CHARACTERIZATION OF PROINFLAMMATORY CYTOKINE PRODUCTION AND CD14 EXPRESSION BY MURINE ALVEOLAR MACROPHAGE CELL LINES

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SUMMARY

Alveolar macrophages, which play a central role in lung defense, produce cytokines that help orchestrate local inflammatory responses. In sepsis and other pathological conditions, bacterial lipopolysaccharide endotoxin can induce alveolar macrophages (AM) to release proinflammatory cytokines, including tumor necrosis factor-alpha, interleukin-1, and interleukin-6. Studying the mechanisms that control alveolar macrophage cytokine production may lead to better therapies for conditions involving inflammatory lung injury. We and others have noted significant differences between alveolar macrophages and peritoneal macrophages, but large numbers of human or murine alveolar macrophages are rarely available for detailed mechanistic studies. We have obtained three murine alveolar macrophage cell lines (AMJ2C8, AMJ2C11, and AMJ2C20) and have begun to characterize their cytokine responses to proinflammatory stimuli. We measured the effects of endotoxin, interferon gamma, and the combination of the two on production of tumor necrosis factor, interleukin-1 beta, and interleukin-6 in each line. We also studied the expression of the endotoxin receptor CD14 by these cells, and investigated the effect of serum on their endotoxin responsiveness. We show here that all three of the cell lines responded in a manner comparable to that of primary murine alveolar macrophages. Observed variations between these lines may reflect the documented heterogeneity seen in populations of primary alveolar macrophages. These cell lines should expand the repertoire of tools available to investigators studying regulation of murine alveolar macrophage responses.

Key words: lipopolysaccharide (LPS); endotoxin; tumor necrosis factor (TNF); interleukin-1 (IL-1); interleukin-6 (IL-6).

INTRODUCTION

Alveolar macrophages (AM) play a central role in lung defense against inhaled irritants and pathogens. AM produce cytokines that are important in orchestrating an inflammatory response. In many pathological conditions, the AM is a major source of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) (25). One of the most potent inducers of these proinflammatory cytokines is bacterial lipopolysaccharide endotoxin (LPS) (4). LPS is an important etiologic agent in sepsis syndrome and adult respiratory distress syndrome (ARDS) (7,11), as well as in toxic alveolitis due to the inhalation of organic dusts (9,22). Studying the mechanisms that control AM cytokine production may reveal important insights that lead to better therapies.

Evidence indicates that cytokine production by AM can be regulated differently than in other types of mononuclear phagocytes, such as monocytes and peritoneal macrophages (1,13,20,24). However, the study of regulatory mechanisms of cytokines in AM has been hampered by the limited number of AM that can be obtained from mice or from human volunteers. When available, cell lines can be valuable tools for studying cytokine regulation. At present, no human AM lines have been reported. Recently, however, AM cell lines from mice have been described (8,19).

Our interest in studying posttranscriptional regulatory mechanisms of TNF production in AM led us to obtain three mouse AM lines recently described by Palleroni et al.: AMJ2C8, AMJ2C11, and AMJ2C20 (19). These cell lines had been produced by immortalizing primary AM from C57BL/6j mice with the J2 retrovirus, a recombinant virus carrying the v-raf and v-myc oncogenes (3). In the report describing the creation of these lines, some aspects of proinflammatory cytokine production were characterized for AMJ2C11 (19).

Unlike primary AM, AMJ2C11 were reported to require both interferon-γ (IFN-γ) and LPS stimulation for induction of TNF and IL-1. This characteristic suggested to us a potentially interesting defect in the posttranscriptional regulation of these cytokines (2). When we sought to reproduce the dual LPS and IFN-γ requirement, we employed a range of LPS concentrations, without IFN-γ, and then combined the maximum LPS concentration with 100 U/ml IFN-γ. In contrast to the earlier report (19), we found that all three of the AM cell lines could produce TNF and IL-1β mRNA as early as 3 h after challenge with 10 μg/ml LPS alone, suggesting that these lines could produce proinflammatory cytokines without costimulation by IFN-γ (23). We extended our study to characterize these AM lines and their production of TNF, IL-1β, and IL-6 following stimulation with LPS and/or IFN-γ, their TNF production in response to varying concentrations of LPS with and without serum, and their constitutive ex-
pression of the membrane LPS receptor, CD14 (mCD14) (33). Our results indicate that these cell lines respond comparably to primary AM, including displaying some variation from one another. They should expand the repertoire of tools available to investigators studying regulation of AM responses.

