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J Immunol 2001; 166:5704-5711; ;
doi: 10.4049/jimmunol.166.9.5704
http://www.jimmunol.org/content/166/9/5704

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Neonatal T Cells in an Adult Lung Environment Are Competent to Resolve *Pneumocystis carinii* Pneumonia[1]

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

Initiation of the pulmonary inflammatory response to *Pneumocystis carinii* is delayed by 3 wk in mice infected as neonates compared with adults. There was no difference in the proliferative response of draining lymph node T cells from mice infected as neonates compared with adults when stimulated in vitro with either Con A or anti-CD3 mAb. However, TNF-α and IFN-γ mRNA expression in the lungs of *P. carinii*-infected neonates was significantly lower than in adults indicating a lack of appropriate activation signaling in the local environment. This may have been due to active suppression because TGF-β mRNA expression was significantly elevated in neonatal lungs compared with adults. To determine whether T cells from 10-day-old mice would effect resolution of *P. carinii* if harbored in an adult lung environment, cells were adoptively transferred to SCID mice with established *P. carinii* infections. There was no difference in the kinetics of T cell migration into the lungs or of clearance of *P. carinii* organisms when SCID mice were reconstituted with splenocytes from young mice as compared with adult mice. Furthermore, splenocytes from young mice stimulated both TNF-α and IFN-γ mRNA expression to levels that were similar to that in the lungs of SCID mice reconstituted with adult cells. These data indicate that neonatal lymphocytes are competent to resolve *P. carinii* infection when harbored in an adult lung environment, suggesting that the neonatal lung environment, and not the T cells, is ineffective at responding to *P. carinii* infection. *The Journal of Immunology,* **2001, 166: 5704–5711.**

Pneumocystis carinii is an opportunistic fungal pathogen that affects predominantly immunocompromised hosts including those with AIDS (1). *P. carinii* pneumonia (PCP)[3] remains the most frequently reported serious opportunistic infection in AIDS patients and the second highest cause of mortality among persons with AIDS in the U.S., despite the availability of effective chemoprophylaxis (2). Primary *P. carinii* infection is not common because immunocompetent individuals develop Ab to *P. carinii* by 2–3 years of age (3). Recently, it has been reported that there is a relatively high incidence of *P. carinii* infection in autopsy specimens of children who die from sudden infant death syndrome. Although this does not necessarily indicate that PCP is a factor in sudden infant death syndrome it may reflect the fact that children frequently carry subclinical *P. carinii* infection (4). However, very young patients with AIDS suffer a more fulminate course of PCP than older children with AIDS (5).

In general, the ability of the neonatal immune system to confer protection against viral, bacterial, and fungal infection is inadequate compared with adults (6). This limitation has been attributed to the immaturity of both innate and adaptive immunity of neonates (6–8). Naive neonatal T cells have been shown to be functionally different from adults (7). Human cord blood T cells produce low levels of IL-2, IFN-γ, TNF-α, IL-4, and IL-5 in response to endogenous APC or anti-CD3 mAb in vitro (9, 10). Murine neonatal cells also produce low IL-2 and IFN-γ in response to anti-CD3 mAb but, unlike human cells, yield high levels of IL-4 (11, 12). However, addition of adult professional APC, exogenous IL-12, or anti-CD28 mAb along with anti-CD3 mAb can elicit an adult-like cytokine profile both in human and murine T cells (13–16). Even though a considerable amount of information is available regarding the function of neonatal T cells, many of these studies are in vitro and may not reflect their function in vivo.

Several in vivo studies have reported adult-like protective responses in neonatal mice in different experimental conditions. Using DNA vaccines, Bot et al. (17) found that mice immunized as neonates mounted primary T helper type (Th) responses equivalent to those of adults. Use of protein Ag in PBS also elicited an adult-like primary Th response in neonates (18). Thus, in the mouse a primary Th response may be achieved through the use of conditions that strongly promote Th responses in adults.

Animal experiments indicate that CD4$^+$ T lymphocytes are crucial for *P. carinii* resolution. Clinical studies also demonstrate an inverse relationship of CD4$^+$ T cell count with PCP incidence among AIDS patients (19, 20). Furthermore, the proinflammatory cytokines, TNF-α and IFN-γ, have been shown to be important for resolution of PCP (21, 22). Thus a complex signaling system seems to be involved in recruitment and activation of the effector cells for *P. carinii* resolution.

Studies from this laboratory reported that resolution of *P. carinii* infection is delayed in neonatal mice as compared with adults (23). In mice infected as neonates, the onset of the inflammatory response against *P. carinii* was delayed by 3 wk as compared with a more immediate response in adults. A sluggish recruitment and activation of T lymphocytes, predominantly CD4$^+$ phenotype, characterized this delay (23). This was consistent with other studies demonstrating the pivotal role of CD4$^+$ cells in protection of

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Received for publication October 5, 2000. Accepted for publication February 28, 2001.

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[1] This work is supported by National Institutes of Health Grant HL-62053.

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[3] Abbreviations used in this paper: PCP, *Pneumocystis carinii* pneumonia; TBLN, tracheobronchial lymph node; RPA, RNase protection assay; i.n., intranasal(ly); CD62L, CD62 ligand; MCP-1, monocyte chemotactic protein-1; MIP, macrophage inflammatory protein.

