data not shown). Because the appearance of $CD4^+$ cells in the alveolar spaces precedes clearance of the organisms, these results suggest that failure of $CD4^+$ cells to appear in the lungs contributes to the delayed clearance of *P. carinii*.

The appearance of $CD4^+$ cells that were activated and produced cytokines such as IFN-γ and IL-10 was delayed in the lungs of reconstituted SCID pups compared with that in adults (data not shown). This was due to the overall low numbers of cells that migrated into the lungs of the SCID pups. However, the proportion of $CD4^+$ cells that produced IFN-γ (>40%) or IL-10 (5–10%), as determined by flow cytometry, was similar in pups and adult mice (data not shown). This suggested that the small numbers of cells that were able to migrate into the lungs of reconstituted SCID pups were activated and producing cytokines.

Migration of APCs from the lungs to the draining lymph nodes is impaired in neonatal mice

One possible reason why there is a delay in the migration of $CD4^+$ cells to the lungs of neonatal mice might be that Ag presentation

is inadequate, so only a small number of cells become activated and express the proper adhesion molecules for extravasation into the infected tissues. To test whether phagocytic cells from the lungs of neonatal mice are capable of transporting particles to the draining lymph nodes, we inoculated mice with fluorescent 1-μm beads. Neonatal and adult BALB/c mice were given i.n. inoculations of 1.2×10^7 beads/g of body weight. Two days later, TBLN were isolated from the mice, and the fluorescent bead-associated cells were enumerated using a fluorescent microscope. The percentage of TBLN cells that were bead-associated is shown in Fig. 3. There was a significant difference in the proportion of bead-positive cells in the TBLN of adult mice compared with neonatal mice. For adult mice, an average of >2% of cells contained beads, whereas four of six neonatal mice had no detectable bead-positive cells in the TBLN (Fig. 3). In contrast, adult and neonatal mice had 36.8 ± 6.1 and 40.0 ± 7.8% bead-positive cells in lung lavages, respectively, suggesting that the cells in the alveolar spaces were able to phagocytose particles with the same efficiency. Similar results were found when *P. carinii* organisms were labeled with the fluorescent dye carboxyfluorescein succinimidyl ester before inoculation into pups (data not shown). These data suggest that due to either low numbers of APCs or inefficient migration to draining lymph nodes, inadequate APC activity may explain the delayed clearance of *P. carinii* in neonatal mice.

The prevalence of dendritic cells and Ia-expressing myeloid cells in the lungs of *P. carinii*-infected adult and neonatal mice was determined using flow cytometry. Normal BALB/c mice were infected with *P. carinii* within 48 h of birth or as 8-wk-old adults. Eight days later the lungs were excised and digested, and TBLN were pushed through mesh to form single-cell suspensions. The proportion of nonlymphoid cells expressing the dendritic cell marker CD11c or expressing MHC class II (Ia) was determined by flow cytometry. As shown in Fig. 4, there was a significantly higher percentage of $CD11c^+$ cells in the lungs of adult mice compared with pups on day 7 postinfection. Similarly, the percentage of nonlymphoid cells that expressed Ia was significantly higher in the lungs and TBLN of adult mice compared with neonatal mice (Fig. 4). These data suggest that migration of dendritic cells and Ag presentation in the TBLN of neonatal mice may be much reduced compared with that in adults.

