Effect of alveolar epithelial cell plasticity on the regulation of GM-CSF expression

Mustafa Mir-Kasimov, Anne Sturrock, Michael McManus, and Robert Paine III

Department of Veterans Affairs Medical Center; and Division of Respiratory, Critical Care and Occupational Pulmonary Medicine, University of Utah School of Medicine, Salt Lake City, Utah

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Mir-Kasimov M, Sturrock A, McManus M, Paine R III. Effect of alveolar epithelial cell plasticity on the regulation of GM-CSF expression. Am J Physiol Lung Cell Mol Physiol 302: L504–L511, 2012. First published January 6, 2012; doi:10.1152/ajplung.00303.2010.—Local pulmonary expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) is critically important for defense of the pulmonary alveolar space. It is required for surfactant homeostasis and pulmonary innate immune responses and is protective against lung injury and aberrant repair. Alveolar epithelial cells (AEC) are a major source of GM-CSF; however, the control of homeostatic expression of GM-CSF is incompletely characterized. Increasing evidence suggests considerable plasticity of expression of AEC phenotypic characteristics. We tested the hypothesis that this plasticity extends to regulation of expression of GM-CSF using 1) MLE-12 cells (a commonly used murine cell line expressing some features of normal type II AEC, 2) primary murine AEC incubated under standard conditions [resulting in rapid spreading and loss of surfactant protein C (SP-C) expression with induction of the putative type I cell marker (T1α)], or 3) primary murine AEC on a hyaluronic acid/collagen matrix in defined medium, resulting in preservation of SP-C expression. AEC in standard cultures constitutively express abundant GM-CSF, with further induction in response to IL-1β but little response to TNF-α. In contrast, primary cells cultured to preserve SP-C expression and MLE-12 cells both express little GM-CSF constitutively, with significant induction in response to TNF-α and limited response to IL-1β. We conclude that constitutive and cytokine-induced expression of GM-CSF by AEC varies in concert with other cellular phenotypic characteristics. These changes may have important implications both for the maintenance of normal pulmonary homeostasis and for the process of repair following lung injury.

lun; growth factors; innate immunity; cell-cell interaction; alveolar macrophages; granulocyte-macrophage colony-stimulating factor

GRANULOCYTE-MACROPHAGE COLONY-stimulating factor (GM-CSF) is essential for normal pulmonary homeostasis (10, 28). In the absence of GM-CSF or its receptor, alveolar macrophage (AM) function is significantly altered, resulting in decreased surfactant turnover and greatly impaired pulmonary innate immune responses. In particular, mice deficient in GM-CSF develop a condition very similar to pulmonary alveolar proteinosis in humans, where surfactant accumulation in the alveoli is primarily related to the inability of AM to participate in surfactant clearance (30). Mice lacking GM-CSF receptors develop a similar condition, underscoring the essential role of GM-CSF for the normal maturation of AM (16, 24). In addition to the disrupted catabolism of surfactant, the lack of local alveolar GM-CSF results in impaired pathogen clearance by the AM (14, 18–19). Expression of GM-CSF exclusively in the lung is sufficient to restore both surfactant homeostasis (11, 21–22) and host defense in mice otherwise lacking GM-CSF (18, 27). Transient loss of GM-CSF activity in the lung, either following intratracheal inoculation with neutralizing anti-GM-CSF antibodies (4) or in the setting of acute lung injury (1–2), results in impairment of AM function within hours to days. Thus GM-CSF is essential for AM maturation and must be present continuously to maintain normal AM function and alveolar homeostasis.

Recent studies from our laboratory and others have demonstrated that GM-CSF plays an important role in the context of lung injury and repair. In the absence of GM-CSF, either via gene targeting or through the use of neutralizing antibodies, normal repair following acute lung injury with bleomycin is significantly impaired. Conversely, provision of GM-CSF in the lung enhances repair and limits fibrosis (7). Similarly, in a model of acute lung injury in response to hyperoxia, overexpression of GM-CSF or administration of recombinant GM-CSF significantly limits AEC apoptosis and improves survival (3, 20). Thus understanding the details surrounding the control of expression of GM-CSF by AEC in the context of acute and chronic inflammation may offer opportunities to influence the process of repair.