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mice against *P. carinii* infection (24–26). However, in vitro studies have demonstrated up-regulated cytokine production by neonatal cells in response to strong costimulatory signals (13–16), indicating that neonatal cells are not inherently unresponsive. Given observations that neonatal T cells produce cytokines that can be normalized to adult levels when stimulated with appropriate costimulatory molecules, it is possible that a lack of orchestrated cytokine/chemokine signaling in the neonatal lung explains the inability of neonatal lymphocytes to resolve infection.

In these studies we have used an adoptive transfer model of *P. carinii*-infected SCID mice reconstituted with splenocytes from 10-day-old (pup) or adult BALB/c donors. This allows comparison of pup and adult lymphocytes in a comparable lung environment. Our data demonstrate that pup lymphocytes competently resolve *P. carinii* infection in adult lungs by recruitment of $CD4^+$ cells in association with cytokine mRNA up-regulation. In vitro proliferation and cytokine production are also similar to those of adults. These study results suggest that neonatal lymphocytes are not intrinsically hyporesponsive to *P. carinii* challenge when placed in an appropriate lung environment.

Materials and Methods

Mice

Five- to 6-wk-old BALB/c SCID and BALB/c mice were purchased from the National Cancer Institute (NCI). Mice were maintained at the Veterinary Medical Unit (VMU) of the Veterans Administration Medical Center (VAMC) under pathogen-free conditions. Mid-term pregnant and 6- to 7-wk-old BALB/c mice were purchased from NCI or Harlan Breeders (Indianapolis, IN). The neonates born of the pregnant mothers and the adult BALB/c mice served as donors for reconstitution. CB-17 SCID mice, used to maintain a source of *P. carinii*, were bred at the VAMC VMU in microisolator cages containing sterilized food and water.

P. carinii *infection*

Six-week-old female BALB/c SCID mice were co-housed with *P. carinii*-infected CB-17 SCID mice for 4 wk before reconstitution. In some experiments, mice were infected by inoculation of *P. carinii*. Lungs were excised from *P. carinii*-infected SCID mice and pushed through steel mesh in HBSS. *P. carinii* was isolated and enumerated by microscopy as described (23). Eight-week- and 48- to 72-h-old BALB/c mice were inoculated intranasally (i.n.) with different doses of *P. carinii* ranging from 1×10^6 to 5×10^7 organisms as indicated.

Reconstitution of infected SCID mice

Ten-day-old pups and 8- to 9-wk-old BALB/c mice were used as the source of splenic T cells for reconstitution. Spleens were pushed through mesh screens in HBSS to form single cell suspensions. Erythrocytes were removed using a hypotonic lysis buffer, and cells were washed and resuspended in PBS. *P. carinii*-infected mice were given i.v. injections of splenocytes adjusted so that all mice received 2×10^6 $CD4^+$ cells. The proportion of $CD4^+$ cells in spleen cell preparations was determined by flow cytometry before injection.

Isolation of lung alveolar and interstitial cells

Lung cells were prepared as described elsewhere (23). Briefly, lung airways were lavaged using an intratracheal cannula with 5×1 ml washes of cold HBSS containing 3 mM EDTA. Right lungs were excised, minced, and enzyme-treated at 37°C for 60 min in RPMI 1640 containing 3% FCS, 50 U/ml DNase (Sigma, St. Louis, MO), and 1 mg/ml collagenase A (Sigma). Digested lung tissue was pushed through mesh screens, and RBC were lysed by treatment with a hypotonic buffer. Lung cells were resuspended in HBSS for enumeration.

Enumeration of P. carinii

Aliquots of digested lung tissue were diluted and spun on glass slides, fixed in methanol, and stained with Diff-Quik (Dade International, Miami, FL). *P. carinii* nuclei were enumerated microscopically as described (23). The number of *P. carinii* organisms was expressed as $\log_{10}$ nuclei/right lungs. To examine the cytokine and chemokine mRNA induction in the same animals of which we are estimating the lung burden, enumeration of organisms was performed in the right lobes of lungs and the left lung lobes were snap frozen for RNase protection assays (RPAs). The lung *P. carinii* burden determined from the right lobes is proportional to counts obtained using whole lung. The limit of detection of *P. carinii* was $\log_{10}3.2$.

Preparation of lung cells for flow cytometric analysis

Cells derived from lung lavage and digested lung tissue were enumerated, and $\sim 5 \times 10^5$ to 1×10^6 cells were used for staining with fluorochrome-conjugated Abs specific for murine CD4, CD8, CD62 ligand (CD62L), CD44 (PharMingen, San Diego, CA). Before and after Ab staining, cells were washed once with PBS containing 0.1% BSA and 0.02% sodium azide. Finally, cells were resuspended in PBS for multiparameter analysis using a FACSCaliber cytofluorometer (BD Biosciences, Mountain View, CA).

In vitro culture of tracheobronchial lymph node (TBLN) cells

TBLNs were collected on day 14 postinfection from *P. carinii*-infected mice. Lymph node tissues were pushed through a mesh screen and treated with a hypotonic lysis buffer to eliminate RBC. Cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin-G (100 U/ml), streptomycin (100 mg/ml), and gentamicin (20 μg/ml). Cells (2×10^5/well) were cultured in triplicate in 96-well flat-bottom tissue culture plates at 37°C in a 5% CO_2-humidified atmosphere for 5 days. Culture conditions were 200 μl of RPMI 1640 with or without Con A (1, 2.5, and 10 μg/ml; Sigma, St. Louis, MO), or plate-bound anti-CD3 mAb (1, 2.5, and 5 μg/ml; R&D Systems, Minneapolis, MN) as indicated. Two days after initiating the cultures, Alamar blue (BioSource International, Camarillo, CA) was added at 10% of the culture volume. Alamar blue contains an oxidation-reduction indicator that changes color in proportion to cell proliferation. On day 5 of culture, plates were read by a microplate reader at wavelengths of 570 and 600 nm. Cell proliferation corresponds to the magnitude of dye reduction and is expressed as percent reduction.

