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Modulation of Proinflammatory Responses to *Pneumocystis carinii f. sp. muris* in Neonatal Mice by Granulocyte-Macrophage Colony-Stimulating Factor and IL-4: Role of APCs

Mahboob H. Qureshi, Kerry M. Empey, and Beth A. Garvy

Clearance of *Pneumocystis carinii f. sp. muris* (PC) organisms from the lungs of neonatal mice is delayed due to failure of initiation of inflammation over the first 3 wk after infection. The ability of neonatal lung CD11c+ dendritic cells (DCs) to induce Ag-specific T cell proliferative responses was significantly reduced compared with adult lung DCs. However, neonatal bone marrow-derived DCs were as competent at presenting PC Ag as were adult bone marrow-derived DCs. Because GM-CSF mRNA expression and activity were significantly reduced in neonatal lungs compared with adults, we treated neonates with exogenous GM-CSF and IL-4 and found enhanced clearance of PC compared with untreated neonates. This was associated with increased lung TNF-α, IL-12p40, and IL-18 mRNA expression, indicating enhanced innate immune responses. Cytokine-treated mice had marked expansion of CD11c+ DCs with up-regulated MHC-II in the lungs. Moreover, increased numbers of activated CD4+ CD44hiCD62Llow cells in the lungs and draining lymph nodes suggested improved Ag presentation by the APCs. Together these data indicate that neonatal lungs lack maturation factors for efficient cellular functioning, including APC maturation.

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Clearance of *Pneumocystis carinii f. sp. muris* (PC) organisms from the lungs of neonates compared with adults (6–8). Delayed clearance was associated with failure to up-regulate lung chemokines, cytokines, and adhesion molecules, resulting in a significant delay in infiltration of inflammatory cells into the lungs of neonatal mice (6–8). However, T cells isolated from the draining lymph nodes were able to produce IFN-γ and proliferate ex vivo in response to anti-CD3 stimulation. These data led us to conclude that some aspect of the neonatal lung environment was lacking in its ability to respond to PC. One possibility is that APC functions are ineffective in PC-infected neonatal lungs, resulting in inadequate Th cell responses and PC clearance.

Ag presentation to specific T cells is a key step in the induction of an inflammatory response in the lungs. Even though dendritic cells (DCs), macrophages, and B cells are professional APCs, DCs are the major APCs of the respiratory tract. Therefore, functional immaturity of DCs in neonates may account for their inability to mount an inflammatory response to PC. In this connection, it has been reported that in neonatal rats there is delayed maturation of respiratory tract DCs characterized by decreased expression of MHC-II and other maturation markers in response to heat-killed *Moraxella catarrhalis* (8, 9). Recently, it has been demonstrated that in vivo administration of Flt-3 ligand enhanced neonatal innate immune responses and defense against HSV-1 and *Listeria monocytogenes*, presumably by expanding neonatal splenic DC populations and enhancing IL-12 and type I IFN production (10).

Although there are indications that neonatal DCs are limited in numbers and lack phenotypic and functional maturity, it is still unclear whether this inadequacy is due to intrinsic or extrinsic factors. These studies address whether neonatal DC have underlying intrinsic defects resulting in inadequate Ag-presenting capabilities.

GM-CSF is a pluripotent cytokine that influences the development and function of myelomonocytic lineage cells, including macrophages and DCs (11, 12). GM-CSF also mediates protective innate immune responses to PC in adult mice and in other infectious disease models (13–17). Furthermore, exogenous administration of GM-CSF alone or in combination with IL-4 has been shown to expand DCs in mice (18). In the present studies, we have used a murine model of PC infection to investigate DC function in neonatal lungs. We have also examined how modulation of the neonatal lung environment by exogenous GM-CSF and IL-4 treatment influences cellular responses, including APC functions. Both in vivo and in vitro assays were used to examine neonatal lung and bone marrow-derived DC (BMDC) functions in the context of a
fungal lung infection. These studies demonstrate that GM-CSF is limiting in neonatal lungs, and that exogenous administration induces enhanced APC functions and augmented innate immune responses, resulting in expedited PC resolution.