The pulmonary alveolar space is lined by thin, squamous type I alveolar epithelial cells (AEC) and cuboidal, surfactant producing type II alveolar epithelial cells. Recent studies have suggested that there is considerable plasticity in the expression of classic phenotypic characteristics by alveolar epithelial cells in response to stimuli in the microenvironment both in vitro and in vivo. AEC are thought to be a major source of GM-CSF in the alveolar space. Initial in vitro studies have shown that AEC respond to inflammatory cytokines. In different systems they have been shown to produce GM-CSF in response to IL-1β (29) or to TNF-α expressed by LPS-stimulated AM (5). However, it has not been determined whether regulation of GM-CSF expression is itself subject to variation in response to changing context influencing other AEC phenotypic characteristics. We hypothesized that patterns of GM-CSF expression would be influenced in parallel with phenotypical changes in AEC. To investigate the plasticity of alveolar epithelial expression of this key growth factor, we compared constitutive and cytokine-stimulated GM-CSF expression in a commonly used epithelial cell line derived from murine lung (MLE-12 cells), in primary murine AEC cultivated under standard conditions, and in primary murine AEC cultured on a hyaluronic acid-gelatin matrix in serum free medium to maintain expression of surfactant protein-C (SP-C).
MATERIALS AND METHODS

Animals. Wild-type (WT) C57BL/6 (Ly5.1; CD45.2) mice were obtained from Jackson Laboratory (Bar Harbor, ME) and were used at 6–10 wk of age. Mutant mice lacking GM-CSF (GM-CSF−/− mice) were obtained from Dr. Jeffrey Whitsett and had been extensively backcrossed against the C57BL/6 background. Mice were housed under specific pathogen-free conditions and were monitored daily by veterinary staff. The Animal Care Committee at the Salt Lake City Veterans Affairs Medical Center approved these experiments.

Isolation and culture of primary murine type II AEC. Murine type II AEC were isolated and purified using a modification of published methods (8). Following anesthesia with Avertin (Sigma, St. Louis, MO) and heparinization, mice were exsanguinated and the pulmonary vasculature was perfused with saline. The trachea was cannulated, and the lungs were filled with 1–2 ml dispase (BD-Bioscience, San Jose, CA). Subsequently, low melting point agarose (Sigma; 1%, 0.45 ml) was infused via the trachea and the lungs were placed in iced PBS for 2 min to harden the agarose. The lungs were incubated in dispase (BD-Bioscience), and parenchymal tissue was teased away from agarose-embedded airway tissue. Lung parenchymal tissues were minced in DMEM with 0.01% DNAse I (Sigma), and the resultant cellular suspension was filtered successively through 100-, 40-, and 25-μm nylon mesh. The cells were incubated with biotinylated anti-CD16/CD32 and anti-CD45 (BD-Bioscience) followed by streptavidin-coated magnetic particles (Promega, Madison, WI) for magnetic removal of leukocytes. Mesenchymal cells were removed by overnight adherence in DMEM + 10% FCS to tissue culture-treated plastic (=day 0). The nonadherent cells were plated in DMEM with penicillin/streptomycin and 10% FCS in wells coated with fibronectin (=day 1; Millipore) or in bronchial epithelial growth medium (BEGM; Lonza, Walkersville, MD) in wells coated with a thin layer of a hyaluronic acid-gelatin matrix (Extracell, described below). AEC were allowed to attach for 48 h, after which nonadherent cells and debris were removed by gentle washing with several changes of room temperature sterile PBS. Fresh growth medium was added, and the AEC were utilized for experiments on day 3 unless otherwise specified. Purity of the cultures was 90–94% after adherence, as reported in our previous work (29).

AEC culture on Extracell matrix. In selected experiments, AEC were cultured on a semisynthetic extracellular matrix (Extracell) composed of thiol-modified hyaluronic acid (Glycosil) and gelatin (Gelin-S) co-cross-linked with polyethylene glycol diacrylate (Extralink). Extracell was used as a thin layer covering the surface of a plastic culture dish and was a kind gift from Glycosan Biosystems (Salt Lake City, UT).