IFN-γ measurement by ELISA

Culture supernatants were collected 5 days after initiating cultures of TBLN cells, as described above. IFN-γ content was measured by using a commercially available sandwich ELISA kit (PharMingen), according to the manufacturer's instruction.

Extraction of RNA and RPA

Total RNA was prepared from frozen lung tissue by using TRIzol (Life Technologies, Gaithersburg, MD) according to manufacturer instructions. Briefly, lung tissue was homogenized in 1 ml of TRIzol reagent followed by chloroform extraction and ethanol precipitation. Air-dried RNA pellets were dissolved in diethyl pyrocarbonate-treated water. Multiprobe DNA templates for cytokines (IFN-γ, TNF-α, TGF-β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and IL-15) and housekeeping genes, L32 and GAPDH, were purchased from PharMingen. RPA was performed using the RiboQuant in vitro transcription and RPA kits (PharMingen) according to the manufacturer's protocol. The 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. The remaining RNase-protected RNA duplexes were extracted with phenol/chloroform/isoamyl alcohol and resolved on 5% denaturing polyacrylamide gels. Dried gels were exposed to PhosphorImager screens, and images were developed using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The intensity of each specific cytokine band was measured by using ImageQuant software (Molecular Dynamics). The cytokine mRNA levels were corrected for RNA loaded by dividing the cytokine hybridization signal by the L32 signal for the same sample.

Statistical analysis

The results of these studies were tested statistically by Student's *t* tests and ANOVA using commercially available software (Sigmastat; SPSS, Chicago, IL). Results were determined to be statistically significant when $p < 0.05$ was obtained.

Results

Differential P. carinii *clearance from neonatal and adult lungs*

The ability of neonatal mice to resolve infection after challenge with varying doses of *P. carinii* organisms was compared with that of adult mice. Eight-week- and 48-h-old BALB/c mice were given i.n. inoculations of various doses of *P. carinii* nuclei. Adult mice

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had significantly reduced *P. carinii* burden by day 21 postinfection for all doses (Fig. 1). In contrast, mice infected as neonates (pups) had significantly higher *P. carinii* burdens in the lungs than adults. Pups were unable to mount an inflammatory response by day 21 irrespective of the challenge dose (data not shown) and so had delayed clearance of *P. carinii* compared with adult mice.

Differential cytokine mRNA expression in pup and adult lungs in P. carinii *infection*

Recently, it has been reported that clearance of *P. carinii* organisms from lungs of reconstituted adult SCID mice was preceded by increased mRNA expression of cytokines including TNF-α, IFN-γ, IL-1, and IL-6 (27). One possible explanation for the differential inflammatory cell migration and clearance of *P. carinii* from pup lungs as compared with adults may be inadequate cytokine up-regulation in the pup lungs. Therefore, we examined kinetics of cytokine mRNA expression in the lungs of immunocompetent BALB/c pups and adults using RPAs. Adult lungs had considerably enhanced mRNA expression of IL-4, IL-5, TNF-α, IL-13, IL-6, and IFN-γ by day 15 postinfection compared with lungs from uninfected mice (Fig. 2 and data not shown). In contrast, the lungs of mice infected as neonates had comparatively reduced expression of TNF-α, IL-13, and IFN-γ, and no expression of IL-4, IL-5, and IL-6 at the same time points (Fig. 2 and data not shown). IFN-γ and TNF-α expression had peaked in the adult lungs on days 8 and 15 postinfection, respectively (Fig. 2). In contrast, expression of these cytokines in pup lungs increased gradually over time and did not peak until day 42 postinfection. In pup lungs, there was a >2-fold increase in TNF-α and IFN-γ expression between days 29 and 42. Expression of these cytokines corresponded to the timing of clearance of *P. carinii*. Adults had no detectable *P. carinii* at day 21 postinfection, whereas pup lung burden reached $\log_{10}5.9$ at day 29 postinfection before the beginning of clearance (data not shown and Ref. 23). These data indicate that up-regulation of cytokines in the lungs of mice infected as neonates are delayed as compared with adult mice. Most importantly, differential kinetics of cytokine mRNA up-regulation in pup and adult lungs corresponded to the ability to clear *P. carinii* in these two groups.

We could not exclude the possibility that the delayed inflammatory response in the neonatal lungs is a consequence of some form of down-regulatory mechanism in the neonatal lung environment. Therefore, we examined the expression of anti-inflammatory cytokines IL-10 and TGF-β mRNA in the neonatal lungs. Interestingly, expression of IL-10 mRNA was elevated in the adult lungs compared with the neonates at days 6 and 13 postinfection (Fig. 3*A*). However, neonatal mice had significantly elevated expression of TGF-β2 and 3 in the lungs compared with the adults at days 6 and 13 postinfection (Fig. 3, *C* and *D*). There was no significant difference in the expression of TGF-β1 in the neonatal