Materials and Methods

Mice

Five- to 6-week-old and mid-term pregnant BALB/c mice were purchased from the National Cancer Institute or Harlan Sprague Dawley. Mice were maintained at the Veterinary Medical Unit of the Veterans Administration Medical Center under specific pathogen-free conditions. C.B-17 SCID mice, used to maintain a source of PC, were also bred at the Veterans Administration Medical Center Veterinary Medical Unit in microisolator cages containing sterilized food and water. Protocols for the usage of mice were approved by the Veterans Administration Medical Center Institutional Animal Care and Use Committee.

PC infection

Lungs were excised from PC-infected SCID mice and pushed through stainless steel mesh in HBSS. PC organisms were isolated and enumerated by microscopy, as described (6). The 8-week-old and 48- to 72-hour BALB/c mice were inoculated intranasally with 1 x 10⁶ and 2 x 10⁶ PC organisms, respectively.

Isolation of lung alveolar, interstitial, and tracheobronchial lymph node (TBLN) cells

Lung and TBLN cells were prepared, as described elsewhere (7). Adult and pup lungs were lavaged with 5 x 1 and 5 x 0.2 ml washes of cold HBSS/EDTA (3 mM), respectively. Right lung lobes were excised, minced, and enzyme treated at 37°C for 60 min in RPMI 1640 containing 3% FCS, 50 U/ml DNase (Sigma-Aldrich), and 1 mg/ml collagenase A (Sigma-Aldrich). Digested lung tissue and lymph nodes were pushed through mesh screens, and images were developed using a Storm 860 PhosphorImager. The magnitude of dye reduction and is expressed as percent reduction.

Preparation of cells for flow cytometric analysis

Cells derived from lungs and TBLN tissue (5 x 10⁶ to 1 x 10⁷ cells) were used for staining with fluorochrome-conjugated Abs specific for murine MHC-II (BD Pharmingen). Multiparameter analysis was performed using a FACSCalibur (BD Biosciences). Greater than 50,000 events were routinely acquired.

Phenotypic characterization of harvested DC by flow cytometric analysis

DCs were collected according to the manufacturer’s protocol. Results were determined to be statistically significant when p < 0.05 was obtained.

Ag presentation assay

DCs were generated in vitro from bone marrow cells or were isolated from 10-day-old pup or adult lungs, as described above. T lymphocytes were isolated from the TBLNs of PC-infected adult mice. Adult mice were given two inoculations of PC organisms at 4-week intervals, and TBLNs were collected from infected mice on day 5 of the secondary challenge. Cells isolated from lymph nodes were stained with anti-B220 Ab, and negative sorting was performed using a MoFlo cell sorter (DakoCytomation) to eliminate B cells. More than 95% negatively sorted cells were CD3⁺ T cells, as determined by FACS. PC Ag was prepared from purified PC organisms isolated from infected mouse lungs by using a sonic cell disruptor (VirTis). Ag presentation was measured by culturing PC-specific T cells (2 x 10⁶/well) with neonatal or adult DCs at different ratios (e.g., at 1:20, 1:10, and 1:5, DC to T cell ratio) in 96-well flat-bottom tissue culture plates in the presence or absence of PC Ag (1:50, v/v) and mouse rGM-CSF (20 ng/ml; BioSource International) and mouse rIL-4 (20 ng/ml; BioSource International) at 0.5 μg/g/day in two divided doses. Cytokines were given every day for 5 days, and expansion of DCs was examined 2 days later. In selected experiments, mice were infected with PC, and cytokines were given from days 0 through 5 postinfection.

In vivo expansion of DCs

Expansion of DC, in vivo, was performed, as described by Basak et al. (18), with slight modifications. The 48- to 72-hour mice were given i.p. injections of recombinant murine GM-CSF (BioSource International, and PeproTech) and IL-4 (BioSource International and PeproTech) at 0.5 μg/g/day in two divided doses. Cytokines were given every day for 5 days, and expansion of DCs was examined 2 days later. In selected experiments, mice were infected with PC, and cytokines were given from days 0 through 5 postinfection.

