Tumor Necrosis Factor- α from Macrophages Enhances LPS-Induced Clara Cell Expression of Keratinocyte-Derived Chemokine

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Tumor necrosis factor (TNF)- α is a cytokine produced by alveolar macrophages in response to LPS in the lung. Clara cells are bronchiolar epithelial cells that produce a variety of proinflammatory cytokines in response to LPS but not to TNF- α . In this study, we examined whether TNF- α affects Clara cell cytokine production in the setting of LPS stimulation. Using a transformed murine Clara cell line (C22), we observed that both LPS and TNF- α induced production of keratinocyte-derived chemokine (KC) and monocyte chemoattractant protein (MCP)-1. We also found that simultaneous LPS and TNF- α stimulation is synergistic for KC production, but additive for MCP-1 production. By using a Transwell coculture system of RAW264.7 macrophages and Clara cells isolated from C57Bl/6 mice, we found that macrophages produce a soluble factor that enhances Clara cell KC production in response to LPS. Cocultures of Clara cells from mice deficient in TNF- α receptors with RAW264.7 macrophages demonstrated that the effect of macrophages on Clara cells is mediated primarily via TNF- α . To determine whether these findings occur *in vivo*, we treated wild-type and TNF receptor-deficient mice intratracheally with LPS and examined the expression of KC. LPS-treated, TNF receptor-deficient mice showed much less KC mRNA in airway epithelial cells compared with wild-type mice. In contrast, a similar number of KC-expressing cells was seen in the lung periphery. Thus, upregulation of KC by Clara cells in the setting of LPS stimulation is largely dependent on TNF- α originating from alveolar macrophages. These findings shed light on macrophage-Clara cell interactions in regulating the pulmonary inflammatory response to LPS.

Keywords: airway; tumor necrosis factor- α ; synergism; macrophages

Tumor necrosis factor (TNF)- α is a pleiotropic cytokine that displays potent neutrophil chemotactic activity and plays a critical role in various inflammatory processes (1). In the lungs, it induces early neutrophilic infiltration and eosinophil recruitment into the airways and airspaces, increases alveolar-capillary permeability, and induces late-phase airway hyperresponsiveness and airway inflammation in animal models of bronchial allergic inflammation (2–4). Inhaled TNF- α increases airway responsiveness and sputum neutrophilia in healthy subjects (5). It is upregulated in the airways of patients with asthma, and

CLINICAL RELEVANCE

This research provides new information about interactions between inflammatory cells in the lung and airway epithelial cells. Understanding these interactions will help in the development of new strategies for controlling pulmonary inflammation.

blocking its activity with soluble TNF receptor (TNFR) improves symptoms and lung function in patients with refractory asthma (6, 7). TNF- α has been implicated in inflammation associated with emphysema, as smoke-induced matrix metalloproteinase production, connective tissue breakdown, neutrophilic inflammation, and an increase in mean linear intercept of distal airspaces are significantly reduced in TNFR knockout (KO) mice (8, 9). Acute lung injury and acute respiratory distress syndrome are also associated with TNF- α (10). Although plasma and bronchoalveolar lavage (BAL) levels of TNF- α have not consistently correlated with clinical outcomes in patients at risk for, or already diagnosed with, acute lung injury (11, 12), increased levels of TNFR1 and TNFR2 in the plasma of patients with acute lung injury have been associated with morbidity and mortality in a multicenter trial (13).

TNF- α binds to two cell-surface receptors, TNFR1 (P55) and TNFR2 (P75), which are present on most cell types. These receptors are structurally related, but lead to distinct biological activities (14). TNFR1 signaling induces most of the inflammatory responses attributed to TNF- α (15, 16); TNFR2 signaling is associated with cell proliferation and apoptosis (17). In addition, the inflammatory response mediated via TNFR1 may be enhanced by TNFR2 due to binding of TNF- α to the cell surface, making it more available for TNFR1, or as a result of overlapping intracellular signaling of the two receptors (18).

We have shown that LPS stimulation of Clara cells induces the upregulation of several cytokines, predominantly keratinocyte-derived chemokine (KC) (19). In addition, we have shown that after naphthalene-induced Clara cell injury, not only is KC expression by Clara cells reduced, but cytokine production by alveolar macrophages is also reduced, suggesting that Clara cells modulate the macrophage response to LPS (19). Although Clara cells express several NF- κ B-regulated cytokines, and appear to play a significant role in the lung innate immune response to LPS, they do not express TNF- α (19).