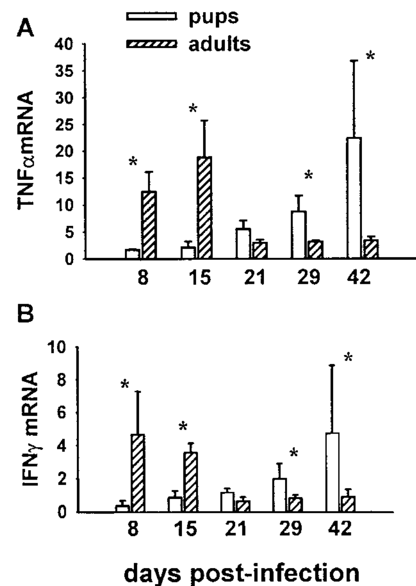

FIGURE 2. Cytokine messenger RNA expression is delayed in the lungs of normal BALB/c mice infected as neonates. Eight-week- and 48- to 72-h-old BALB/c mice were infected i.n. with 1×10^7 and 2×10^6 *P. carinii* organisms, respectively. Lungs were snap frozen and expression of cytokine mRNA was quantified using an RPA. *A,* Expression of TNF-α mRNA. *B,* Expression of IFN-γ mRNA. Data represent the mean ± SD of three to four mice per group and is representative of two separate experiments. *, $p < 0.05$ compared with adults at the same time point.

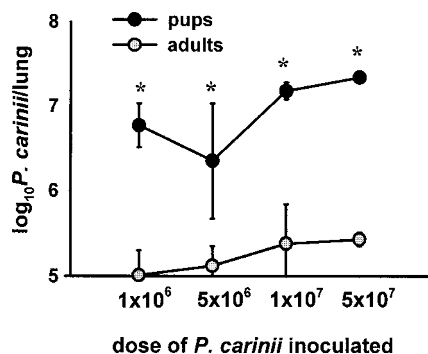

FIGURE 1. Resolution of *P. carinii* is delayed in mice infected as neonates compared with adults. Neonatal or adult BALB/c mice were inoculated i.n. with varying doses of *P. carinii*, and lung burden was determined at day 21 postinfection. Data are expressed as $\log_{10}$ *P. carinii* per right lung lobes. Data represent mean ± SD of three to four mice per group. *, $p < 0.05$ compared with adults at the same time point.

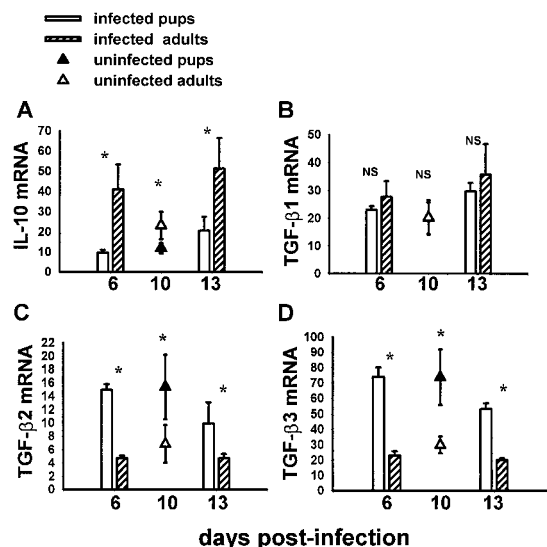

FIGURE 3. Expression of TGF-β but not IL-10 mRNA is significantly up-regulated in the lungs of neonatal mice compared with adults. Mice were infected with *P. carinii*, and left lung lobes were snap frozen at the times indicated and used for RPA. *A,* Expression of IL-10 mRNA. *B, C,* and *D,* Expression of TGF-β1, 2, and 3 mRNA, respectively. Data represents the mean ± SD of three to four mice per group and is representative of three separate experiments. *, $p < 0.05$ compared with adults at the same time point, NS, no statistically significant difference.

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lungs compared with the adults (Fig. 3*B*). Moreover, TGF-β expression in pups was not driven by *P. carinii* infection because uninfected pups also had elevated TGF-β2 and 3 compared with adults (Fig. 3). This elevated TGF-β mRNA corresponded to the time frame during which expression of IFN-γ and TNF-α mRNA was depressed in neonates, suggesting that TGF-β signaling may modulate the pro-inflammatory cytokine induction in the neonatal lungs.

Proliferation of pup and adult TBLN cells in response to Con A and anti-CD3 mAb

Next we addressed the question of whether the observed inefficient clearance of *P. carinii* and inadequate cytokine up-regulation in the lungs of neonates was due to an intrinsic defect in neonatal lymphocytes. We examined, ex vivo, the responsiveness of lymphocytes isolated from TBLN of mice infected with *P. carinii* as neonates. Adult or 48-h-old neonatal BALB/c mice were infected with *P. carinii* and $CD3^+$ T cells obtained from TBLN 14 days after infection. Cells were stimulated with various doses of Con A and plate-bound anti-CD3 mAb. Five days after the initiation of culture, the proliferative response was evaluated by Alamar Blue reduction. Stimulation with Con A at 1, 2.5, and 10 μg resulted in proliferation of both pup and adult lymphocytes (Fig. 4*A*). Adult lymphocyte proliferation caused only 2–4% more Alamar Blue reduction than did proliferation of pup cells. Stimulation was maximal with 1 or 2.5 μg of Con A in both pup and adult lymphocytes. In addition, both pup and adult lymphocytes proliferated after stimulation with plate-bound anti-CD3 mAb at 1, 2.5, and 5 μg/ml. Stimulation of pup lymphocytes with anti-CD3 mAb resulted in 2–4% more Alamar Blue reduction than the adult lymphocytes. These data indicate that the lymphocytes from mice infected as neonates can proliferate, ex vivo, as efficiently as adult lymphocytes when appropriately activated through a TCR-dependent or independent pathway.