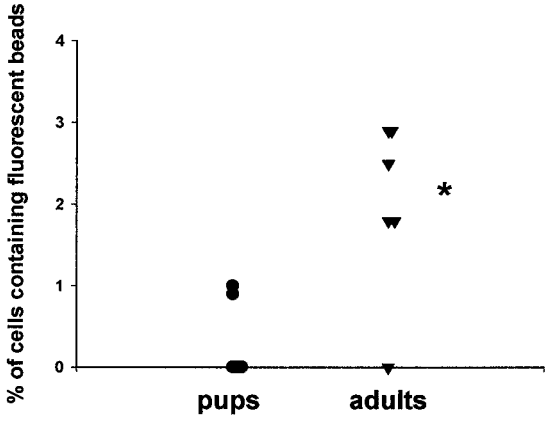
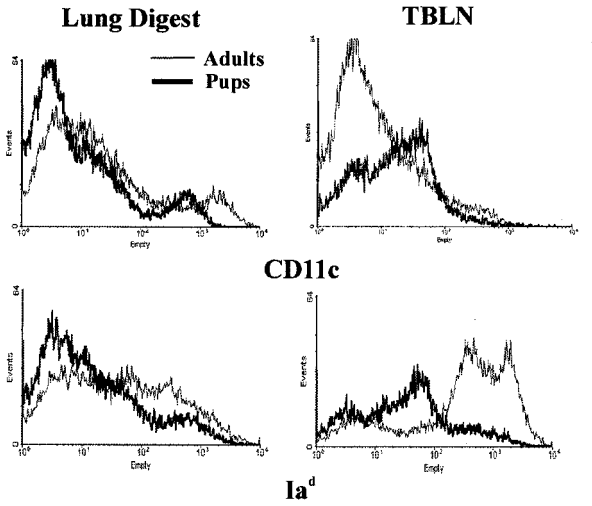


FIGURE 3. Neonatal mice are less efficient than adult mice at phagocytosing fluorescent beads and migrating with them to the draining lymph nodes. Adult and neonatal BALB/c mice were given i.n. inoculation of 1-μm fluorescent beads. Forty-eight hours later, draining lymph nodes were excised, and the proportion of cells containing fluorescent beads was determined microscopically. The proportion of bead-positive cells in the TBLN is shown for individual mice. *, $p < 0.05$ compared with neonatal mice.



Group	Lung digest cells		TBLN cells	
	% $CD11c^+$	% Ia^+	% $CD11c^+$	% Ia^+
Pups	17.2±5.8	3.6±2.1	3.9±2.1	3.6±2.1
Adults	25.2±4.6	14.7±2.0	3.7±1.5	14.7±2.9
p value	0.053	0.009	0.901	<0.001

FIGURE 4. The proportions of $CD11c^+$ and Ia^+ nonlymphoid cells in the lungs and TBLN of neonatal mice are significantly lower than those in adults. BALB/c mice were given i.n. inoculations of *P. carinii* as neonates or adults. Lungs and TBLN were excised on day 8 postinfection, and the proportions of $CD11c^+$ dendritic cells and Ia^+ myeloid cells were determined by flow cytometry. Shown are histograms of fluorescence intensity for CD11c and Ia. Cells were gated on live, nonlymphoid cells. *Left*, lung digest cells; *right*, TBLN cells. The table below the histograms shows the mean ± SD of four or five mice per group and is representative of two separate experiments. Note that all pup values are significantly less than adult values, $p \leq 0.05$.

Expression of cytokine and chemokine mRNA is delayed in the lungs of neonates

It has been reported that *P. carinii* infection stimulates increased expression of cytokine and chemokine mRNA in the lungs of adult reconstituted SCID mice (18, 19). Increases in lung TNF-α, IL-1, IL-6, and IFN-γ mRNA as well as increased lung lymphotactin, RANTES, MCP-1, MIP-1β, and MIP-1α mRNA have been demonstrated. This increased cytokine and chemokine production precedes clearance of the organisms and corresponds to lung inflammation (18, 19).

It is possible that the lack of an inflammatory response in the lungs of *P. carinii*-infected neonates is due to the lack of cytokine and/or chemokine signals for migration to the site of infection. Chemokine mRNA expression over time in SCID mice infected with *P. carinii* as neonates or adults and reconstituted with splenocytes from immunocompetent adult BALB/c donors was detected by RPA. Consistent with the delayed inflammatory response in mice infected as neonates, there was delayed expression of cytokine and chemokine mRNA in *P. carinii*-infected SCID pups reconstituted with adult lymphocytes compared with reconstituted adults (Fig. 5). On day 13 postreconstitution, low, but detectable, amounts of mRNA for RANTES, MIP-1α, MIP-1β, and MCP-1 was found in the pup lungs (Fig. 5). In contrast, lung lymphotactin,