Extraction of RNA and RPA

Total RNA was prepared from frozen lung tissue by using TRizol (Invitrogen Life Technologies), according to manufacturer instructions. Multi-probe DNA templates for cytokines (GM-CSF, IFN-γ, TNF-α, IL-1β, IL-12, IL-18) and housekeeping genes, L32 and GAPDH, were purchased from BD Pharmingen. RPA was performed using the RiboQuant in vitro transcription and RPA kits (BD Pharmingen), according to the manufacturer’s protocol. RNase-protected RNA duplexes were resolved on 3% denaturing polyacrylamide gels. Dried gels were exposed to PhosphorImager screens, and images were developed using a Storm 860 PhosphorImager (Amersham Pharmacia Biotech). The intensity of each specific cytokine band was measured by using ImageQuant software (Amersham Pharmacia Biotech). The cytokine mRNA levels were corrected for gel RNA loading by dividing the cytokine hybridization signal by the L32 signal for the same sample.

Measurement of GM-CSF, IL-12, IL-18, and TNF-α in the bronchoalveolar lavage fluid (BALF)

GM-CSF levels in BALF were measured by ELISA (eBioscience), according to manufacturer protocol. IL-12, IFN-γ, and TNF-α levels in the BALF were measured by cytometric bead assay (BD Pharmingen), according to manufacturer protocol.

Statistical analysis

The results were tested statistically by Student’s t tests or ANOVA, followed by Student-Neuman-Keul’s posthoc test, where appropriate, using commercially available software (Sigmastat; SPSS). Results were determined to be statistically significant when p < 0.05 was obtained.
Results

Inefficient Ag presentation by neonatal lung DCs

We have previously reported that there is a significant delay in the infiltration of activated T cells into the lungs of PC-infected neonatal mice compared with adults (8). Because neonatal lung DCs have been reported to be phenotypically immature (20), we wanted to know whether the delayed T cell responses to PC in neonates are due to less efficient APC function compared with adult DCs. We examined the in vitro Ag presentation ability of lung DCs obtained from 7-day-old and adult mice (Fig. 1). Neonatal lung DCs did not induce a T cell proliferative response to PC Ag at DC to T cell ratios of 1:20 and 1:10. However, there was a moderate T cell proliferation at a DC to T cell ratio of 1:5. In contrast, DCs isolated from adult lungs stimulated significant T cell proliferation at ratios as low as 1:20 (Fig. 1). Moreover, T cell proliferation was significantly higher when adult DCs were used in cultures instead of neonatal DCs at a ratio of 1:5 (DCs to T cells), indicating that neonatal lung DCs are less efficient APCs compared with the adult lung DCs.

Neonatal bone marrow progenitor cells have the potential to differentiate into mature, competent DC

The results described in Fig. 1 suggested that neonatal lung DCs do not have the same functional efficiency as adult DCs. Therefore, we next examined whether this defect is developmental and whether neonatal bone marrow progenitor cells have the potential to differentiate into DCs when stimulated, in vitro, with GM-CSF and IL-4. Bone marrow cells obtained from 5-day-old and adult mice were cultured, in vitro, with GM-CSF and IL-4, as described by Sato et al. (19). FACS analysis confirmed that ~90% of cells harvested from either neonatal or adult bone marrow cultures were CD11c+ BMDCs (Fig. 2A). Moreover, maturation of neonatal and adult BMDCs, as determined by expression of MHC-II and costimulatory molecules, including CD40, CD80, and CD86, was also comparable (Fig. 2A).

We next examined the APC function of these BMDCs. T lymphocytes isolated from TBLN of PC-infected adult mice were cultured with PC Ag and either neonatal or adult BMDCs. Proliferation of T cells in response to PC Ag presented by neonatal BMDCs was not significantly different compared with stimulation with adult DCs after 5 days of culture (Fig. 2B). This indicated that
neonatal BMDCs were efficient at presenting PC Ag to T lymphocytes and thereby capable of inducing proliferative responses comparable to stimulation with adult DCs.