Isolation and culture of AM. To obtain AM, mice were euthanized, the trachea was cannulated, and the lungs were lavaged with a total of 5 ml of PBS in 1-ml aliquots. The lavage aliquots for each animal were pooled, and the cell pellet was collected by centrifugation and counted. AM then were resuspended in DMEM + 10% FCS and added to epithelial cells in culture. In selected experiments, AM were incubated with anti-TNF-α neutralizing antibodies (3 μg/ml; R&D Systems, Minneapolis, MN) for 1 h before their use in cocultures with epithelial cells.

Real-time PCR. Total cellular RNA was isolated from cultured cells using RNAeasy (Qiagen, Valencia, CA). First strand cDNA was reverse transcribed from 1 μg of total RNA using a high capacity cDNA kit (ABI; Applied Biosystems, Foster City, CA). GM-CSF transcripts were quantified using the primer pairs previously described (1). Specific PCR products were generated from cDNA (100 ng) using Brilliant SYBR Green QRT-PCR 2-step (Stratagene) and an Mx3000P real-time computerized cycler from Stratagene. The two-step cycle program (Tm = 60°C) with a dissociation analysis was used as recommended by the manufacturer. Appropriate controls (no template control and Rox reference dye) were included in each experiment. Each biological sample was amplified in duplicate, and the average of the duplicates was taken as a single data point for statistical analysis. The threshold cycle from GAPDH was used as a calibrator to normalize the specific RNA quantitation. Results are expressed relative to control values after correcting for GAPDH and setting the first biological control at 100.

GM-CSF and TNF-α protein measurements. GM-CSF and TNF-α protein in cell-free supernatants were measured by ELISA (R&D Systems), following the manufacturer’s recommendations.

Confocal microscopy. Primary murine AEC were plated in eight-well Lab-Tek Permanox chamber slides (Thermo-Scientific) coated with either Extracell or fibronectin and in either DMEM 10% FCS or BEGM. Cells were incubated for 4 days, with the culture medium changed after 48 h. At the end of incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Cells were then washed, permeabilized with 10% Triton X-100 in 5% BSA, and incubated with primary antibody overnight at 4°C. The following primary antibodies were used: pro-SPC polyclonal rabbit AB (Millipore; 1:400) and T1α polyclonal goat AB (R&D; 1:200). Normal rabbit and goat IgG was used as a control. At the conclusion of the overnight incubation, slides were washed and appropriate secondary antibodies (Alexa Fluor) were applied (1:2,000 for 1 h). Slides were washed, and nuclei were stained with DAPI before mounting with Fluoromount-G (SouthernBiotech). Microscopy was performed using a Nikon AR-1 confocal microscope with ×40 oil-immersion objective, utilizing NIS-Elements Advanced Research software. Images shown are maximum projection images obtained with the NIS-Elements software (Nikon).

Statistical analysis. Data are presented as means ± SE. Statistical analysis was carried out using GraphPad Prism v4C software (GraphPad). Differences between two groups were compared with the unpaired Student’s t-test. Two-tailed tests of significance were used. Differences between multiple groups were compared using one-way ANOVA. Comparisons were deemed statistically significant for P values <0.05.

RESULTS

GM-CSF expression by MLE-12 cells. MLE-12 cells are an immortalized murine cell line that exhibits some characteristics of type II AEC in vivo (32). In the absence of inflammatory stimulation, MLE-12 cells did not express significant GM-CSF mRNA (Fig. 1A) or detectable GM-CSF protein (Fig. 1B). GM-CSF expression was vigorously induced in these cells by treatment with TNF-α. However, in contrast to our prior studies (1) with primary AEC, there was little GM-CSF induction in response to IL-1β (Fig. 1).