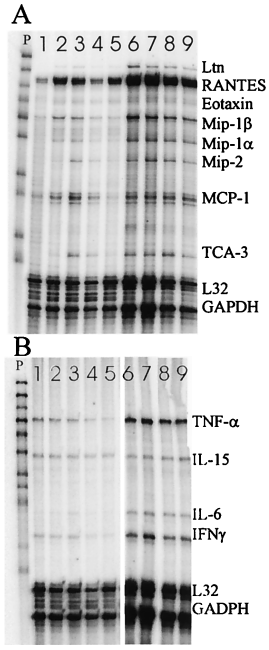


FIGURE 5. RPAs indicate that there are delays in the expression of chemokine and cytokine mRNA in the lungs of reconstituted neonatal SCID mice compared with adults. Mice were infected with *P. carinii* and reconstituted with immunocompetent splenocytes as described in Fig. 2. Left lung lobes were excised and snap-frozen before extraction of RNA and RPA. Protected mRNA was detected using a polyacrylamide gel followed by analysis with a Storm 860 PhosphorImager. *A*, RPA for chemokines on day 13 postreconstitution. *B*, RPA for cytokines on day 13 postreconstitution. *Lane P*, chemokine or cytokine unprotected probe; *lanes 1–5*, mRNA from individual pups; *lanes 6–9*, mRNA from individual adults. Individual bands that were visible are labeled on the *right side* of the gel. Note that IL-4, IL-5, IL-13, IL-9, and IL-2 are not labeled in *B* because they were not detected.

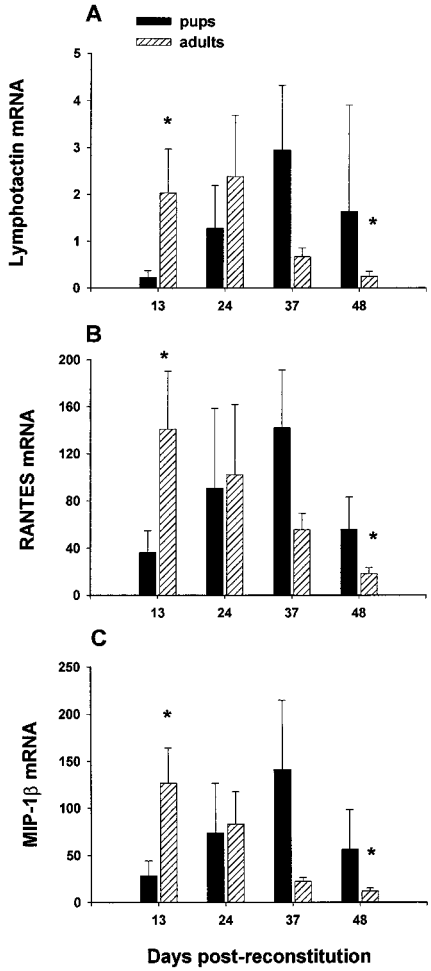


FIGURE 6. Expression of lymphotactin, RANTES, and MIP-1β mRNA expression is delayed in the lungs of neonatal mice compared with that in adults. Mice were infected with *P. carinii* and reconstituted with immunocompetent splenocytes as described in Fig. 2. Left lung lobes were snap-frozen at the times indicated and used for RPA as described in Fig. 5. *A*, Lymphotactin; *B*, RANTES; *C*, MIP-1β. The density of mRNA bands was determined using ImageQuant software. The density of each band was normalized by dividing by the density of L32 mRNA expression in the same lung and multiplying by 100 to obtain whole numbers. Data are the mean $\pm$ SD for three to five mice per group. $*$, $p < 0.05$ compared with adults at the same time point.