**Differential GM-CSF mRNA expression and activity in neonatal and adult lungs**

Neonatal lung DCs were immature, as evidenced by less efficient APC functions. However, neonatal bone marrow progenitor cells, when stimulated with GM-CSF, differentiated into efficient DCs. Moreover, the involvement of GM-CSF in the development of myelomonocytic lineage cells, including DCs, and its role in enhancing innate immune responses in PC-infected mice have been well documented (11–13). To determine whether delayed DC maturation and delayed PC clearance in neonates are associated with GM-CSF deficiency, we examined the kinetics of GM-CSF mRNA expression and protein activity in PC-infected neonatal and adult lungs. As shown in Fig. 3A, expression of GM-CSF mRNA in neonatal lungs in response to PC was significantly lower than that in adult lungs on day 8 postinfection. However, expression of GM-CSF was up-regulated in neonatal lungs over the next week and was comparable to that of adults by day 15 postinfection (Fig. 3A). Furthermore, BALF levels of GM-CSF were also significantly lower in neonates than that in adults on days 7 and 10 postinfection (Fig. 3B). Consistent with the GM-CSF mRNA expression kinetics, BALF levels of GM-CSF gradually increased in neonates and became comparable to that in adults by day 17 postinfection. By day 21 postinfection, GM-CSF levels were significantly higher in neonates than that in adults (Fig. 3B).

**GM-CSF does not influence in vitro Ag-presenting function of neonatal lung DCs**

On the basis of our understanding that GM-CSF is crucial for growth and maturation of cells of the myelomonocyte lineage, including DCs, and that neonatal lungs have reduced levels of GM-CSF mRNA expression, we examined the effects of GM-CSF on in vitro neonatal DC function as APC. As shown in Fig. 4, addition of GM-CSF to neonatal lung DC and T cell cultures did not improve T cell proliferative responses to PC Ag. Interestingly, GM-CSF also had no effect on adult lung DC function (Fig. 4).

**In vivo expansion of neonatal DCs by exogenous administration of GM-CSF and IL-4**

Because in vitro treatment with GM-CSF failed to enhance Ag-presenting function of neonatal lung DCs (Fig. 4), we considered that GM-CSF may not influence function as much as expansion of DC populations. Therefore, we examined whether exogenous administration of GM-CSF could induce expansion and/or maturation of neonatal DCs. Forty-eight- to 72-h-old mice were administered i.p. injections of either GM-CSF and IL-4 or PBS twice daily for 5 days. Expansion of neonatal DC populations was examined by determining the proportions of CD11c+ cells in the spleens of cytokine- vs PBS-treated neonates by flow cytometry. Neonates receiving GM-CSF and IL-4 had an almost 5-fold increase in the proportion of CD11c+ splenic DCs compared with that in the control neonates (Fig. 5A). Moreover, cytokine treatment increased neonatal splenic DC numbers to levels comparable with that of adult spleens (Fig. 5A). Consistent with our in vitro observations, proportions of neonatal splenic DCs expressing MHC-II molecules were also significantly increased with GM-CSF and IL-4 treatment as compared with control neonates (Fig. 5B). Moreover, significantly higher proportions of CD11c–MHC-II– cells were also found in the cytokine-treated neonatal lungs (Fig. 5C). In addition, there was no significant difference in the proportion of CD11c+ MHC-II+ cells in the cytokine-treated neonatal lungs (data not shown).
Exogenous GM-CSF and IL-4 enhance clearance of PC organisms from neonatal lungs through augmented proinflammatory responses