GM-CSF is essential to maintain the normal function of AM. Previous work (5) has suggested that AM may directly stimulate GM-CSF expression by AEC. Coincubation of MLE-12 cells with freshly isolated AM resulted in significant induction of GM-CSF expression (Fig. 2, A and B). We also found that coincubation of AM with MLE-12 cells resulted in significant TNF-α expression (Fig. 2C). The induction of GM-CSF expression in these cocultures was largely reversed when AM had been preincubated with neutralizing anti-TNF-α antibodies. It was possible that AM were a significant source of GM-CSF in these experiments. However, similar results were obtained in experiments using AM from mutant mice lacking GM-CSF, confirming that MLE-12 cells were the source of GM-CSF in cocultures with AM (data not shown). Thus type II-like MLE-12 cells express little GM-CSF at baseline, but GM-CSF expression is induced by contact with resting AM via TNF-α.
GM-CSF expression by primary murine AEC under standard conditions. We next extended these studies to primary murine type II cells cultured on fibronectin-coated dishes in the presence of 10% FCS. Primary AEC cultured under these standard conditions will be designated as sAEC. Under these conditions, primary AEC spread rapidly and assume a flattened, squamous morphology. They lose many type II cell-specific characteristics, while acquiring some of the characteristics of type I AEC (23). In interesting contrast to MLE-12 cells, sAEC constitutively express abundant GM-CSF protein (Fig. 3B, compare Fig. 1B). Expression can be further increased by treatment with IL-1β; however, treatment with TNF-α, at a dose that significantly stimulated expression in MLE-12 cells, resulted in little change in GM-CSF expression by sAEC (Fig. 3, A and B). Of note, this does not represent a global failure of response to TNF-α by sAEC. We (1) have found previously that AEC cultured under these conditions respond vigorously to TNF-α by secreting the chemokine MCP-1. Given the limited induction of GM-CSF in response to TNF-α, it was not surprising that coculture with AM failed to induce increased GM-CSF expression by primary AEC (data not shown). Thus primary AEC differ from MLE-12 cells in both baseline expression and in the pattern of response to inflammatory cytokines.

We next determined the pattern of GM-CSF mRNA expression of primary murine AEC in response to these inflammatory cytokines at earlier time points during the cell isolation. The adherence-mediated removal of nonepithelial cells from the initial cell suspension is an integral part of the isolation procedure. Cells were exposed to TNF-α or IL-1β for 4 h

Fig. 1. Expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA and protein in MLE-12 cells. MLE-12 cells were treated with medium alone, TNF-α (20 ng/ml), or IL-1β (10 ng/ml). After 4 h, total cellular RNA was extracted, and relative GM-CSF mRNA expression was determined by real-time PCR (A). After overnight incubation, GM-CSF protein expression was determined in cell-free supernatants by ELISA (B). *P < 0.001 vs. control and vs. IL-1β in A and B.

Fig. 2. Effect of coculture with alveolar macrophages (AM) on GM-CSF expression by MLE-12 cells. Freshly isolated AM (2 × 10⁶ cells/well) from C57Bl/6 wild-type mice were coincubated with MLE-12 cells. TNF-α neutralizing antibody (3 μg/ml; TNF-Ab) was added to AM for 1 h before addition of AM to wells containing MLE-12 cells. A: total cellular RNA was extracted after 4 h and relative GM-CSF mRNA expression determined by real-time PCR. *P < 0.001 vs. AM. B: GM-CSF protein expression was determined in cell-free supernatants after overnight co-incubation. **P < 0.01 vs. AM. C: TNF-α protein levels were measured in MLE-12 cells in the presence or absence of AM (with and without addition of TNF-α neutralizing antibodies at 3 μg/ml). *P < 0.001 vs. medium alone or AM + TNF-AB.
immediately after collection from the panning plate, before full adherence. After routine overnight panning, primary AEC demonstrated a preferential GM-CSF response to IL-1β/H9252 with more limited response to TNF-α/H9251 (Fig. 4A). Although the response was more limited, we observed this same pattern even when the AEC cell suspension was collected after a period for panning as short as 1 h (Fig. 4B). Thus, immediately following removal from the mouse, primary AEC resembled sAEC in established culture and differed from MLE-12 cells.