RANTES, MIP-1β, MIP-1α, and MCP-1 mRNA had peaked in the lungs of adult mice by day 13 or 24 postreconstitution, whereas chemokine mRNA expression did not peak until day 37 in the lungs of reconstituted pups (Figs. 5 and 6). This delayed expression of chemokine mRNA in mice infected as neonates was consistent with the delayed inflammatory response and resolution of the infection.

Lung cytokine mRNA expression in the lungs of *P. carinii*-infected pups and adults after reconstitution with adult splenocytes was also assessed by RPA. TNF-α is of particular interest because it has been shown that this proinflammatory cytokine is critical for resolution of PCP in murine models of infection (20–22). As shown in Figs. 5 and 7*A*, TNF-α mRNA expression in adult mice had already peaked by day 13 postreconstitution. TNF-α mRNA expression decreased almost 2-fold between days 13 and 24 postreconstitution in adult SCID mice and remained level thereafter. In contrast, TNF-α levels in neonatal mice were low on day 13 and increased significantly through day 37 postreconstitution (Fig. 7*A*). The delayed changes in TNF-α mRNA expression observed in pups corresponded to the delayed inflammation in the lungs of *P. carinii*-infected pups compared with adults as well as the delayed clearance of the infection. Similarly, delayed expression of IL-6 mRNA was seen in reconstituted pups compared with adults (data not shown). Lung IFN-γ mRNA expression had already peaked in reconstituted adult mice by day 13 (Fig. 7*B*). However, mice infected as neonates had increasing levels of IFN-γ mRNA expression through day 48 postreconstitution (Fig. 7*B*). Expression of IL-4 and IL-5 mRNA was so low so as to not be confidently quantitated in both pups and adults after reconstitution (data not shown). The kinetics of cytokine mRNA expression corresponded with those of chemokine mRNA expression in the lungs of pups and adults. Furthermore, the delayed cytokine and chemokine responses in the lungs of pups were consistent with the delayed inflammatory response and resolution of *P. carinii*, suggesting that altered cytokine and/or chemokine responses may contribute to the defects in the neonatal lung environment that result in delayed *P. carinii* clearance.

Discussion

The data presented here demonstrated that the lung environment in neonatal mice was not conducive to clearing *P. carinii* even in the presence of adult immunocompetent lymphocytes. The kinetics of clearance in adult cell reconstituted SCID pups reflected a delay similar to that observed previously in immunocompetent pups (3). Adult mice were able to resolve *P. carinii* infection a full 2 wk

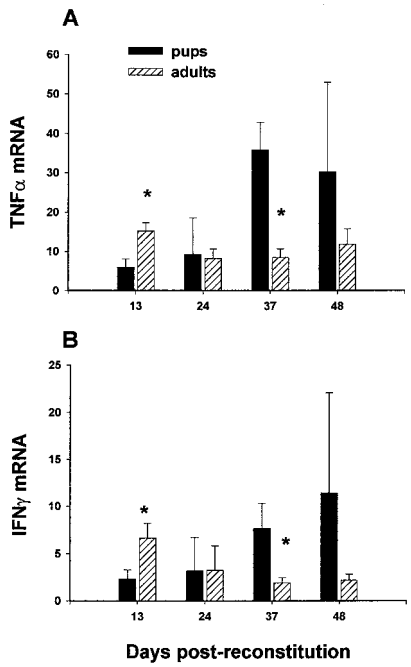


FIGURE 7. Expression of TNF-α and IFN-γ mRNA expression is delayed in the lungs of neonatal mice compared with that in adults. Mice were infected with *P. carinii* and reconstituted with immunocompetent splenocytes as described in Fig. 2. Left lung lobes were snap-frozen at the times indicated and used for RPA as described in Fig. 5. *A*, TNF-α; *B*, IFN-γ. The density of each band was normalized to L32 expression in the same lung as that described in Fig. 6. Data are the mean $\pm$ SD for three to five mice per group. $*$, $p < 0.05$ compared with adults at the same time point.

earlier than mice infected as neonates. Similarly, reconstitution of infected SCID adults resulted in clearance of *P. carinii* 2 wk earlier than in reconstituted pups. These data indicated that the delayed inflammatory response to *P. carinii* was not strictly due to immaturity in the lymphocyte pool, but that there was something inherent in the lung environment that allowed the organisms to grow unchecked until an inflammatory response was mounted around weaning age. In this regard, unreconstituted SCID mice had similar numbers of *P. carinii* organisms in the lungs 7 wk after inoculation even though adults were infected with 5-fold more organisms than neonates, also suggesting a more permissive environment in the lungs of young mice.