Given that Clara cells are exposed to macrophage-derived TNF- α in the lung during LPS stimulation, and secrete proinflammatory cytokines in response to TNF- α stimulation, we hypothesized that TNF- α triggering of Clara cells would impact the pulmonary innate immune response to LPS. We report that LPS and TNF- α synergistically induce KC production by Clara cells. Using a Transwell coculture system, we found that macrophages augment Clara cell production of KC in response to LPS largely via TNF- α . *In vivo* studies supported these findings.

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MATERIALS AND METHODS

Mice

C57Bl/6 mice (6- to 8-wk-old; Taconic, Hudson, NY) or TNF double-receptor KO mice (B6:129S-TNFRSF1a^{TM1IMX}TNFRSF1b^{TM1IMX}/J mice; Jackson Laboratory, Bar Harbor, ME) were housed in a barrier facility under veterinary care of the Department of Comparative Medicine at Washington University School of Medicine. All procedures involving mice were approved by the Washington University School of Medicine Animal Studies Committee and were performed in accordance with the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals*.

Cell Culture

C22 cells. C22 cells were maintained as previously described (20). Briefly, the cells were maintained in permissive conditions (Dulbecco's modified Eagle's medium supplemented with 2% FBS, penicillin [100 U/ml], streptomycin [100 μ g/ml], amphotericin B [250 μ g/ml], endothelin-1 [0.25 μ g/ml], IFN- γ [100 U/ml], insulin [10 μ g/ml], transferrin [5 μ g/ml], endothelial cell growth supplement [7.5 μ g/ml], epidermal growth factor [0.025 μ g/ml], hydrocortisone [0.36 μ g/ml], and T₃ [0.02 μ g/ml]) at 33°C.

Clara cells. Clara cells were isolated from C57Bl/6 or from TNFR-KO mice using a protocol modified from Corti and colleagues (21), as previously described (19).

RAW264.7 macrophages. Murine RAW264.7 macrophages, obtained from American Type Culture Collection (Rockville, MD), were maintained in low-bicarbonate (1.5 g/liter) Dulbecco's modified Eagle's medium, supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 4 mM L-glutamine.

Stimulation of C22 Cells with LPS or TNF- α

C22 cells were plated at a density of 4.5×10^5 cells per 60-mm plate and incubated overnight in nonpermissive conditions (media not containing IFN- γ at 39°C to inactivate the major histocompatibility complex-driven large T-antigen) in a 10% CO₂ incubator (20). After incubation, C22 cells were washed three times in serum-free media and incubated for 24 hours with serum-free media alone or with media containing various concentrations of *Escherichia coli* LPS (L-4005) or mouse TNF- α (Sigma, St. Louis, MO). In some experiments, C22 cells were treated simultaneously with LPS (10 ng/ml) and TNF- α (25 ng/ml).

Detection of Cytokine Production

Conditioned media of C22 cells treated with 20 ng/ml TNF- α for 24 hours were collected and cytokine levels were assayed using the Raybio Mouse Cytokine Antibody Array (MA6060 TranSignal; Panomics, Redwood City, CA), according to the manufacturer's recommendations. Blots were scanned and analysis of optical densities (OD) was performed on a personal computer using the public domain program, NIH Image (National Institutes of Health, Bethesda, MD; available online at <http://rsb.info.nih.gov/ni-image/>). The average OD of each cytokine was normalized to the average OD of the biotin-conjugated control samples (upper left and lower right corners of each membrane) on the same membrane, to eliminate loading differences between membranes. Fold change was then determined as the difference between normalized untreated and TNF- α -treated conditions.

Conditioned media of C22 cells treated for 24 hours with various concentrations of LPS, TNF- α , or both were collected, and levels of KC, monocyte chemoattractant protein (MCP)-1, TNF- α , TNFR1, or TNFR2 in the conditioned media were determined by ELISA, according to the manufacturers' recommendations (mouse KC DuoSet, mouse TNF- α OptEIA Set, mouse MCP-1 OptEIA set; BD Biosciences, San Jose, CA; and mouse TNFR1 DuoSet, and mouse TNFR2 DuoSet; R&D Systems, Minneapolis, MN). ELISA data represent at least three independent experiments performed in triplicate.

Intratracheal Administration of LPS

Wild-type (WT) or TNFR-KO mice were anesthetized by intraperitoneal injection of 87 mg/kg ketamine plus 13 mg/kg xylazine. Under sedation, the trachea was exposed and 25 μ l of PBS alone or containing

5 μ g LPS was instilled directly into the trachea via an insulin syringe. After 2 hours, mice were killed by carbon dioxide narcosis and BAL fluids were obtained, as previously described (22). The BAL fluids were assayed for cytokines by ELISA. The lungs were inflation-fixed with formalin and paraffin embedded, as previously described (23).