**Materials and Methods**

**Reagents.** Phenol-extracted *Escherichia coli* 0111:B4 LPS and propidium iodide were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant murine IFN-γ was obtained from Amgen Biologicals (Thousand Oaks, CA) (specific activity was 10^6 U/mg). Rat monoclonal anti-murine CD14 antibodies #555 and #609 were a generous gift from Dr. Shunsuke Yamamoto, Department of Pathology, Oita Medical University, Japan (20). Nonimmune rat IgG was purchased from Sigma. Goat anti-rat IgG conjugated to fluorescein isothiocyanate (FITC) was from Organon-Teknika (Durham, NC).

**Cell lines.** AMJ2C8, AMJ2C11, and AMJ2C20 cells were generously provided by Dr. Alicia Palleroni, Hoffman LaRoche (Nutley, NJ). These cell lines had been derived from C57Bl/6J mouse AM transformed with recombinant J2 retrovirus (19). Cells were grown in Dulbecco’s minimal essential medium (DMEM, BioWhittaker, Walkersville, MD) supplemented with 10% defined fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) and 5 mM HEPES buffer (GIBCO, Grand Island, NY) at 37 °C in 5% CO2 and 95% humidity. Cells were routinely passaged every other day by gently shaking the flasks to detach the cells, and removing enough cells to dilute them in new medium to a density of approximately 1 × 10^6 cells/ml in 75 cm^2 culture flasks (Falcon, Becton Dickinson, Lincoln Park, IL).

**Endotoxin-free solutions.** Media and phosphate-buffered saline solution (PBS) were from BioWhittaker, free of detectable LPS (< 0.1 EU/ml). All other solutions were prepared using pyrogen-free water and sterile polypropylene plasticware, or glassware that had been baked at 190 °C for at least 4 h. No solutions contained detectable LPS, as determined by the Limulus amoebocyte lysate assay (sensitivity limit 12 pg/ml; Associates of Cape Cod, Woods Hole, MA).

**AM cell line culture and stimulation.** Experiments determining NF-κB activation and upregulation of mCD14. Either 1 gl of rat monoclonal antibody directed against murine IFN-γ, or 10 pg/ml LPS for an additional 24 h. For determination of NF-κB activation, experiments were performed on two different cell passages to produce a total of six cell cultures. Cells were cultured in 1 ml in 24-well plates (Linbro, Flow Laboratories, McLean, VA) at a density of 1 × 10^6 cells/ml for 24 h, then stimulated with 0–10 μg/ml LPS for an additional 24 h. For determination of IL-1β production, experiments were performed on two different cell passages to produce a total of six cell cultures. Cells were cultured in 1 ml in 24-well plates (Linbro, Flow Laboratories, McLean, VA) at a density of 1 × 10^6 cells/ml for 24 h, then stimulated with 100 U/ml recombinant murine IFN-γ and/or 10 μg/ml LPS for either 3 or 24 h, after which culture supernatants were collected. For determination of NF-κB release in response to LPS stimulation, triplicate cultures of 5.9 × 10^6 cells/ml in 10 ml in 100 × 20 mm culture dishes (Falcon) at 37 °C, in 5% CO2 and 95% humidity. Cultures were then passaged every other day by gently shaking the flasks to detach the cells, and removing enough cells to dilute them in new medium to a density of approximately 1 × 10^6 cells/ml in 75 cm^2 culture flasks (Falcon, Becton Dickinson, Lincoln Park, IL).

**Cytokine assays.** Murine TNF in culture supernatants was quantified by ELISA (Factor Test-X, Genzyme, Cambridge, MA), which has a sensitivity of 15 pg/ml. Murine IL-6 was measured in supernatants and also in cell lysates by ELISA (InTest-IIL, Genzyme); in our hands, the sensitivity limit of this assay was 60 pg/ml. Murine IL-6 was quantified in supernatants by ELISA (Cytoscreen, Bioscience International, Camarillo, CA), which has a sensitivity limit of 8 pg/ml.