**GM-CSF expression by primary AEC incubated on an extracellular matrix of hyaluronic acid/gelatin in serum-free defined medium.** Based on the differences between primary AEC under standard conditions and MLE-12 cells, we hypothesized that the context influencing expression of classic AEC phenotypic characteristics might determine the pattern of AEC GM-CSF expression. Therefore, we developed an in vitro system promoting preservation of SP-C expression in primary cells. In contrast to sAEC (plated on fibronectin in the presence of 10% FCS), primary type II AEC plated in dishes coated with a semisynthetic extracellular matrix composed of cross-linked hyaluronic acid and gelatin in BEGM (designated eAEC), maintained SP-C mRNA expression at levels similar to those in MLE-12 cells (Fig. 5). To further characterize cells on the hyaluronic acid/gelatin matrix in BEGM, we examined the expression by these cells of T1α, a marker commonly associated with type I AEC phenotype. Somewhat surprisingly, primary AEC maintained in culture for 4 days under both conditions expressed T1α mRNA at similar levels (Fig. 6). Because both SP-C and T1α mRNA were found in cultures of AEC on hyaluronic acid/gelatin in BEGM, we performed dual

![Fig. 3. Expression of GM-CSF mRNA and protein in alveolar epithelial cells (AEC) cultured under standard conditions.](image1)

**Fig. 3.** Expression of GM-CSF mRNA and protein in alveolar epithelial cells (AEC) cultured under standard conditions. AEC cultured on fibronectin (FN) in DMEM 10% FCS (sAEC) for 3 days were exposed to medium alone, TNF-α (20 ng/ml), or IL-1β (10 ng/ml). After 4 h, total cellular RNA was extracted and relative GM-CSF mRNA expression determined by real-time PCR (A). After overnight incubation, GM-CSF protein expression was determined in cell-free supernatants (B). *P < 0.001 vs. medium alone or TNF-α in A and B.

![Fig. 4. Expression of GM-CSF mRNA in AEC culture in standard conditions at early time points after isolation.](image2)

**Fig. 4.** Expression of GM-CSF mRNA in AEC culture in standard conditions at early time points after isolation. Immediately following overnight panning (A) or panning for only 1 h (B), AEC were exposed to medium alone, TNF-α (20 ng/ml), or IL-1β (10 ng/ml) for 4 h. Total cellular RNA was then extracted and relative GM-CSF mRNA expression was determined by real-time PCR. **P < 0.005 vs. medium alone. *P < 0.01 vs. medium alone or TNF-α in A and B.

![Fig. 5. Expression of surfactant protein C (SP-C) mRNA in MLE-12 cells and primary murine AEC.](image3)

**Fig. 5.** Expression of surfactant protein C (SP-C) mRNA in MLE-12 cells and primary murine AEC. Relative mRNA expression of SP-C was determined by real-time PCR in MLE-12 cells, primary murine AEC cultured under standard conditions (on fibronectin-coated dishes in DMEM + 10% FCS) for 4 days (sAEC) and primary murine AEC cultured on hyaluronic acid/gelatin in bronchial epithelial growth medium (BEGM) for 4 days (eAEC). *P < 0.01 vs. MLE-12 or eAEC.
staining of these cultures for pro-SP-C and T1α protein. Interestingly, this staining revealed a mixed cell culture, with most cells expressing predominantly one protein or the other (Fig. 7).

In contrast to sAEC in standard culture, eAEC (cultured on hyaluronic acid/gelatin matrix in BEGM) expressed little GM-CSF at baseline and were preferentially induced in response to TNF-α, with more limited response to IL-1β (Fig. 8). Thus the pattern of baseline expression and cytokine response in eAEC that have preserved SP-C expression was similar to that in the MLE-12 cell line and differed significantly from that in primary AEC under standard conditions.

To demonstrate that the difference in the pattern of GM-CSF expression is related to AEC plasticity in response to the local microenvironment, we compared the pattern of GM-CSF response to cytokines on the day following isolation (day 1) in primary cells cultured either in DMEM 10% FCS on fibronectin or in BEGM on dishes coated with hyaluronic acid/gelatin. Cells were exposed to TNF-α or IL-1β for 4 h immediately after collection from the panning plate, before full adherence. The pattern of GM-CSF expression in response to the cytokines was similar in both culture conditions (Fig. 9) and differed from that found in eAEC after several days in culture (compare Fig. 8). This result suggests that the difference in pattern of expression of GM-CSF is likely due to plasticity of AEC characteristics and is not related to the immediate effects of either medium or matrix.