In Situ Hybridization

Expression of KC in response to LPS was determined by *in situ* hybridization using a 315-bp fragment corresponding to nucleotides 533–847 of the mouse KC gene (24) that was generated as previously described (19). To determine the number of airway epithelial cells expressing KC, digoxigenin (DIG)-positive cells within 200 μ m of basement membrane of the bronchoalveolar region in distal airways were counted in five random high-power fields (HPF; 40 $\times$) in one lung section per mouse. To determine the number of peripheral lung cells expressing KC, DIG-positive cells were counted in five random HPF in one lung section per mouse. Lung sections were obtained from at least seven mice per condition. Results for airway epithelial cells are expressed as the average number of KC-expressing cells per 200 μ m length of basement membrane. Results for peripheral lung cells are expressed as the average number of KC expressing cells per HPF.

Transwell Coculture System and LPS Stimulation

RAW264.7 macrophages were plated at a density of 1×10^6 in the upper chamber of 22-mm diameter, 0.4- μ m pore size Costar Transwell Permeable Supports (Corning Inc., Corning, NY). C22 cells were plated in media not containing IFN- γ at a density of 2.5×10^5 cells in the lower chamber in a six-well plate. After incubation at 37°C, the media in the upper chamber was replaced with serum-free media alone, while the media in the lower chamber was replaced with either serum-free media alone or containing 10 ng/ml LPS. We have shown previously that RAW264.7 macrophages plated in the upper chamber produce TNF- α in response to LPS-containing media placed in the lower chamber, indicating that LPS diffuses across the Transwell membrane (19). Therefore, LPS was placed only in the lower chamber of the Transwell in all experiments. After 24 hours, the conditioned media from both the lower and the upper chambers were pooled and assayed for KC and MCP-1 by ELISA, as described previously here. In some studies, Clara cells from WT or TNFR-KO mice were isolated as previously described (19) and plated in equal numbers in the lower chamber of a six-well plate. In all experiments, C22 or Clara cells treated with LPS in monocultures served as controls.

RNA Isolation and Quantitative Real-Time PCR

RNA was isolated from C22 cells using the PureLink Micro-to-Midi isolation kit (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed using random hexamers and the GeneAmp RNA PCR Core kit (Roche, Branchburg, NJ). After reverse transcription, real-time PCR reactions were performed using Mx3000p thermocycler (Stratagene, La Jolla, CA), CYBR green, and primers specific for KC, TNFR1, or TNFR2. The primers for mouse KC were 5'-GGG CGCTATCGCCAAT-3' and 5'-ACCTCAAGCTCTGGATGTT CTTG-3' (25). The primers for mouse TNFR1 were 5'-GGGGCACC TTTACGGCTTCC-3' and 5'-GGTTCTCCTTACAGCCACACA-3' (26). The primers for mouse TNFR2 were 5'-GTCGCGCTGG TCTTCGAAGTG-3' and 5'-GGTATACATGCTTGCCCTCACA GTC-3' (27). The primers for the mouse housekeeping gene, L32, were 5'-CAGGGTGCGGAGAAGGTTCAAGGG-3' and 5'-CTTA GAGGACACGTTGTGAGCAATC-3' and were used as a control. Samples were run for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The cycle threshold (C_T) for each condition was determined and normalized to that of the L32 housekeeping gene for loading (Δ C_T). Differences between C_T values of LPS-treated and untreated ($\Delta\Delta$ C_T) were used to calculate the fold change (fold change = $2^{-\Delta\Delta C_T}$) (28). Data represent at least three independent experiments performed in duplicate.

Small Interfering RNA Transfection

C22 cells were plated at a density of 1×10^5 in a six-well plate. Cells were transfected with 50 nM small interfering (si) RNA for TNFR1, TNFR2, or a scrambled sequence as control (Sigma), using Lipofect-

amine 2000 (Invitrogen, Carlsbad, CA) as the transfecting agent and according to the manufacturer's recommendations. Three duplexes of siRNA were tested for each receptor. Two duplexes per receptor demonstrated knockdown of 60–80% of the corresponding receptor and were thus used for further experiments. After 48 hours, siTNFR1, siTNFR2, or scramble transfected cells were treated with LPS as a monoculture or in the Transwell coculture system with RAW264.7 macrophages, as described previously here. After 24 hours of LPS treatment, RNA was isolated from siRNA-transfected cells, reverse transcribed, and subjected to quantitative PCR (qPCR) reactions, as described previously here, to determine the percentage of knockdown of TNFR1 and TNFR2 expression, and expression of KC.