Next, we addressed the question of whether expansion of neonatal DC populations and increased MHC-II molecule expression following GM-CSF and IL-4 treatment have any functional significance. Neonatal mice were infected with PC and received GM-CSF and IL-4 or PBS for 5 days starting on the day of infection, as described in Materials and Methods. Lung burdens of PC organisms were determined on days 10 and 20 or, in separate experiments, on day 30 postinfection. As shown in Fig. 6A, lung PC burdens were reduced significantly by day 20 postinfection in GM-CSF- and IL-4-treated neonates compared with PBS-treated neonates. At this time point, cytokine-treated neonatal mice had a median lung PC burden of log_{10} 6.38 as opposed to log_{10} 6.78 in control neonates representing a greater than 60% decrease in lung PC burden upon treatment with GM-CSF and IL-4. Lung burdens of PC were further reduced with GM-CSF and IL-4 treatment by day 30 postinfection (Fig. 6B). In this experiment, two of four cytokine-treated neonates had lung PC burdens below the detection limit (median lung burden log_{10} 4.57). In contrast, all PBS-treated neonatal mice had lung PC burdens that were >25-fold higher than in cytokine-treated pups (median lung burden 6.08) (Fig. 6B). These data indicate that exogenous administration of GM-CSF and IL-4 not only expanded neonatal DCs, in vivo, but also significantly enhanced resolution of PC organisms.

To further investigate the underlying mechanisms of GM-CSF- and IL-4-mediated protective effects against PC, we examined whether there was altered TNF-α expression and/or CD4 cellular responses in the neonatal lungs, which are critically involved in effective PC clearance (21, 22). As shown in Fig. 7A, expression of TNF-α mRNA was significantly higher in the cytokine-treated neonatal lungs compared with the control lungs on day 10 postinfection. At day 20 postinfection, TNF-α mRNA expression was up-regulated in the PBS-treated neonates in response to PC and became significantly higher than that in the cytokine-treated mice (Fig. 7A). Expression patterns of IL-12 p35 and IL-18 mRNA in the PC-infected neonatal lungs were similar to those observed for TNF-α (Fig. 7A). In contrast, levels of IL-12 and TNF-α protein in BALF of cytokine-treated neonates were not significantly different compared with PBS-treated mice at days 10 and 20 postinfection (Fig. 7B). Interestingly, mRNA expression of another important proinflammatory cytokine, IFN-γ, was not influenced by GM-CSF and IL-4 treatment at any time points (Fig. 7A). Consistently, BALF levels of IFN-γ were also comparable in two groups (Fig. 7B).

Activated (CD44^{high}CD62^{low}) CD4^{+} T cells were present in significantly higher numbers in the TBLNs of cytokine-treated neonates on days 10 and 20 postinfection (Fig. 8A). Very few activated CD4^{+} cells were found in alveolar spaces in either the cytokine-treated or control lungs on day 10 postinfection (Fig. 8B). However, at day 20, the numbers of activated CD4^{+} cells in the alveolar spaces of the cytokine-treated neonatal lungs were 4-fold greater than in control lungs (Fig. 8B). Increased infiltration of CD4^{+} cells into the lungs of GM-CSF- and IL-4-treated mice corresponded to controlled growth of PC organisms in the lungs of these mice. Total numbers of activated CD4^{+} T cells in alveolar spaces and TBLNs of cytokine- and PBS-treated neonates were comparable at day 30 postinfection (data not shown). However, B cell function was not augmented by GM-CSF and IL-4 treatment. PC-specific serum IgG levels were minimal and comparable in the

FIGURE 5. In vivo expansion of DCs stimulated by treatment with GM-CSF and IL-4. Neonatal mice were given daily i.p. injections of either GM-CSF and IL-4 or PBS for 5 days. Untreated adults are shown for comparison. Three days later, the proportion of CD11c$^+$ cells (A) in the spleens and the proportion of CD11c$^+$ cells expressing MHC-II$^+$ in the spleens (B) and lungs (C) were determined using flow cytometry. Data are the mean ± SD of three to four mice per group and are representative of two separate experiments. *, p < 0.05 compared with PBS-treated pups.

FIGURE 6. Lung PC burden in neonatal mice treated with exogenous GM-CSF and IL-4. The 48- to 72-h-old mice were infected with PC organisms and treated with exogenous GM-CSF and IL-4 or PBS as in Fig. 5, and lung burdens were determined at the indicated time points. Separate experiments were performed to determine lung PC burdens at days 10 and 20 postinfection (A) or at day 30 postinfection (B). Data points are the log_{10} PC per right lung lobes of individual mice (four mice per group). *, p < 0.05 when compared with PBS-treated neonates at the same time points.