We also examined the ability of TBLN cells from pups and adults to produce IFN-γ in response to anti-CD3 mAb. The level of IFN-γ in the TBLN cultures of pup origin was >2-fold higher compared with that of cultures of adult origin (Fig. 4*B*). These findings suggest that, when activated appropriately, lymphocytes from mice infected as neonates are capable of producing equivalent or even greater amounts of cytokines than do adult lymphocytes. Therefore, the delayed up-regulation of cytokines in neonatal lungs in response to *P. carinii* may not be due to incompetent neonatal lymphocytes but rather a lack of appropriate activation signaling or, alternatively, active suppression.

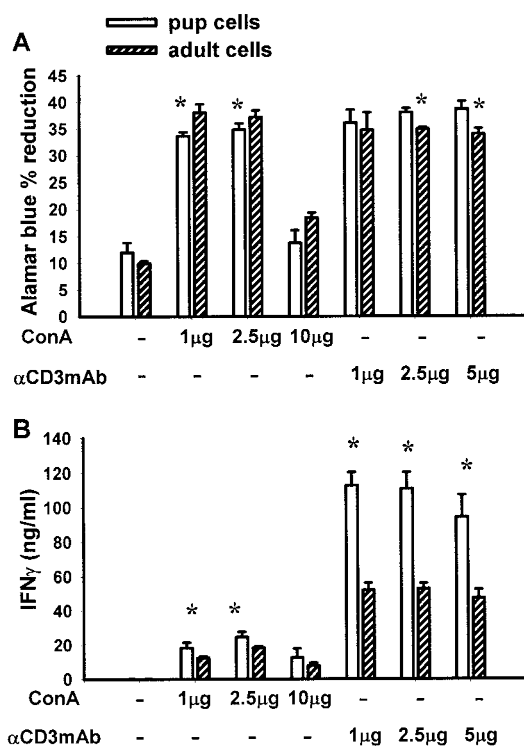

FIGURE 4. $CD3^+$ T lymphocytes from TBLN of mice infected as neonates respond, ex vivo, to Con A and anti-CD3 mAb as efficiently as those from adult mice. 48-h- and 8-wk-old BALB/c mice were infected i.n. with 2×10^6 and 1×10^7 *P. carinii* organisms, respectively. At day 14 postinfection, TBLNs were excised from both groups, and 1×10^6 $CD3^+$ T lymphocytes were cultured in triplicate for 5 days with varying doses of Con A and anti-CD3 mAb as shown. *A*, Proliferative responses were measured by Alamar Blue assays and expressed as percent reduction of the dye. *B*, Amount of IFN-γ production in the culture supernatants as determined by ELISA. Data are expressed as mean ± SD of triplicate cultures. The data shown are representative of two independent experiments. $*$, $p < 0.05$ compared with adults.

P. carinii *resolution from adult SCID lungs after reconstitution with pup lymphocytes*

To determine whether the delayed resolution of *P. carinii* in neonatal mice was due to functionally immature lymphocytes as opposed to an immature lung environment, the functional competence of neonatal lymphocytes when harbored in an adult lung environment was examined. *P. carinii*-infected adult SCID mice were reconstituted with splenocytes isolated from adults or 10-day-old pups. $CD4^+$ cells represented on average 20% of adult splenocytes but only 5–10% of pup splenocytes (data not shown). Therefore, we adjusted the number of splenocytes injected into the adult SCID mice to ensure that 2×10^6 $CD4^+$ T cells were transferred. Reconstitution with pup or adult lymphocytes resulted in a significant reduction in lung organisms as compared with unreconstituted mice (Fig. 5). The organisms grew until day 14 in all the groups before decreasing in the reconstituted groups beginning at day 21 (Fig. 5). Mice reconstituted with pup or adult cells had over 100 times fewer *P. carinii* organisms as compared with the unreconstituted mice at day 45 postreconstitution (Fig. 5*B*). There was no statistically significant difference in the *P. carinii* burden at any time point between the pup and adult cell-reconstituted groups.

Because the number of $CD4^+$ cells transferred was kept constant, mice given pup cells also received significantly more B and accessory cells than mice given adult cells. To control for the number of B cells, mixing experiments were performed. *P. carinii*-infected adult SCID mice were reconstituted with 4×10^6 neonatal or adult $CD3^+$ T lymphocyte-enriched (by panning with polyvalent Ig) cells mixed with 6×10^6 adult B lymphocyte-enriched (by treatment with anti-Thy1.2 Ab and complement) cells. There was no difference in lung *P. carinii* burden in mice reconstituted with neonatal T cells compared with those reconstituted with adult T cells by day 21 postreconstitution (data not shown). These data indicate that the pup T lymphocytes were equally efficient as those of adults in clearing the *P. carinii* organisms when harbored in an adult lung environment.