There are several possible reasons for the inability of mice infected as neonates to resolve PCP with the same kinetics as adults. These include delayed migration of immature dendritic cells into the lungs, inability of immature dendritic cells to mature from phagocytic cells to mature APCs, inability of mature dendritic cells to migrate to the draining lymph nodes, and inability of T cells to respond to Ag presentation and migrate back to the lungs. Because neonatal mice were reconstituted with competent adult splenocytes, they should have been fully able to respond to appropriate stimuli for activation and migration to the site of inflammation. These data indicate that the lung phagocytic cells did not recognize the infectious agent, they were not sufficiently stimulated to maturity, and/or they did not migrate properly. Additionally, it is possible that lung chemoattractants or adhesion molecules were not sufficiently expressed in neonatal lungs for extravasation of mature T cells to the site of infection.

It has been reported in neonatal rats that there is delayed maturation of respiratory tract dendritic cells characterized by decreased expression of Ia, decreased Ag presentation activity in mixed lymphocyte reactions, and decreased recruitment of dendritic cells into the airway epithelium in response to heat-killed *Moraxella catarrhalis* (16). Consistent with these previous findings, our data indicated that there was a lower percentage of dendritic cells in the lungs and TBLN of *P. carinii*-infected neonatal mice compared with adult mice. In addition, there was decreased expression of Ia on nonlymphoid cells of both lungs and TBLN of neonatal mice compared with adults. Phagocytic cells from neonatal lungs were significantly less efficient at migrating to draining lymph nodes than were cells from adult lungs. Two days after i.n. challenge with either fluorescent beads or fluorescent *P. carinii* organisms, there were detectable numbers of fluorescent cells in the TBLN of the adult mice, but not in the neonatal mice. The decreased migration was not necessarily due to decreased phagocytosis, because the percentages of lung lavage cells containing beads were similar in neonatal and adult mice. It is possible that the signals necessary for migration were either not present or not at a sufficient concentration for attracting phagocytic cells to the TBLN. These signals could include TNF-α, up-regulation of chemokine receptors such as CCR7, or expression of adhesion molecules. We are currently examining the expression of chemokine receptors and adhesion molecules in the lungs and immune cells of neonatal mice.

It is not possible to determine unequivocally from the data presented whether dendritic cells in the TBLN of neonatal mice were capable of effective Ag presentation. However, there were 200-fold fewer activated $CD4^+$ T cells in the TBLN of neonates than in adults on day 13 postreconstitution, even though fully competent adult splenocytes had been adoptively transferred into both adults and pups. This may have been due to decreased expression of adhesion molecules in the lymph nodes or decreased chemokine production, such as lymphotactin. Both could be a result of the inability of Ag-loaded dendritic cells or macrophages to reach the draining lymph nodes. Additionally, the reduced numbers of $CD11c^+$ dendritic cells in the lungs, the reduced expression of Ia in nonlymphoid cells of the lungs and TBLN, as well as literature reports from rat models (15, 16) suggest that Ag presentation is depressed in neonatal mice compared with that in adults. The depressed Ag presentation is likely to be at least partially responsible for the delayed host response to *P. carinii* in mice infected as neonates compared with that in adults.