Statistical Analysis

All analysis was performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL). An independent, two-sided *t* test was used to analyze the relationship between continuous variables. A *P* value of less than 0.05 was considered significant. All data represent at least three independent experiments performed in triplicate for ELISA and in duplicate for qPCR.

RESULTS

Cytokine Profile of C22 Cells in Response to TNF-α

We have previously used a mouse cytokine antibody array to determine the main cytokines produced by Clara cells in response to LPS (19). This array contained antibodies to 32 cytokines, including CXC chemokines (KC and macrophage inflammatory protein [MIP]-2), CC chemokines (RANTES [regulated upon activation, normal T-cell expressed and secreted], MIP-1α, MIP-3β, MCP-1, MCP-5, 6-cysteine chemokine, cutaneous T-cell-attracting chemokine, eotaxin, thymus and activation-regulated chemokine), colony stimulating factors (CSF) (G-CSF, GM-CSF, stem cell factor, vascular endothelial growth factor, thrombopoietin), a pleiotropic cytokine (TNF-α), and receptor (sTNFRI), an IFN (IFN-γ), ILs (IL-2, -3, -4, -5, -6, -9, -10, -12p40, -12-p70, -13, -17), a metalloproteinase inhibitor (tissue inhibitor of metalloproteinase-1), and a hormone (leptin). Of the 32 cytokines, only a few were up-regulated by C22 cells in response to LPS: KC, 14.6-fold; MCP-1, 2.8-fold; MIP-2, 2.3-fold; and RANTES, 1.4-fold (19). The same cytokine antibody array was used to examine the C22 cell cytokine production profile in response to TNF-α. Similar to the response to LPS, KC, MCP-1, and RANTES were the main cytokines upregulated in response to TNF-α (Figure 1). However, the magnitude to which each cytokine was upregulated was different (KC, 8.2-fold; MCP-1, 12.7-fold; and RANTES, 3.0-fold). TNF-α was not secreted into the culture media of TNF-α-treated C22 cells, nor in response to LPS (19). These

data indicate that TNF-α indeed triggers C22 cells to produce cytokines, and that there is a quantitative difference in cytokine production between LPS and TNF-α.

KC and MCP-1 Production by C22 Cells in Response to LPS and TNF-α

To confirm and quantify the distinct profiles of KC and MCP-1 production in response to LPS and TNF-α stimulation, C22 cells were treated with a range of concentrations of LPS or TNF-α for 24 hours. KC and MCP-1 production peaked at 40 ng/ml and 50 ng/ml, respectively, in response to 1 ng/ml LPS (Figure 2A). KC and MCP-1 production peaked at 24 ng/ml and 64 ng/ml, respectively, in response to 25 ng/ml TNF-α (Figure 2B). Thus, the upregulation of KC is more significant in response to LPS, whereas that of MCP-1 is higher in response to TNF-α. A 24-hour time course demonstrated that the secretion of both KC and MCP-1 was elevated by 2 hours of LPS (10 ng/ml; Figure 2C) or TNF-α (25 ng/ml; Figure 2D) stimulation of C22 cells, and continued to increase over the entire 24-hour period of study.

LPS and TNF-α Have a Synergistic Effect on KC Production by C22 Cells

Because KC and MCP-1 exhibit quantitatively different responses to LPS and TNF-α, we examined whether the combined stimulation would exert different responses as well. C22 cells were treated with LPS (10 ng/ml) or TNF-α (25 ng/ml) alone, or in combination, using concentrations shown to elicit maximal KC and MCP-1 production (Figure 2). The production of both KC and MCP-1 increased significantly in response to the combined LPS and TNF-α stimulation compared with each stimulus alone, suggesting that induction of both cytokines can occur via more than one signaling pathway (Figure 3). However, the production of MCP-1 in response to the combined LPS and TNF-α stimulation was only slightly higher (1.1-fold) than the sum of the responses of the two alone. In contrast, KC production by C22 cells in response to simultaneous LPS and TNF-α treatment was significantly higher (2.5-fold) than the sum of KC produced in response to each of the stimuli alone. These data suggest that LPS and TNF-α act synergistically to induce KC production from C22 cells. In addition, they demonstrate that different cytokines respond in different ways to combined LPS and TNF-α stimulation.

Macrophages Augment C22 Cell Production of KC in Responses to LPS

Previously, we have shown that the predominant source of TNF-α in the lung in response to LPS is the alveolar macro-

Figure 1. Cytokine profile of C22 cells in response to tumor necrosis factor (TNF)-α. C22 cells were treated with 20 ng/ml TNF-α for 24 hours. The conditioned media from three independent experiments were collected, and cytokine production was assayed using an antibody array. Blots were scanned, op-

tical densities adjusted based on positive controls (upper left, lower right), and the fold change, from untreated to TNF-α-treated, calculated as described in MATERIALS AND METHODS. A representative blot is shown with its quantification. Solid ellipses, keratinocyte-derived chemokine (KC); long black dashed ellipses, monocyte chemoattractant protein (MCP)-1; long gray dashed ellipses, regulated upon activation, normal T-cell expressed and secreted (RANTES); short black dashed ellipses, TNF-α.