**DISCUSSION**

GM-CSF serves an important role in defense of the pulmonary alveolar space. Its expression in the lung is essential for normal maturation of AM for both surfactant homeostasis and host defense (30). GM-CSF also promotes healing in the setting of lung injury that can lead to fibrosis (7). Conversely, overexpression of GM-CSF in the wrong context may promote aberrant healing (6, 34). AEC are a major source of GM-CSF in the lung. Expression of GM-CSF is tightly controlled, but little information is available concerning the regulation of baseline GM-CSF expression in the normal lung. In the present study, we have shown that primary murine AEC cultured under standard conditions express abundant GM-CSF at baseline, in the absence of stimulation by inflammatory cytokines. GM-CSF expression by these cells is further increased by stimulation with IL-1β, with little response to TNF-α or to coculture with resting AM. In interesting contrast, primary AEC placed in culture under conditions preserving SP-C expression and type II cell-like MLE-12 cells express little constitutive GM-CSF without stimulation. In these cells, GM-CSF expression is preferentially induced by TNF-α. Thus both baseline expres-

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**Fig. 6.** Time course of expression of T1α mRNA in primary AEC cultured under standard conditions and on Extracell/BEGM. Relative mRNA expression of T1α was determined by real time PCR on days 1, 2, 3, and 4 in primary AEC cultured under 2 different culture conditions. *P < 0.01 vs. control.

**Fig. 7.** Immunofluorescence microscopy of putative type I cell marker (T1α) and SP-C in AEC cultured with Extracell/BEGM image. Primary murine AEC were cultured on Extracell/BEGM for 3 days. Cells were then fixed and stained for confocal microscopy (maximum intensity projection). A: AEC stained for T1α (green) and pro-SP-C (red). B: negative control (rabbit and goat normal IgG) staining of AEC cultured on Extracell/BEGM.

**Fig. 8.** Effects of inflammatory cytokines on GM-CSF expression by AEC cultured on hyaluronic acid/collagen in BEGM. AEC (eAEC) were cultured on hyaluronic acid/gelatin (Extracell) in BEGM for 6 days. eAEC were exposed to medium alone, TNF-α (20 ng/ml), or IL-1β (10 ng/ml). After 4-h incubation total cellular RNA was extracted and relative GM-CSF mRNA expression determined by real-time PCR (A). *P < 0.001 vs. medium alone or IL-1β. After overnight incubation, GM-CSF protein expression was determined in cell-free supernatants (B). *P < 0.05 vs. medium alone.
sion of GM-CSF and the pattern of response to inflammatory cytokines demonstrate significant plasticity, varying with other aspects of AEC phenotype.

Studies of gene targeted mice lacking either GM-CSF (10, 28) or its receptor (16, 24) and studies of humans with pulmonary alveolar proteinosis (30) have demonstrated the critical role of GM-CSF in normal pulmonary homeostasis. In each instance, it is clear that surfactant turnover is dependent on AM maturation, which is in turn dependent on adequate local GM-CSF in the alveolar space. It is now clear that GM-CSF must be continually present in the alveolar space to maintain AM function. Inhibition of lung GM-CSF by intratracheal instillation of neutralizing antibodies (4) or suppression of AEC GM-CSF expression in the setting of lung injury results in loss of AM functional characteristics within 24 h (1–2). Although GM-CSF protein may be difficult to measure in bronchoalveolar lavage fluid due to binding to AM and endogenous antibodies (15), GM-CSF mRNA is readily detected in normal lung. Importantly, studies (1) using laser capture microdissection indicate that GM-CSF mRNA is continually produced by cells in the alveolar wall. However, information concerning the details of homeostatic regulation of GM-CSF expression by AEC is lacking.

There is considerable plasticity of AEC phenotypic expression along a spectrum of classic type II cell and type I cell characteristics. Our studies were originally undertaken to investigate the hypothesis that GM-CSF expression varies along this same spectrum. The mammalian lung is a structurally complex organ with ~40 different types of cells. To this point, we have had little success in immunolocalization of this secreted protein at the level of the individual cell, either in the intact lung or in cell culture. Therefore, we have used in vitro models to evaluate the regulated expression of GM-CSF by AEC. Primary AEC cultured under standard conditions rapidly lose expression of SP-C mRNA and are induced to express the putative type I AEC marker T1α. These cells constitutively secrete GM-CSF, with further induction by IL-1β and little response to TNF-α. Interestingly, this pattern of cytokine response is found even in cells examined very early after isolation from the mouse, before culture conditions would be expected to modulate cellular behavior. Constitutive GM-CSF expression by these primary AEC in vitro is consistent with the observation using laser-capture microdissection showing baseline GM-CSF mRNA expression by alveolar wall cells in vivo (1). In contrast, GM-CSF expression was distinctly different when primary cells were maintained in culture in BEGM on a hyaluronic acid-gelatin matrix. These conditions, which led to maintenance of SP-C expression, were associated with low basal expression of GM-CSF and preferential induction in response to TNF-α. These results are most consistent with the notion that GM-CSF expression by AEC is determined by microenvironmental influences on the cell that also may influence classic phenotypic characteristics.