To further investigate the underlying mechanisms of GM-CSF- and IL-4-mediated protective effects against PC, we examined whether there was altered TNF-α expression and/or CD4 cellular responses in the neonatal lungs, which are critically involved in effective PC clearance (21, 22). As shown in Fig. 7A, expression of TNF-α mRNA was significantly higher in the cytokine-treated neonatal lungs compared with the control lungs on day 10 postinfection. At day 20 postinfection, TNF-α mRNA expression was up-regulated in the PBS-treated neonates in response to PC and became significantly higher than that in the cytokine-treated mice (Fig. 7A). Expression patterns of IL-12 p35 and IL-18 mRNA in the PC-infected neonatal lungs were similar to those observed for TNF-α (Fig. 7A). In contrast, levels of IL-12 and TNF-α protein in BALF of cytokine-treated neonates were not significantly different compared with PBS-treated mice at days 10 and 20 postinfection (Fig. 7B). Interestingly, mRNA expression of another important proinflammatory cytokine, IFN-γ, was not influenced by GM-CSF and IL-4 treatment at any time points (Fig. 7A). Consistently, BALF levels of IFN-γ were also comparable in two groups (Fig. 7B).
cytokine- and PBS-treated neonates on days 10 and 19 postinfection (data not shown).

Discussion

In the present studies, we demonstrated that neonatal lung DCs were less efficient at APC function compared with adult DCs. However, this defect was not inherent to the DC progenitor cells as they differentiated into mature DCs with competent APC functions after appropriate cytokine stimulation. More importantly, exogenous administration of GM-CSF and IL-4 induced in vivo expansion and maturation of neonatal DC populations with enhanced APC clearance, which was associated with up-regulated TNF-α, IL-12 p35, and IL-18 mRNA expression and increased numbers of activated CD4+ T cells in the neonatal lungs. Together, these data confirm that the neonatal lung APCs, including DCs and possibly macrophages, lack appropriate cytokine stimulation for effective cellular functioning, which may be the basis for delayed inflammatory responses to \( P. \) carinii infection.

Initiation of a protective inflammatory response is largely dependent on efficient Ag presentation to Ag-specific T cells. In our present studies, we demonstrated that neonatal DCs were significantly less efficient than adult DCs at presenting \( P. \) carinii Ag to T cells (Fig. 1). Our data are consistent with some other recently published studies (22, 23). In those studies, splenic DCs obtained from 3- and 7-day-old mice were severely defective in presenting Ag to Ag-specific T cell clones (23). In addition, human cord blood-derived DCs stimulated significantly less alloreactivity compared with adult peripheral blood-derived DCs (24). However, Dadaglio et al. (25) have recently reported that neonatal splenic DCs are capable of inducing in vivo CTL responses comparable to that of adult DCs. In their experimental system, neonatal DCs were pulsed with peptide ex vivo and then were transferred to adult mice, which may have provided a more favorable environment for induction of immune responses, including the CTL responses. An important difference between our studies and those of Dadaglio et al. (25) is that we have used 7- to 10-day-old lung DCs to examine APC function as opposed to splenic DCs. Lung DCs, particularly neonatal lung DCs, are unique in that they reside in the neonatal lung environment under the influence of predominantly anti-inflammatory conditions induced by IL-10, TGF-β, and glucocorticoids (7, 26, 27). In the study by Dadaglio et al. (25), it was demonstrated that uptake of dextran particles, which involves DEC-205 receptors, by neonatal splenic DCs was comparable to that of adult splenic DCs. In this regard, receptor involvement in the uptake of \( P. \) carinii by DCs is a less well-studied area. Multiple studies suggest that mannose and \( \beta \)-glucan receptors are involved in nonopsonic uptake of \( P. \) carinii by macrophages (28–30). Involvement of different receptors during Ag uptake may be an important factor in inducing differential DC responses. Involvement of receptor-mediated \( P. \) carinii uptake by neonatal DCs and its effect on subsequent T cell responses in neonates are under investigation in our laboratory.