Figure 2. KC and MCP-1 secretion by C22 cells in response to LPS or TNF- α . C22 cells were treated with various concentrations of LPS (A) or TNF- α (B) for 24 hours, and the levels of KC (open bars) and MCP-1 (shaded bars) in the conditioned media were measured by ELISA. Alternatively, C22 cells were treated with 10 ng/ml LPS (C) or with 25 ng/ml TNF- α (D) for various times up to 24 hours. Cells treated with serum-free media alone served as control. Solid triangles, KC control; solid squares, KC treated; shaded triangles, MCP-1 control; shaded squares, MCP-1 treated. Data represent means ($\pm$ SEM) of at least three independent experiments done in triplicate.

phage (19). As production of KC by C22 cells is significantly enhanced by the combination of LPS and TNF- α , we examined whether C22 cells produce more KC in response to LPS in the presence of macrophages. Therefore, C22 cells were cocultured with RAW264.7 macrophages in a Transwell system. No KC was detected in the culture media of RAW264.7 macrophages grown in monocultures at baseline or in response to LPS. This is consistent with the findings of Ohmori and colleagues (29), who used RAW264.7 cells to determine the transcriptional regula-

Figure 3. Secretion of KC by C22 cells in response to combined LPS and TNF- α is synergistic. C22 cells were treated with 10 ng/ml LPS alone, 25 ng/ml of TNF- α alone, or LPS and TNF- α combined for 24 hours and the levels of KC (open bars) and MCP-1 (shaded bars) in the conditioned media were measured by ELISA. Data represent means ($\pm$ SEM) of at least three independent experiments done in triplicate. * $P < 0.05$.

tion of LPS-induced KC expression, as they do not express endogenous KC mRNA. Therefore, KC levels measured in the culture media using ELISA reflected KC production by C22 cells only. C22 cells produced KC in low levels at baseline, and its production increased in the presence of RAW264.7 macrophages, even in the absence of LPS stimulation, although statistical significance was not met ($P = 0.07$). In response to LPS, C22 cells produced 5.3-fold more ($P < 0.001$) KC when cocultured with RAW264.7 cells than when treated alone (Table 1). Together, these data suggest that RAW264.7 cells secrete a soluble factor(s) that enhances the epithelial cell production of KC at baseline, and more so in response to LPS.

Macrophages Impact Clara Cell Response to LPS via TNF- α

To examine whether TNF- α is the soluble factor secreted by RAW264.7 macrophages that augments the Clara cell response to LPS, freshly isolated Clara cells from WT or TNFR-KO mice were plated in a Transwell coculture system with RAW264.7 macrophages. Similar to C22 cells, Clara cells isolated from WT mice produced KC at baseline (Figure 4A), and its production was significantly higher in the presence of RAW264.7 macrophages ($P = 0.01$), even in the absence of LPS stimulation. Consistent with our previous findings (19), LPS stimulated KC production by WT Clara cells. However, KC production by LPS-treated WT Clara cells was significantly enhanced (5.9-fold; $P < 0.001$) in the presence of RAW264.7 macrophages (Table 1). This is similar to the upregulation observed in LPS-treated C22 cells (5.3-fold) in the presence of RAW264.7 macrophages (Table 1).

Clara cells isolated from TNFR-KO mice produced KC in response to LPS to a similar level as WT Clara cells, which was significantly enhanced (2.6-fold; $P < 0.001$) by the presence of

TABLE 1. KC PRODUCTION BY C22 CELLS IN RESPONSE TO LPS IS AUGMENTED IN COCULTURE WITH RAW264.7 MACROPHAGES

Cell Type	KC (ng/ml)	
	Control	LPS
C22	0.4 $\pm$ 0.06	17.7 $\pm$ 1.2
RAW	N/D	N/D
C22 + RAW	0.8 $\pm$ 0.2	93.3 $\pm$ 10.5

Definition of abbreviations: KC = keratinocyte-derived chemokine; N/D = not detected.

Data represent mean ($\pm$ SEM) from at least three independent experiments.