**Expression of CD14.** Indirect fluorescent antibody staining, followed by flow cytometry (FACSscan flow cytometer, Becton Dickinson, San Jose, CA) was used to determine the presence of the LPS receptor mCD14 on the surface of each cell line. Two separate cell isolations were obtained for microfluorometry using similar culture conditions. Cells were kept on ice to prevent activation and upregulation of mCD14. Either 1 μl of rat monoclonal antibody #609 or #526 specific for murine CD14 (14), or 1 μl of nonimmune rat IgG (all stock solutions approximately 1 mg/ml) were incubated with 2 × 10^6 cells in 25 μl medium for 30 min at 4 °C. Cells were washed twice with PBS supplemented with 2% FBS, pelleted by centrifugation at 600 × g, then stained with goat anti-rat IgG-FITC [1:100 (vol/vol)] for 30 min at 4 °C. Cells were again washed twice with PBS-FBS and then resuspended in 1 μg/ml propidium iodide in PBS-FBS for FACSscan analysis. A total of 10,000 events (gated to exclude nonviable cells) were collected in the log mode and expressed as histograms of relative fluorescence intensity.

**Statistics.** Individual culture supernatants were assayed for TNF, IL-1β, or IL-6 in duplicate. Experiments with each cell line included three to six replicates of each condition. Results represent the mean ± standard error (SE). Data were analyzed using Statview Student, a statistical analysis software package (Abacus Concepts, Berkeley, CA). Analysis of variance, followed by multiple comparison analysis was performed. Dunnett's t-test was used to compare cytokine production by treated cells with untreated control cells. Fisher's exact test was used for multiple comparisons between cells treated with LPS, IFN-γ, or both. Statistical significance was taken as P < 0.05.

**Results**

**LPS induction of TNF in alveolar macrophage cell lines.** LPS, without the aid of IFN-γ, induced TNF production in all cell lines. The dose-response curves for each cell line, with and without 10% FBS, are shown in Fig. 1. The presence of 10% FBS greatly augmented LPS-induced TNF production. With 10% FBS in the medium, LPS induced significant TNF production at 2 h in a dose-dependent manner in all three AM cell lines. Untreated cells from each line spontaneously produced small amounts of TNF in the presence of serum. In the presence of serum, the lines differed from one another in sensitivity to LPS: AMJ2C8 cells required approximately 2 μg/ml LPS, AMJ2C20 cells required approximately 4 μg/ml LPS, and AMJ2C11 cells required approximately 10 μg/ml LPS to produce 17 ng TNF/10^6 cells (the 50% response point in the AMJ2C8 dose-response curve). Maximal TNF production differed among the three lines as well. The maximum amount of TNF produced by AMJ2C11 cells was 18.5 ± 2.7 ng/10^6 cells, in response to 20 μg/ml LPS, which was the lowest among the three lines. AMJ2C11 cells, however, were the most responsive to lower concentrations of LPS, requiring only 0.05 μg/ml LPS to increase TNF production significantly above controls. AMJ2C8 and AMJ2C20 required at least 2.5 μg/ml LPS to achieve significant TNF production.

Without serum, AMJ2C11 could be stimulated by LPS in a dose-dependent manner. As little as 500 ng/ml LPS stimulated a small but significant increase in their serum-free TNF production (1.4 ± 0.06 ng/10^6 cells) compared with unstimulated cells (1.07 ± 0.06 ng/10^6 cells). Without serum, AMJ2C20 cells induced a significant amount of TNF (3.1 ± 0.6 ng/10^6 cells) only in response to the highest LPS concentration tested (10 μg/ml LPS). AMJ2C8 cells required at least 2.5 μg/ml LPS for significant TNF induction (2.9 ± 0.2 ng/10^6 cells) in the absence of serum. In spite of these variations in sensitivity to LPS in the absence of serum, maximal TNF output did not differ significantly among the three lines.