MLE-12 cells are an immortalized cell line derived from the alveolar epithelium of transgenic mice expressing the large T antigen of SV40 under control of the SP-C promoter. Although often used as models of normal AEC, our study again emphasizes the importance of correlating observations in transformed cell lines with information from primary cells and intact animals.

The plasticity of response to inflammatory cytokines is of interest in the transition from acute inflammation to chronic inflammation and wound repair. Preferential response to TNF-α might be important for enhanced GM-CSF expression in the context of acute inflammation and recruitment of new mononuclear phagocytes into the lung. This would be consistent with the observations of Cakarova et al. (5). Those investigators found that primary AEC cultured under the side of a porous support secreted GM-CSF in response to TNF-α produced by LPS-stimulated AM, although the precise phenotypic characteristics of the AEC in this system were not defined. In contrast, IL-1β-stimulated expression might be of particular value in the setting of wound repair. The mechanisms to explain why cells under one set of conditions express GM-CSF preferentially in response to IL-1β, while under other conditions they preferentially release GM-CSF in response to TNF-α, have not been defined. We (1) have previously found that AEC cultured under standard conditions do respond to TNF-α with robust induction of MCP-1 expression, indicating that a TNF receptor is present and functional. Further studies are underway to define this selective responsiveness.

We have found previously that GM-CSF mRNA is expressed in thin cells of the alveolar wall of normal, unjured mice (1), suggesting that this may be a feature of normal type I AEC. This is consistent with our observation of constitutive GM-CSF expression in primary AEC cultured under conditions associated with loss of SP-C expression and induction of T1α expression. Conversely, we have also considered the possibility that expression of GM-CSF by AEC in vitro in the absence of cytokine stimulation is a stress response. If true, this might have important implications for promotion of GM-CSF-mediated alveolar repair in the setting of lung injury.

Extensive literature describes the influence of various extracellular matrix preparations and media formulas on in vitro differentiation of primary AEC (9, 13, 31). Three-dimensional matrices that promote cuboidal shape and close cell-cell interaction have been found to support preservation of type II cell
phenotypic characteristics in vitro (17, 25–26, 35). This is the first report of AEC culture on plates coated with a thin layer of cross-linked hyaluronic acid and gelatin. This combination of semisynthetic extracellular matrix and a defined, serum-free medium preserved populations of cells with cuboidal morphology and SP-C expression for at least 10 days in culture, while allowing ready access to the cell surface in a planar culture configuration. This approach also limited the emergence of mesenchymal cells within the cultures (12). We anticipate that this combination will prove useful for future studies of AEC biology.

In conclusion, we have shown that GM-CSF expression by murine AEC is dependent on the phenotype expressed by the cells. A cell line expressing some characteristics of normal type II cells and primary cells cultured under conditions to preserve type II characteristics express low-level baseline GM-CSF but respond to TNF-α with increased GM-CSF production. In contrast, primary cells that have undergone transition away from the type II cell phenotype and developed some characteristics associated with the type I cell phenotype express abundant GM-CSF constitutively, with further induction preferentially with IL-1β. These observations provide new insights into the homeostatic regulation of expression of this critical growth factor within the alveolar space.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.M.-K., A.S., and R.P. conception and design of research; M.M.-K., A.S., and M.M. performed experiments; M.M.-K., M.M., and R.P. analyzed data; M.M.-K., A.S., and R.P. interpreted results of experiments; M.M.-K. and R.P. prepared figures; M.M.-K. drafted manuscript; M.M.-K., M.M., and R.P. approved final version of manuscript.

REFERENCES


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