Figure 4. The effect of macrophages on the Clara cell secretion of KC to LPS is mediated via TNF- α . Freshly isolated Clara cells from wild type (WT) (A) or TNF receptor knockout (TNFR-KO) (B) mice were placed in the bottom chamber of a Transwell system alone or with RAW264.7 cells in the upper chamber, as described in MATERIALS AND METHODS, and were treated with 10 ng/ml LPS for 24 hours. The levels of KC in the conditioned media were measured by ELISA. Data represent means ($\pm$ SEM) of at least three independent experiments done in triplicate. * $P < 0.05$.

RAW264.7 macrophages (Figure 4B). However, KC production by LPS-treated TNFR-KO Clara cells in the presence of RAW264.7 macrophages was significantly lower (by 66%) than the production by WT cells ($P < 0.001$). This indicates that TNF- α is the predominant factor produced by RAW264.7 cells in response to LPS that augments the C22 cell response. These data also show that there is another macrophage-derived soluble factor(s) that impacts the Clara cell response to LPS.

KC Expression Is Significantly Reduced in Distal Airway Epithelium, but Not Alveolar Macrophages, of TNFR-KO Mice in Response to LPS

To examine whether our findings apply *in vivo*, WT and TNFR-KO mice were treated with intratracheal LPS for 2 hours and examined for KC expression. The 2-hour time point was chosen because we have previously shown that TNF- α is present at significant levels in BAL fluid 2 hours after LPS exposure (19). No KC expression was noted in either WT or TNFR-KO mice treated with PBS (Figures 5A and 5D). KC expression was prominent both in airway epithelial and peripheral lung cells of WT mice 2 hours after LPS stimulation (Figures 5B and 5C), consistent with previous findings (19). In TNFR-KO mice (Figures 5E and 5F), KC expression in distal airways was significantly reduced compared with that in WT mice (Figure 5G) ($P < 0.001$), confirming that TNF- α plays an important role in augmenting airway epithelial expression of KC in response to LPS. In contrast, expression of KC in the lung periphery was similar between WT and TNFR-KO mice ($P = 0.19$) (Figure 5H), suggesting that other factor(s) can compensate for the lack of TNF- α signaling in cells in the lung periphery.

TNF- α Augments Clara Cell Responses to LPS via TNFR1

TNF- α can exert its effect by signaling via two different receptors, TNFR1 and TNFR2. To examine the expression of these receptors by Clara cells, RNA isolated from untreated and LPS-treated C22 cells was subjected to qPCR using primers specific for TNFR1 or TNFR2. Both receptors were expressed constitutively by C22 cells, and were not upregulated in response to LPS (fold change, 0.95 ± 0.01 and 1.05 ± 0.07 , respectively) (data not shown). To determine which TNF-receptor is involved in macrophage-derived TNF- α augmentation of Clara cell responses to LPS, the expression of TNFR1 or TNFR2 in C22 cells was selectively inhibited using siRNA. C22 cells transfected with TNFR1 or TNFR2 siRNA duplexes showed significant reductions in TNFR1 ($64.2 \pm 2.5\%$) or TNFR2 ($69.8 \pm 3.6\%$) messages, respectively (data not shown). C22 cells were separately transfected with scrambled siRNA duplexes as a negative control. In scrambled-transfected LPS-treated C22 cells, the expression of KC was significantly higher (6.4-fold; $P = 0.03$) when treated in coculture with RAW264.7 macrophages than when treated alone (Figure 6), suggesting that KC expression was not affected by the transfection procedure. KC expression in LPS-treated C22 cells in which TNFR2 was knocked down was also markedly elevated (5.7-fold; $P < 0.001$) in the presence of RAW264.7 macrophages compared with LPS-treated C22 cells alone, similar to scrambled-transfected cells ($P = 0.72$) (Figure 6). In contrast, in LPS-treated C22 cells in which TNFR1 was knocked down, although KC expression was higher (twofold) in coculture with macrophages than in LPS-treated C22 cells alone ($P = 0.002$), this increase was much less than for LPS-treated, TNFR2-transfected ($P < 0.001$) or LPS-treated, scrambled-transfected ($P = 0.04$) cells. Accordingly, macrophage-derived TNF- α augments KC production in LPS-treated C22 cells by signaling primarily via TNFR1. These data also support our observations made previously here, indicating that another macrophage-derived factor(s), besides TNF- α , augments the Clara cell response to LPS.

DISCUSSION

In this article, we describe that Clara cells respond to TNF- α stimulation *in vitro* by producing cytokines—primarily, KC and MCP-1. We also demonstrate that LPS and TNF- α are synergistic, in that stimulation of Clara cells with both agents leads to significantly elevated production of KC compared with the sum of the responses to the two stimuli separately. Further, we show the importance of this synergism by demonstrating *in vitro* and *in vivo* that the response of Clara cells to LPS is augmented in the presence of macrophages, largely mediated via TNF- α signaling through TNFR1.