**CD14 expression.** Surface expression of mCD14 on each cell line is shown in Fig. 2. All three lines expressed mCD14, as determined by their enhanced fluorescence following incubation with rat anti-mouse mCD14 antibody compared with cells incubated with normal rat IgG. In replicate experiments, the absolute levels of mCD14 appeared to vary slightly, but in each case the overall pattern of mCD14 expression was consistent for each line.

**Effect of IFN-γ on TNF production.** The results presented in Fig. 3 show that, by itself, IFN-γ stimulation did not induce significant TNF in any of the lines at either 3 or 24 h. In contrast, at 24 h, LPS alone (10 μg/ml) induced significant amounts of TNF in all three AM lines. Combining the two stimuli for 24 h led to distinct results for each cell line. In AMJ2C20 cells, IFN-γ significantly increased LPS-induced TNF production, whereas it significantly decreased it in
AMJ2C8 cells (relative to LPS treatment alone). In AMJ2C11 cells, IFN-γ had no significant effect on LPS-induced TNF production. No additive or synergistic effects of the combined stimuli were observed in any of the lines at 3 h.

Induction of IL-1β by LPS and IFN-γ: The 24 h supernatants in which TNF was quantified (Fig. 3) were also tested for the presence of IL-1β, but none was detected. Subsequently, $1 \times 10^6$ cells from each line were stimulated for 24 h in the same manner, then lysed to release any intracellular IL-1β. The results from two identical experiments producing six cultures per condition are shown in Fig. 4. None of the three cell lines contained detectable constitutive levels of IL-1β. IFN-γ alone did not increase IL-1β levels above those observed in control unstimulated cells in any line. However, all three lines produced significant quantities of IL-1β in response to LPS treatment and, again, the effect of combined treatment with LPS and IFN-γ was different for each line. LPS (10 μg/ml) induced 893 ± 103 pg IL-1β/10⁶ cells and 544 ± 11 pg IL-1β/10⁶ cells in AMJ2C8 cells and AMJ2C20 cells, respectively. IFN-γ dampened these LPS-induced elevations in IL-1β considerably, diminishing the responses approximately 30-fold and 500-fold in AMJ2C8 and AMJ2C20, respectively. Compared with the other cell lines, AMJ2C11 cells produced little IL-1β (117 ± 10 pg/10⁶ cells) in response to LPS, including IFN-γ was without additional effect.
Effect of IFN-γ on LPS-induced IL-6 production. IL-6 was not detected in culture supernatants from unstimulated cells. IFN-γ and/or LPS had differential effects on each AM cell line with regard to IL-6 production (Fig. 5). Cells treated with 100 U/ml IFN-γ produced very small but measurable amounts of IL-6: 97 ± 14 pg/10⁶ cells in AMJ2C8; 91 ± 41 pg/10⁶ cells in AMJ2C11; and 210 ± 22 pg/10⁶ cells in AMJ2C20. LPS (10 μg/ml) induced copious and significant production of IL-6 from AMJ2C8 and AMJ2C20 compared with unstimulated cells; there was a trend toward an LPS-induced IL-6 increase in AMJ2C11 cells as well. The relative quantities of IL-6 induced by LPS were significantly different between each cell line. Again, the effect of 100 U/ml IFN-γ on LPS-induced IL-6 was different for each line. In AMJ2C11 cells, IFN-γ significantly increased LPS-induced IL-6 (approximately fivefold), whereas in AMJ2C8 cells, IFN-γ significantly decreased it (more than twofold). IFN-γ had no effect on LPS-stimulated IL-6 production by AMJ2C20 cells.

DISCUSSION

TNF responses of AM cell lines. Palleroni et al. reported creating these cell lines to facilitate studies of the mechanisms through which AM exert their biological activities, such as cytotoxicity and cytokine production, since primary AM are difficult to obtain in sufficient numbers for such studies (19). In the report describing the derivation of these AM cell lines, they were shown to be similar to their primary progenitors with respect to cytotoxicity and expression of major histocompatibility complex (MHC) class II antigens, and with respect to IL-6 production by AMJ2C11 cells.