Competent migration of lymphocytes of pup origin to site of infection

Flow cytometric analysis of lung lavage and lung digest cells revealed similar kinetics of $CD4^+$ and $CD8^+$ T cell infiltration in the pup compared with the adult cell-reconstituted mice (Fig. 6 and data not shown). $CD4^+$ and $CD8^+$ T cells were detected in the

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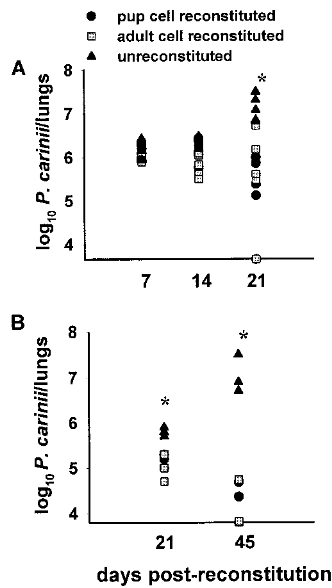

FIGURE 5. Neonatal lymphocytes harbored in an adult lung environment resolve *P. carinii* infection as competently as adult lymphocytes. *P. carinii*-infected adult SCID mice were reconstituted with splenocytes containing 2×10^6 $CD4^+$ cells isolated from adult or 10-day-old mice. Lung *P. carinii* burden of two separate experiments is shown. *A,* Mice were sacrificed on days 7, 14, or 21 postreconstitution. *B,* Mice were sacrificed on days 21 and 45 postreconstitution. Data are expressed as $\log_{10}$ *P. carinii* per right lung lobes. Data are shown for individual mice. *, $p < 0.05$ compared with unreconstituted mice at the same time point. There are no statistically significant differences between the reconstituted groups.

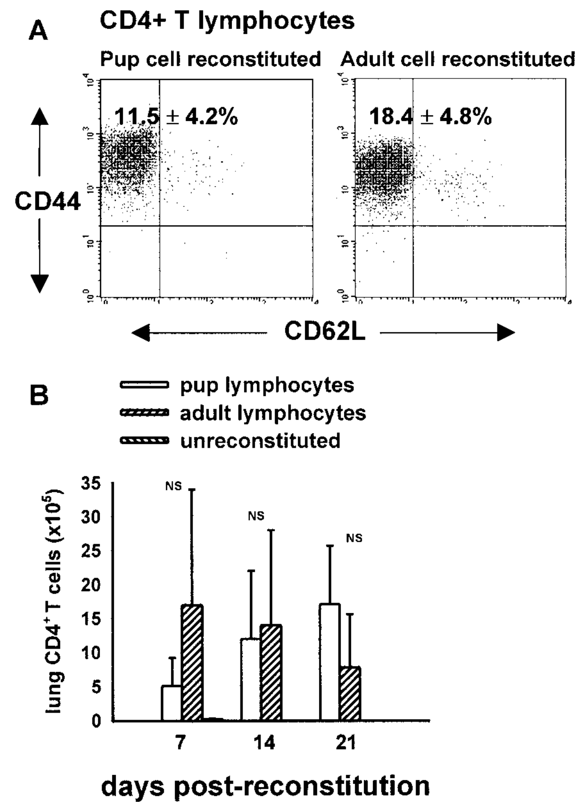

FIGURE 6. $CD4^+$ T cell response in the lungs of SCID adults reconstituted with pup splenocytes are comparable to that of adult cell-reconstituted SCID mice. *P. carinii*-infected adult SCID mice were reconstituted as per Fig. 5. Single lung cell suspensions were analyzed for the presence of activated and total $CD4^+$ T cells by flow cytometry. *A,* Proportion of $CD44^{high}CD62L^{low}$ $CD4^+$ T lymphocytes present in the lung lavage at day 21 postreconstitution. *B,* Total $CD4^+$ T cells present in the lung interstitium. Data represent the mean ± SD of six mice per group and is representative of three separate experiments. There are no statistically significant differences between the reconstituted groups.

lungs of both pup and adult cell-reconstituted mice at day 7 postreconstitution. Lung T cells increased over time and peaked by day 21 postreconstitution. There were no statistically significant differences in the total number of $CD4^+$ or $CD8^+$ T cells present in the lung digests between the reconstituted groups at any time points examined (Fig. 6*B* and data not shown).

Comparable numbers of activated (CD44 ^{high}CD62L^{low}) $CD4^+$ and $CD8^+$ cells were present in the lung lavages of mice reconstituted with lymphocytes of pup or adult origin (Fig. 6*A* and data not shown). Migration of $CD4^+$ and $CD8^+$ cells into the alveolar spaces increased by as much as 40-fold in the pup and adult cell-reconstituted mice from day 7 through day 21 postreconstitution (data not shown). This cellular infiltration into alveolar spaces preceded clearance of lung *P. carinii* burden that was observed by day 21 postreconstitution. Notably, the presence of activated ($CD44^{high}CD62L^{low}$) $CD4^+$ and $CD8^+$ cells in the lung lavages was comparable in both pup and adult cell-reconstituted groups at day 21 postreconstitution (Fig. 6*A* and data not shown). This corresponded with the significant decrease in *P. carinii* lung burden over the same time frame.

Cytokine mRNA expression in SCID lungs after reconstitution

Because our previous results demonstrated that the inflammatory response to *P. carinii* corresponds to up-regulation of lung cytokine mRNA expression, we examined cytokine mRNA expression in the lungs of SCID mice reconstituted with pup or adult splenocytes. Expression of cytokine mRNA in the infected lungs of mice reconstituted with pup or adult cells was not different at any of the time points examined. The expression of TNF-α, IFN-γ, IL-2, and IL-6 mRNA in the lungs was comparable in pup and adult cell-reconstituted animals at days 7 and 14 postreconstitution (Fig. 7 and data not shown). Quantitative analysis of mRNA expression confirmed that there was no significant difference in TNF-α, and IFN-γ expression between the two groups during this time frame (Fig. 7, *A* and *B*). TNF-α and IFN-γ expression peaked at day 14 postreconstitution and then declined through day 45 in both the reconstituted groups (Fig. 7 and data not shown). The kinetics of cytokine mRNA expression in the lungs of pup and adult cell-reconstituted mice corresponded with the timing of *P. carinii* resolution (Fig. 5). Thus pup lymphocytes, when harbored in an adult lung environment, were associated with adult-like cytokine mRNA up-regulation and *P. carinii* clearance.