Immature dendritic cells are dependent on inducible chemokines such as RANTES, MIP-1α, MIP-1β, and MCP-1 to migrate from the circulation to sites of inflammation (23). Once in the lungs, immature dendritic cells mature from phagocytic cells to APCs. The signals required for maturation include proinflammatory cytokines such as IL-1 and TNF-α, viral or bacterial products, and CD40 ligand (23). Mature dendritic cells down-regulate chemokine receptors such as CCR1, -2, and -5 and up-regulate CCR7, causing them to migrate to draining lymph nodes in response to chemokines such as EBV-induced molecule 1 ligand chemokine (ELC)/MIP-3β (24, 25). It is possible that any or all of these steps in mobilization of dendritic cells could be impaired in the lungs of *P. carinii*-infected neonates. Specifically, we found that lung mRNAs from the inducible chemokines RANTES, MIP, and MCP were all at significantly lower levels in neonates compared with adults. This may explain the reduced numbers of $CD11c^+$ dendritic cells in the lungs of neonates compared with adults. Additionally, expression of lymphotactin mRNA, a lymphocyte-specific chemoattractant, was delayed in neonates compared with adults. This may account in part for the delayed lymphocyte response to *P. carinii* in the neonatal mice. It has been shown in adult mice infected with *Crytococcus neoformans* that MCP-1 is critical for attracting $CD4^+$ cells to the lungs (26). Because $CD4^+$ cells are required for host defense against *P. carinii* (6, 7), the

delayed expression of lymphotactin and MCP-1 in neonatal mice probably contributes significantly to delayed resolution of PCP.

In contrast to the data presented here, it has been reported that exposure to hyperoxia significantly increased RANTES, MIP-1α, and MCP-1 mRNA levels in the lungs of 4-day-old C57BL/6 mice (27). These data suggest that given the proper stimulus, the lungs of neonatal mice are capable of increasing chemokine mRNA expression. Consistent with this idea, we have found that neonatal mice infected with *Streptococcus pneumoniae* mount a neutrophil response that is as intense at that of adult mice (28). The delayed response to *P. carinii* infection may be due to low levels of TNF-α or IFN-γ production in neonates. Although IFN-γ is not required for clearance of *P. carinii* (29), it has been shown to be critical for the expression of RANTES and MIP-1β in a model of murine lung infection with *Cryptococcus neoformans* (30), suggesting that IFN-γ may has critical functions in controlling inflammation in the lungs.

TNF-α has been shown to be a stimulus for maturation of dendritic cells resulting in increased CCR7 expression and migration toward EBV-induced molecule 1 ligand chemokine (ELC)/MIP-3β (24). In addition, both TNF-α and IFN-γ increase expression of MHC class II on APCs and stimulate expression of costimulatory and adhesion molecules. The presence of TNF-α has been shown to be required early in the immune response to *P. carinii* for resolution to take place (20). Furthermore, double IFN-γ and TNF-α receptor knockout mice are susceptible to PCP (21), indicating that in concert TNF-α and IFN-γ have important roles in host defense to *P. carinii* that probably include controlling the inflammatory response.

The data suggest a model in which *P. carinii* infection in neonatal mice fails to stimulate production of proinflammatory cytokines such as TNF-α, resulting in decreased chemokine production in the lungs. As a consequence, immature dendritic cells either do not migrate to the lungs promptly or are not stimulated to maturity once there. Therefore, mature Ag-loaded dendritic cells do not migrate to the draining lymph nodes, and so Ag presentation and activation of $CD4^+$ T cells are delayed. Additionally, delayed chemokine production may further delay migration of $CD4^+$ cells to the lungs. The inability of dendritic cells and T cells to traffic appropriately results in delayed clearance of the organisms. Although these data do not rule out the possibility that immaturity of the lymphoid compartment in neonatal mice also contributes to delayed resolution of PCP, we have found that adult SCID mice reconstituted with $CD4^+$ cells from 10-day-old BALB/c mice are capable of resolving PCP as efficiently as mice reconstituted with equal numbers of $CD4^+$ cells from adult donors (manuscript in preparation). These data clearly indicate that the neonatal lung environment is inadequate for clearance of *P. carinii*, even in the presence of mature T cells.

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