In our in vitro APC functional assays, we observed a moderate T cell response induced by neonatal DC at 1:5 ratios (Fig. 1). The absence of T cell responses at 1 to 10 and 1 to 20 neonatal DC to T cell ratios suggests that there are reduced numbers of mature DCs in neonatal lungs. However, we cannot discount that functionally neonatal lung DCs may not compare with that of adults, because T cell responses to neonatal DCs were significantly lower compared with that of adults at the same DC to T cell ratios. Additional factors that are required for effective pulmonary inflammatory responses include availability of sufficient cytokine/
compared with PBS-treated neonates at the same time points. In the present studies, the total number of cells present in BALF and TBLN was enumerated microscopically. Pheno-
type was determined by flow cytometry. Total num-
bers of CD4^+CD44^highCD62^low cells in the TBLN (A) and the alveolar spaces (B) are shown. Data represent the mean ± SD of three to four mice per group and are representative of two separate experiments. *, p < 0.05 compared with PBS-treated neonates at the same time points.

FIGURE 8. Pulmonary and TBLN cellular responses to PC in the GM-CSF- and IL-4-treated neonates were compared. Neonatal mice were infected with PC and cytokine treated, as described in Fig. 7. At the indicated time points postinfection, lungs were lavaged and TBLN were excised, and the total number of cells present in BALF and TBLN was enumerated microscopically. Phenotype was determined by flow cytometry. Total numbers of CD4^+CD44^highCD62^low cells in the TBLN (A) and the alveolar spaces (B) are shown. Data represent the mean ± SD of three to four mice per group and are representative of two separate experiments. *, p < 0.05 compared with PBS-treated neonates at the same time points.

chemokine support in the local environment (31, 32). Availability of appropriate cytokine(s) significantly influences APC and effector cell functions (33). In the present studies, PC-infected neonatal lungs had significantly lower levels of GM-CSF mRNA expression and protein activity than adults (Fig. 3), which may have delayed the expansion and maturation process of neonatal DCs as well as alveolar macro-
phages. Reduced levels of GM-CSF in neonatal lungs are highly likely to be a developmental phenomenon. Bronchial epithelial cells are the major sources of GM-CSF (34), and these cells are not fully matured in the neonatal lungs (35). Furthermore, high levels of glucocorticoids in the neonatal lungs (35) required for postnatal development and maturation may have inhibitory effects on GM-CSF induction (36, 37). The lack of GM-CSF also influenced TNF-α, IL-12, and IL-18 expression in the neonatal lungs because exogenous GM-CSF administration resulted in up-regulated expression of these cytokines (Fig. 7). Moreover, treatment of neonatal bone marrow progenitors with GM-CSF and IL-4 stimulated maturation of DCs, suggesting that neonatal DCs are capable of maturation given the proper signals. In this connection, human cord blood-derived DCs, when stimulated with LPS or CD40 cross-linking, became mature and induced in vitro Ag-specific CTL responses (38). Therefore, inadequate function of neonatal lung DC is, at least partially, due to lack of appropriate cytokine(s) in the neonatal lung environment. We have reported that a predominance of anti-inflammatory cytokines, including IL-10 and TGF-β, may be a limiting factor for the up-regulation of proinflammatory cytokines in neonatal lungs in response to PC (7).