Discussion

The major finding of this study was that neonatal T lymphocytes, when harbored in a favorable adult lung environment, were equally competent as those of adults in resolving PCP. Reconstitution of *P. carinii*-infected adult SCID mice with lymphocytes from 10-day-old BALB/c mice demonstrated comparable kinetics of clearance of the organisms as observed in mice reconstituted with adult lymphocytes. This phenomenon was associated with a similar kinetics of migration and activation of $CD4^+$ and $CD8^+$ T lymphocytes, as well as comparable lung cytokine mRNA up-regulation.

Host defense against *P. carinii* infection is critically dependent on T lymphocytes, specifically $CD4^+$ T lymphocytes. This has

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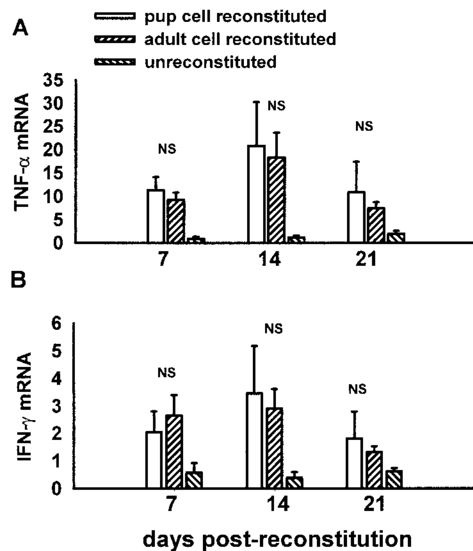

FIGURE 7. Expression of cytokine mRNA in *P. carinii*-infected adult SCID mice was not different after reconstitution with lymphocytes of pup or adult origin. Infected SCID mice were reconstituted as per Fig. 5. Lungs were snap frozen and expression of cytokine mRNA was quantified using RPA. *A,* Expression of TNF-α mRNA. *B,* Expression of IFN-γ mRNA. Data represent the mean ± SD of three to four mice per group and are representative of three separate experiments. There are no statistically significant differences between the two reconstituted groups.

been shown by deletion of $CD4^+$ cells using Ab or targeted mutations (24, 25) or by adoptive transfer of $CD4^+$ cells into immunodeficient mice (26). We also observed resolution of *P. carinii* from the lungs of SCID mice after reconstitution with $CD4^+$ cells from pup and adult BALB/c mice. Lymphocytes of pup origin showed migration kinetics similar to that of adults. Their activation status, as indicated by $CD44^{high}CD62L^{low}$ surface expression, was also similar. Therefore, $CD4^+$ T lymphocytes of pup origin were capable of responding to *P. carinii* challenge when harbored in an adult lung environment. In contrast, pup $CD4^+$ cells in their natural environment are inefficient at resolving PCP as seen in the delayed inflammatory response in infected BALB/c pups (23, 28). Thus the delayed onset of the inflammatory response to *P. carinii* infection in neonates is not necessarily due to an incompetent $CD4^+$ T lymphocyte population but rather due to naiveté or inadequacy of some other factors, or alternatively, to active suppression in the neonatal lung environment. In addition to the above observations, it is relevant to mention that the proportion of $CD4^+$ T cells was much lower in pup as compared with adult splenocytes (5–8% vs 20–25%). Therefore, *P. carinii*-specific $CD4^+$ cells were even fewer. This low frequency of *P. carinii*-specific T cells may contribute to the delayed clearance of the organisms from the neonatal lungs. However, we have found that reconstitution of neonatal SCID mice with adult splenocytes also failed to resolve the PCP compared with adult reconstituted mice (28) indicating inadequacy in the neonatal lung environment.

Using in vitro approaches, many laboratories have compared the functional activities of neonatal and adult T cells. Human and murine neonatal T lymphocytes produce low IL-2 and IFN-γ and proliferate poorly in response to physiologically relevant stimuli, such as anti-CD3 mAb (9–12). By contrast, they are capable of adult-level IL-2 production and proliferation by TCR-independent stimulation (29). In contrast to these previous studies, we observed an adult-like proliferative response in the $CD3^+$ T lymphocytes from TBLN of mice infected as neonates after stimulation with either Con A or plate-bound anti-CD3 mAb. Moreover, production of IFN-γ by the pup lymphocytes was significantly higher than the adult lymphocytes after TCR-dependent stimulation with anti-CD3 mAb (Fig. 4*B*). In our studies, TBLN cells were collected on day 14 postinfection, whereas other studies used naive lymph nodes from 8-day-old or younger mice. We selected this time point on the basis of in vivo kinetics of cell migration in the immunocompetent neonates (Ref. 23 and data not shown). No activated T cells had migrated to the alveolar spaces by day 21 postinfection, indicating inadequate lymphocyte responsiveness or lack of appropriate adhesion molecule expression and chemokine up-regulation. Thus, TCR-dependent proliferation and IFN-γ production indicate that neonatal T cells are not inherently hyporesponsive.