We were intrigued by the initial paper, in which the authors reported finding no production of either TNF or IL-1 by AMJ2C11 cells in response to LPS, and we sought to investigate this phenomenon. We also expanded our study to include production of these proinflammatory cytokines in response to LPS, IFN-γ, or both LPS and IFN-γ by AMJ2C11 and two other AM cell lines derived from the same mice. Our results agreed with Palleroni et al. in that AMJ2C11 cells produced much greater concentrations of IL-6 than of TNF or IL-1β following 24-h stimulation with LPS or LPS plus IFN-γ.

In contrast to the prior report, however, we found that LPS alone could induce TNF production in a dose-dependent manner. There are several possible reasons for the discrepancy. We used an ELISA that was over 500 times more sensitive (limit of 15 pg/ml) than the TNF LM cell cytotoxicity bioassay of the prior study (limit of 8000 pg/ml) (15). Additionally, our culture conditions differed. We stimulated 5.9 × 10⁶ AMJ2C11 cells with up to 10 μg/ml LPS, for 24 h, whereas the other study utilized five times as much LPS to stimulate 25-fold fewer cells for twice as long. The much higher LPS exposure could easily have been toxic (16). By trypan blue exclusion, we observed a 30% loss of viability among AMJ2C8 cells treated with 10 μg/ml LPS for 48 h. When Palleroni et al. decreased the LPS concentration to 5 μg/ml in conjunction with 50 U/ml IFN-γ, they did see induction of both IL-1 and TNF. Probably a combination of these factors (potentially toxic LPS concentration, lower cell numbers, and a less sensitive assay) explain the discrepancy between our results concerning TNF and IL-1β production and those of the previous report (19).

CD14 expression on AM cell lines. Because the AM cell lines were all responsive to LPS, yet differed somewhat in the particulars of their responses, we examined their expression of mCD14. In monocytic and peritoneal macrophages, the best characterized mechanism by which LPS induces cytokine production is through activation of mCD14 by a complex of LPS and the LPS-binding protein (LBP) (12). Binding is thought to trigger a cascade of events leading to the activation of protein kinase C, the phosphorylation of tyrosine kinases and mitogen-activated protein kinases, and then to activation of transcription factors including nuclear factor kappa B (NFκB), and, ultimately, production of cytokines (5,10,17,28,29,31,33). Data presented in Fig. 2 show that each of the three AM cell lines expressed mCD14, although the absolute levels of expression were different.
We have reported as well that AM from LPS-resistant C3H/HeJ mice are comparable to AM from normal (LPS-sensitive) C3HeB/FeJ mice in TNF production in their response to LPS. This property is not shared by peritoneal macrophages from the same animals (24). Together these studies support the hypothesis that AM possess additional pathway(s) of LPS sensing and response that may be independent of CD14 and/or unaffected by the C3H/HeJ Lps mutation. [The specific protein product of the Lps gene is undefined at this writing. For a comprehensive review, see Vogel (30).] In the present study, AMJ2C11 cells displayed the least expression of CD14, but were the most sensitive to low concentrations of LPS and to LPS under serum-free conditions (Fig. 1). These data also may be interpreted as supporting the existence of additional mechanism(s) of LPS sensing and response, independent of mCD14, in these cells.

**Comparison with primary mouse AM: TNF.** In most respects, the cytokine responses of the AM cell lines are comparable to primary AM from mice. Primary C57BL/6 mouse AM have been reported to produce low levels of TNF constitutively, 0.41 ± 0.23 ng/ml/10^6 AM, which is quantitatively similar to the levels we measured from these cell lines (1.0–2.3 ng TNF/ml/10^6 cells), and which was similarly unaffected by stimulation with IFN-γ (26). Although that study assessed lower cell densities than we used, its authors also noted...
When compared with LPS treatment alone, AMJ2C8 represents the mean ± standard error (SE) from four to six replicates. Cell lines: AMJ2C8 (solid bars); AMJ2C11 (striped bars); AMJ2C20 (open bars). Statistical significance: † denotes $P < 0.01$, for differences noted in comparison with unstimulated control cultures; ‡ denotes $P < 0.05$ for differences noted when compared with LPS treatment alone.