Another limiting factor for inducing effective T cell response in neonates may be reduced numbers of DCs available to function as APC (8, 25). This is supported by the fact that T cell responses and PC clearance were augmented upon expansion of DC populations in vivo by exogenous GM-CSF and IL-4 treatment (Figs. 5, 6, and 8). Furthermore, our in vitro data suggest that GM-CSF does not have direct effects on DC APC function, and therefore, it is pos-
sible that GM-CSF is required for expansion of DCs in the lungs. However, another possibility is that the lack of an in vitro effect of GM-CSF, as opposed to the in vivo effects, may be due to a shorter exposure of the cells to GM-CSF, as GM-CSF was added only once at the beginning of culture as opposed to twice per day injection of GM-CSF to the neonatal mice. Positive effects of expansion of neonatal DC populations on antimicrobial activities have also been reported recently by Vollstedt et al. (10). In this study, Flt3 ligand-induced expansion of splenic DCs elicited IL-12-dependent increases in innate immunity and protection against HSV-1 and L. monocytogenes. Our finding of enhanced expression of TNF-α, IL-12, and IL-18 mRNA in neonatal lungs (Fig. 7) suggested that innate immune responses, possibly by alveolar macro-
phages, were augmented by exogenous GM-CSF and IL-4 treat-
ment. However, TNF-α and IL-12 protein levels were not signif-
icantly different between cytokine-treated and control neonates at any time, even though the trend was similar to that of mRNA expression. The kinetics of cytokine production follows that of mRNA expression due to various steps of posttranscriptional pro-
cessing before protein synthesis. Therefore, we cannot discount the possibility that we may have missed the kinetics of increased pro-
duction of TNF-α and IL-12 in response to GM-CSF.

Macrophages and DCs both are potential sources of IL-12 (39). However, it has not yet been determined which cell population(s) is/are involved in the increased IL-12 mRNA expression in the lungs following exogenous GM-CSF treatment. Furthermore, the temporal association of increased IL-12 expression with enhanced T cell responses strongly implicates the involvement of APCs, and DCs are the major APCs in the lungs (40, 41). It is also likely that GM-CSF-driven expression of TNF-α in PC-infected neonatal lungs (Fig. 7A) influenced maturation of neonatal DCs, because TNF-α is required for DC maturation (42). In regard to this, a recent study has shown that availability of TNF-α skews the differentiation of peripheral blood monocytes from macrophages to DCs (43). This may explain why, unlike the Vollstedt study, we observed an associated increase in CD4^+ T cell response (Fig. 8). In addition, activation of macrophages by TNF-α (autocrine and paracrine) may have contributed to form a positive feedback loop for cytokine production with an enhanced effector function for PC resolution.

GM-CSF-induced augmented neutrophil and monocyte/macro-
phage phagocytic functions (36–38) mediate antimicrobial effects against various infectious pathogens, including PC (13–16), Candida albicans, and A. fumigatus (44–46). Paine et al. (13) have demonstrated that mice lacking GM-CSF (GM-CSF^-/- ), when depleted of CD4^+ T cells and infected with PC, suffer from increased infection and inflammation. Alveolar macrophages from GM-CSF^-/- mice had compromised phagocytic activity and produced less TNF-α, which was restored upon selective expression of GM-CSF in the lungs of these GM-CSF^-/- mice (13). In addition, Blylyk and Holt (47) have reported that GM-CSF modulates alveolar macrophage function to induce T cell responses. However, none of these studies have examined the effects of GM-CSF on DC populations in the lungs. Our data suggest that in addition to most likely augmenting macrophage function, treatment of neonates with exogenous GM-CSF induced expansion of lung DC population with up-regulated MHC-II expression resulting in enhanced lymphocytic responses.

The data included in this work demonstrate that the delay of neonatal cellular inflammatory responses to PC is in part due to inefficient Ag presentation by neonatal lung DC. Consistent with this, we and others have shown that neonatal CD4^+ cells are not inherently defective and are capable of producing efficient Th1 responses when provided with strong costimulatory signals, including culture with adult DC (7, 48). In addition, the neonatal lung environment is also responsible for this delayed response in
neonates by lacking appropriate cytokine signaling, which is crucial for DC expansion and maturation. We observed expansion and maturation of DC in neonatal lungs upon exogenous administration of GM-CSF, which enhanced PC clearance. GM-CSF is a Food and Drug Administration-approved drug that has been used successfully in the treatment of neonatal sepsis (49–51). Our data suggest that systemic administration of GM-CSF may also be a viable treatment in conjunction with conventional antimicrobial drugs for treating PCP and other respiratory infections in infants.

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References