Activation of T cells is largely dependent on local cytokine environment, which influences the up-regulation of costimulatory molecules and APC function necessary for effective lymphocyte activation (30–33). Consistent with the absence of activated T cells, mice infected as neonates displayed much less lung cytokine mRNA expression, including IFN-γ and TNF-α, compared with adults at day 15 postinfection (Fig. 2). It is interesting to note that T cells from pups of the same age as those used for in vivo studies produced significantly higher levels of IFN-γ when activated, ex vivo, through a TCR-dependent pathway. These data suggest that the significantly low cytokine mRNA expression in *P. carinii*-infected neonatal lungs was due to unavailability of appropriate activation signaling rather than an incompetent lymphocyte population.

An alternative explanation for delayed T cell responses in the lungs of neonatal mice may be that there is active suppression taking place in the form of expression of anti-inflammatory cytokines. In this regard, we found that neonatal mice expressed large amounts of TGF-β2 and 3 mRNA in the lungs at early time points when compared with adults. TGF-β1, 2, and 3 are pleiotropic cytokines that have been reported to be involved in developmental processes of organs, including lungs, as well as in immunomodulation (34, 35). TGF-β down-regulates T cell function directly and indirectly via down-regulating dendritic cell function (36–38). TGF-β also down-regulates some macrophage functions in favor of infectious pathogens (35, 39). Therefore, elevated TGF-β expression (Fig. 3) may be responsible, directly or indirectly, for delayed inflammation and clearance of PCP. Interestingly, TGF-β expression was also up-regulated in the uninfected neonates compared with adults (Fig. 3), indicating that TGF-β-mediated immunomodulation is not driven by *P. carinii* infection but is a developmental phenomenon.

Local cytokine milieu is critical for determining activation, clonal expansion, and differentiation of naive T cells to effector cells. During this process of activation and differentiation, specific adhesion molecules and chemokine receptors are induced on the surface of activated T cells, enhancing their migration to sites of infection. Rudmenn et al. have demonstrated that mice lacking both the TNF receptor gene (TNFRI and TNFRII), as well as the IFN-γ gene, develop severe *P. carinii* infection despite the presence of $CD4^+$ T lymphocytes (22). Chen et al. reported that TNF-α is required early in the immune response to *P. carinii* (21). In these studies, lungs from immunocompetent BALB/c neonates displayed a delayed induction of cytokines, including TNF-α and IFN-γ (Fig. 2), which corresponded to the delayed *P. carinii* resolution. Thus, defective cytokine production may explain the inability of neonates to clear *P. carinii*.

TNF-α has been reported to be involved in the induction of several adhesion molecules, including lymphocyte function-associated Ag 1, Mac-1, and ICAM-1 on lymphocytes and macrophages (40); and ICAM-1, VCAM-1, and endothelial leukocyte

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adhesion molecule-1 (ELAM-1) on endothelial cells (41). Furthermore, TNF-α has been reported to enhance production of a variety of chemokines including monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α, β (MIP-1α, β), and RANTES (42, 43). MCP-1 has been shown to be important for protection against pulmonary *Cryptococcus neoformans* infection by recruiting CD4$^+$ T lymphocytes to the lungs (44). RANTES and MIP-1α, β are also strong chemoattractants for T lymphocytes (43). IFN-γ up-regulates induction of IFN-γ-inducible protein 10 (IP-10), monokine induced by IFN-γ (Mig), and IFN-inducible T cell α chemoattractant (I-TAC), all of which have a common receptor, CXC chemokine receptor 3 (CXCR3), expressed on CD4$^+$ T lymphocytes (45). Therefore, inadequate TNF-α and IFN-γ production in the neonatal lungs may have adversely affected the adhesion molecule and chemokine-dependent functions critical for T lymphocyte recruitment. Consistent with this, we have observed that up-regulation of lymphotactin, RANTES, MCP-1, MIP-1α, β was delayed in *P. carinii*-infected neonatal lungs until day 21 postinfection, whereas an enhanced expression of these chemokines was observed in adult lungs as early as day 7 postinfection (our unpublished observation). In this regard, up-regulation of both TNF-α and IFN-γ in postreconstitution adult SCID lungs was comparable in mice given splenocytes from neonates or adults, suggesting that neonatal lymphocytes can up-regulate cytokine expression in a favorable lung environment.

Taken together, these results indicate that neonatal lymphocytes are competent to up-regulate lung IFN-γ and TNF-α mRNA expression and resolve *P. carinii* infection in an adult lung environment. These data are consistent with our observation that transfer of adult splenocytes to *P. carinii*-infected neonatal SCID mice failed to up-regulate cytokine and chemokine mRNA expression in the neonatal lungs (28). Therefore, inefficient resolution of *P. carinii* from neonatal lungs was not due to their naive T lymphocyte population, rather it is possible that the lung environment in neonates was not conducive for cytokine/chemokine up-regulation, lymphocyte migration, and, thereby, *P. carinii* resolution. This could be due to increased anti-inflammatory cytokines seen in neonatal lungs. To have a complete understanding of the delay of *P. carinii* resolution in neonates, more data are needed regarding neonatal lung environment with special reference to expression of adhesion molecules, chemokines and corresponding receptors, and APC functional status. These issues are currently being addressed.

Acknowledgments

We thank Dr. Claire Pomeroy for critical reading of the manuscript and Kevin Schuer and Wayne Young for expert technical assistance.

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