We have previously reported that Clara cells produce several NF- κ B-regulated cytokines in response to LPS, but that they do not produce TNF- α (19). In the present study, we found that, although C22 cells do not produce TNF- α in response to LPS, they respond to TNF- α stimulation, secreting cytokines that are qualitatively similar but quantitatively distinct from those produced in response to LPS. In addition, TNF- α itself is not secreted by C22 cells in response to TNF- α . Computational models demonstrate that LPS-induced NF- κ B activity in mouse fibroblasts is sustained due to an autocrine loop that is dependent on TNF- α induction and secretion (30). As a result, the upregulation of several cytokine genes is higher in response to LPS than in response to TNF- α stimulation (31). As C22 cells do not produce TNF- α in response to LPS (19), they may lack this autocrine loop, so that their cytokine production potential in response to LPS may be limited. However, as they respond to

Figure 5. The number of KC-expressing cells is significantly reduced in distal airways of TNFR-KO mice compared with wild-type (WT) mice in response to LPS. WT (A–C) and TNFR-KO (D–F) mice were given PBS alone (A and D) or containing 5 μ g/ml LPS (B–C, E–F) by intratracheal injection. After 2 hours, the lungs were inflation fixed and paraffin embedded, and lung sections were examined for KC expression by *in situ* hybridization. Images represent at least three independent experiments using at least two mice per condition. The number of KC-expressing cells was determined by counting the number of digoxigenin-positive cells along 200 μ m of basement membrane (BM) of distal airways from five lung sections per mouse (G) or within the peripheral lung (H) in five high-power fields (HPF) of one lung section from at least seven mice per condition. Data represent means ($\pm$ SEM). * P < 0.05.

TNF- α , one may postulate that, upon exposure to TNF- α originating from other cells, the loop may be restored and augmented cytokine expression may be achieved.

Costimulation experiments were performed using LPS and TNF- α concentrations that individually achieved maximal cytokine production. LPS and TNF- α bind to different cell surface receptors coupled to distinct signaling pathways (30–32). However, they both share a common transcription factor, NF- κ B, which translocates to the nucleus to induce gene expression (30). Costimulation with LPS and TNF- α induced cytokine production that exceeded the response of each stimulus alone. Therefore, it seems likely that a maximal response to each stimulus alone is achieved once its individual receptor, rather than the common signaling pathway, is saturated, and that the increased expression to the combined stimulation occurs through

sustained activation of NF- κ B. This concept is supported by Werner and colleagues (31), who found that maximal expression of certain cytokines in response to LPS was achieved in a downstream TNF- α -dependent manner, resulting in sustained NF- κ B activation. However, the expression of other cytokines, including MCP-1, was not TNF- α dependent (31). Similarly, we found that the combined LPS and TNF- α stimulation is synergistic for KC, inducing markedly greater (2.5-fold higher) production of KC than the sum of the two stimuli alone. In contrast to KC, MCP-1 production by combined LPS and TNF- α stimulation was only 1.1-fold higher than the sum of the stimuli alone. Thus, in Clara cells, the expressions of different cytokines are affected differentially by LPS and TNF- α .

Crosstalk between pulmonary epithelial cells and inflammatory cells has been a topic of interest in recent years. Airway and peripheral lung epithelial cells have been shown to enhance cytokine production by macrophages under several injury models (19, 33–37), whereas macrophages have been shown to enhance cytokine production by peripheral lung epithelial cells in response to LPS, ischemia–reperfusion injury, and other stimuli (38–41). Although many of the *in vitro* studies have indicated that the bidirectional effect was dependent on direct contact between the two cell types (33–36, 41), we and others using the Transwell coculture system have indicated that diffusible factors released by lung epithelial cells are capable of activating the macrophages (19, 37). In the present study, we examined whether macrophages release factors that enhance the Clara cell response to LPS stimulation. As MCP-1 is produced by both Clara cells and macrophages, the effect of coculture on the expression of MCP-1 could not be examined using this model. However, because RAW264.7 macrophages do not produce KC (29), we were able to monitor the Clara cell response specifically. We found that coculture of RAW264.7 macrophages and C22 cells, even in the absence of LPS, increased the production of KC (Table 1), indicating that macrophages constitutively release a soluble factor that is

Figure 6. Macrophage-derived TNF- α augments Clara cell responses to LPS via TNFR1. RNA isolated from C22 cells transfected with TNFR1, TNFR2, or scrambled (Scr) small interfering RNAs were treated with LPS alone (open bars) or in coculture with RAW264.7 macrophages (shaded bars). The RNA was reverse transcribed and quantitative PCR reactions were performed for KC. Data represent means ($\pm$ SEM) of at least four independent experiments done in duplicate. * P < 0.05; NS = not significant.