**Fig. 4.** Interleukin-1β (IL-1β) measured in lysates of alveolar macrophage (AM) cell lines stimulated with lipopolysaccharide (LPS) (10 µg/ml), interferon-γ (IFN-γ) (100 U/ml), or both for 24 h. Each bar represents the mean ± standard error (SE) from four to six replicates. Cell lines: AMJ2C8 (solid bars); AMJ2C11 (striped bars); AMJ2C20 (open bars). Statistical significance: † denotes $P < 0.01$, for differences noted in comparison with unstimulated control cultures; †‡ denotes $P < 0.05$ for differences noted when compared with LPS treatment alone.

**Fig. 5.** Interleukin-6 (IL-6) measured in culture supernatants from alveolar macrophage (AM) cell lines stimulated with lipopolysaccharide (LPS) (10 µg/ml), interferon-γ (IFN-γ) (100 U/ml), or both for 24 h. Each bar represents the mean ± standard error (SE) from four to six replicates. Cell lines: AMJ2C8 (solid bars); AMJ2C11 (striped bars); AMJ2C20 (open bars). Statistical significance: † denotes $P < 0.01$, for differences noted in comparison with unstimulated control cultures; †‡ denotes $P < 0.05$ for differences noted when compared with LPS treatment alone.

that increasing AM cell densities from $10^6$ to $0.6 \times 10^6$ (the concentration we used) did not result in a commensurate increase in LPS-induced TNF production (26). In addition, constitutive TNF production appears to vary among different mouse strains. For example, we have not detected any constitutive TNF in primary AM culture supernatants from C3HeB/FeJ or C3H/HeJ mice (24).

Primary C57BL/6 AM have been reported to release 21.7 ± 0.47 ng/ml TNF in response to 24-h culture with 10 µg/ml LPS, which compares well with our findings of 18–34 ng/ml TNF under the same stimulation conditions. The same report noted that in primary AM, costimulation with LPS and IFN-γ marginally increased TNF production, relative to stimulation with LPS alone (26). One of the cell lines we tested, AMJ2C20, shared this trait. We have also found that C3HeB/FeJ primary AM produced comparable levels of TNF (10–18 ng/10^6 cells) under these stimulation conditions (24).

**Comparison with primary mouse AM: IL-1β.** In primary human AM, LPS-induced IL-1β production by AM is usually low; most of the cytokine remains inside the cell (32). We have also previously observed barely detectable levels of IL-1β in culture supernatants from primary AM lavaged from C3HeB/FeJ mice (unpublished data). The three murine AM cell lines reflected this property: IL-1β was undetectable in any of the culture supernatants, and present at low levels in lysates of unstimulated cells (Fig. 4). Two of the lines, AMJ2C8 and AMJ2C20, when treated with LPS, produced IL-1β in amounts comparable to those reported for human primary AM (approximately 1 ng/10^6 cells) (6).

**Comparison with primary mouse AM: IL-6.** Although published data are scarce regarding quantitative IL-6 output from primary murine AM stimulated under the same conditions as our cells, Palleroni et al. reported that the IL-6 response from AMJ2C11 cells treated with 50 µg/ml LPS was comparable to that which they observed with primary cells (19).

**Synopsis.** Our results show that these murine AM cell lines respond to LPS in a manner comparable with primary AM. Interestingly, we found that IFN-γ can influence the cytokine responses to LPS differently in each line. Variation was noted in the original report concerning these cells (19), and probably reflects the situation of normal AM in the lung. It is well known that macrophages from diverse anatomical sites are distinct (21,24). Others have pointed out that additional heterogeneity exists within macrophage populations in a single site. More than 10 yr ago, Murphy and Herscowitz reported functional variability and subpopulations at differing stages of maturity among AM isolated from rabbits (18). From analyzing TNF production by individual porcine AM treated with LPS or phorbol ester, Taylor and colleagues have more recently shown that particular small subsets of AM respond to distinct stimuli (27). Thus, the variations observed with these AM cell lines most likely reflect the heterogeneity of the original pool of primary cells from which they were derived.

These AM cell lines, derived from primary AM lavaged from the lungs of C57BL/6J mice and transformed with the J2 retrovirus, should expand the repertoire of tools available for studying cytokine responses in the lung. Such studies may prove helpful to those seeking to understand the mechanisms that regulate these responses, and may shed new light on basic processes contributing to inflammatory lung injury and ARDS.

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