capable of inducing KC production by C22 cells. Our previous studies showed that the mere coculture of RAW264.7 cells with C22 cells failed to induce the production of TNF- α by the macrophages (19), indicating that this constitutively produced factor is not TNF- α . In addition, in response to LPS, KC production by C22 cells increased more than threefold when cocultured with RAW264.7 macrophages, indicating that macrophages produce soluble, LPS-inducible factors that enhance the production of KC by Clara cells. Using freshly isolated Clara cells from TNFR-KO mice, we found that this effect was significantly reduced, indicating that one of the LPS-induced, macrophage-derived factors is TNF- α . However, the absence of TNF- α signaling failed to totally eliminate the enhanced production of KC by C22 cells, suggesting another unidentified LPS-inducible factor.

TNF- α can signal through one of two receptors: TNFR1 or TNFR2. The 55-kd TNFR1 mediates primarily inflammation and cell death, whereas the 75-kd TNFR2 enhances TNFR1-induced cell death and mediates cell proliferation (42). Mice lacking TNFR1 but not TNFR2 demonstrate deficits in innate immune responses in various models of inflammation (16). For example, only mice deficient in TNFR1 or both TNF receptors, but not those deficient in TNFR2, demonstrated reduced neutrophilic inflammation in response to *Micropolyspora faeni* given intranasally (16). We found that both receptors are expressed by Clara cells at baseline, with no significant change in expression in response to LPS. Using siRNA to downregulate the expression of each receptor, we observed that the TNF- α -dependent effect of macrophages on Clara cell responses to LPS is mediated primarily via TNFR1.

Experiments using mice deficient in either TNF- α or IL-1 receptors, or both, have demonstrated that only mice deficient in both receptors have reduced expression of KC in the BAL and reduced neutrophilic inflammation in the lung in response to LPS or to infection with *E. coli* or *Streptococcus pneumoniae* (43, 44). Mice deficient only in TNF- α signaling had compromised bacterial killing, but inflammation was not reduced (45). We examined whether our *in vitro* findings applied *in vivo* by treating WT and TNFR-KO mice intratracheally with LPS. Unlike RAW264.7 macrophages, alveolar macrophages produce KC in response to LPS, as we have previously shown (19). Using *in situ* hybridization, we were able to examine cell-specific expression of KC in response to LPS *in vivo*. We found that the expression of KC in airway epithelial cells is significantly reduced in the absence of TNF- α signaling, but the alveolar macrophage response remained comparable. This suggests that the epithelial cell-derived factor(s) that augments cytokine production by the inflammatory cells in response to LPS (19) is not TNF- α mediated. One such factor could be IL-1, as TNF- α and IL-1 receptor-deficient mice exhibit reduced inflammatory responses to LPS or bacteria compared with TNF- α -deficient mice (43, 44).

Chimeric mouse models have demonstrated the importance of both resident lung cells and hematopoietic cells for the full production of KC in response to LPS, and the marked reduction in cytokine production and lung inflammation in the absence of macrophages (46, 47). We have previously shown that Clara cells play a significant role in LPS-induced lung inflammation by modulating alveolar macrophage responses to LPS (19). This suggests that these cells must have mechanisms to control and downregulate inflammatory responses. Our data show that maximal KC production by airway epithelial cells in response to LPS is dependent on the presence of macrophage-derived TNF- α . We suggest a model in which, upon LPS stimulation, airway epithelial cells and alveolar macrophages secrete KC and attract neutrophils. Airway epithelial cells also release

soluble factors that augment macrophage production of KC and TNF- α , enhancing neutrophil recruitment (19). TNF- α , along with other macrophage-derived factor(s), augments further KC production by epithelial cells, likely via sustained activation of the NF- κ B pathway. As activation of the airway epithelial NF- κ B pathway is sufficient to drive lung inflammation (48), the crosstalk between alveolar macrophages and airway epithelial cells maximizes neutrophil chemoattraction.

In summary, we report that Clara cell responses to LPS and TNF- α are synergistic. We show that this synergism allows macrophages to amplify Clara cell responses to LPS stimulation *in vitro* and *in vivo*. As Clara cells do not produce TNF- α , activation of macrophages determines the magnitude of cytokine production by Clara cells in response to LPS. Given their role in cytokine production and lung inflammation, together with other factors, macrophage-Clara cell interactions may be significant in regulating the pulmonary inflammatory response to LPS.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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