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Regulation of Chemokine Expression by NaCl Occurs Independently of Cystic Fibrosis Transmembrane Conductance Regulator in Macrophages

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Chronic pulmonary inflammation and infection are the leading causes of morbidity and mortality in cystic fibrosis (CF). While the effect of mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) on airways remains controversial, some groups have demonstrated increases in Na⁺ and Cl⁻ in CF airway surface liquid compared to normal airways. We investigated the consequences of NaCl on pro-inflammatory chemokine and cytokine production by macrophages. Stimulation of mouse macrophages with increasing amounts of NaCl induced macrophage inflammatory protein-2 (MIP-2) and tumor necrosis factor- α (TNF- α) production. Further, co-incubation of macrophages with NaCl in the presence of either lipopolysaccharide (LPS) or TNF- α synergistically increased MIP-2 production. Both the NaCl and NaCl plus LPS responses were partially dependent on endogenous production and autocrine signaling by TNF- α . To investigate the role of CFTR in MIP-2 production, we compared the responses of wild-type and Δ F508 CF mouse macrophages to NaCl and LPS. The responses of macrophages from both strains were indistinguishable. In addition, CFTR mRNA was not expressed in macrophages. Taken together, these findings suggest that NaCl stimulates MIP-2 production by

macrophages through a mechanism that is partially dependent on TNF- α but independent of macrophage CFTR expression. (*Am J Pathol* 2006, 169:12–20; DOI: 10.2353/ajpath.2006.051042)

Cystic fibrosis (CF) is the most common fatal hereditary disorder of the Caucasian population and is the result of the inheritance of mutations in the gene encoding CF transmembrane conductance regulator (CFTR). While many organs are affected in CF, the exuberant inflammatory response and chronic bacterial infections of the airways are major causes of morbidity and mortality.¹ In seeking to investigate the mechanism of CFTR involvement in the pathogenesis of lung disease in CF, we and others have shown that pulmonary inflammation is present in infants as early as 4 weeks after birth.² Furthermore, in more than half of the infants studied, pulmonary inflammation was present even in the absence of detectable infection with common CF pathogens.² These findings led us to propose that inactivation of CFTR function contributes to the initiation of early pulmonary inflammation in CF.^{2,3}

Several hypotheses have been proposed to explain how mutations in the CFTR, a transmembrane protein that maintains airway surface liquid (ASL) function by regulating electrolyte transport across pulmonary epithelial cells,⁴ contribute to airway disease in CF. Based on measurements of the elemental composition of ASL ob-

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tained from CF patients and healthy subjects, together with *in vitro* and *in vivo* models using bronchial epithelial cells, one hypothesis proposes that mutations in CFTR cause abnormalities in electrolyte transport across airway epithelia leading to elevations in NaCl concentration in ASL.^{5–8} Other studies, however, have failed to detect differences in NaCl concentrations in ASL.^{9–12} These studies have led to a second hypothesis in which mutations in CFTR promote increased absorption of isotonic ASL leading to dehydration and volume reduction of the ASL and impaired mucociliary clearance.¹³ Both hypotheses support the view that the ASL is abnormal in CF and arises as a consequence of the loss of CFTR activity. Both hypotheses have also focused attention on the mechanism by which abnormalities in ASL composition affect the function of airway epithelial cells and macrophages in inflammation and innate immunity in CF.

In addition to expression by epithelial cells, sporadic reports have suggested that CFTR is expressed by macrophages and negatively regulates macrophage tumor necrosis factor- α (TNF- α) production. Using nonquantitative polymerase chain reaction (PCR), Yoshimura et al¹⁴ first reported that CFTR mRNA is expressed by human alveolar macrophages. Pfeffer et al¹⁵ showed that monocyte-derived macrophages from CF patients express higher levels of TNF- α than macrophages from normal subjects following lipopolysaccharide (LPS) stimulation. Similar findings were reported by Thomas et al¹⁶ using macrophages from G551D CF mice. Taken together, these findings suggest that macrophages express CFTR mRNA and that loss of CFTR functional activity is associated with increased sensitivity to LPS.

Given that mutations in CFTR may result in increased NaCl concentrations in the ASL of CF patients and that macrophages are an important source of pro-inflammatory chemokines and cytokines, we investigated the consequences of elevated NaCl on the production of pro-inflammatory cytokines by macrophages and the role of CFTR in this response. Here we show that NaCl stimulates the production of macrophage inflammatory protein-2 (MIP-2) by macrophages and amplifies MIP-2 production in response to LPS via a mechanism that is partially dependent on endogenous TNF- α production but independent of CFTR expression. These findings suggest that CFTR function is not required in the response of macrophages to either NaCl or LPS and favor a model in which macrophages respond to the abnormal environment of the lung created by loss of CFTR activity in other cell types.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum were purchased from BioWhittaker (Walkersville, MD) and Atlanta Biologicals (Norcross, GA), respectively. Recombinant mouse TNF- α was purchased from R&D Systems, Inc. (Minneapolis, MN). All enzyme-linked immunosorbent assay (ELISA) kits were pur-

chased from ELISA Tech (Aurora, CO). NaCl, mannitol, sodium gluconate, choline chloride, and *Salmonella typhimurium*-derived LPS were all obtained from Sigma-Aldrich Co. (St. Louis, MO). Sorbitol was obtained from EM Science (Gibbstown, NJ).

Mice

C57Bl/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *tnf- α ^{-/-}* mice were generously donated by Dr. Jack Routes (National Jewish Medical and Research Center, Denver, CO). *tnf-r1/r2^{-/-}* mice and their C57Bl/6 $\times$ 129 controls were generously donated by Dr. Peter Henson (National Jewish Medical and Research Center). All mice were housed at the accredited National Jewish Medical and Research Center Biological Resource Center. Δ F508 mice, originally generated by Dr. William Colledge (University of Cambridge, Cambridge, UK),¹⁷ and their C57Bl/6 wild-type controls were housed at the Health Science Research Facility at the University of Vermont (Burlington, VT).

Macrophage Isolation and Culture

Monolayers of mouse bone marrow-derived macrophages were prepared as previously described.¹⁸ Briefly, bone marrow cells from the tibias, femurs, and pelvises of mice were flushed with, and grown in, DMEM supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10% (v/v) fetal bovine serum, and 10% (v/v) L929 cell-conditioned medium, as a source of macrophage-colony stimulating factor. The bone marrow cells were seeded in 12-well culture dishes at 10^5 cells/well with 2 ml of medium/well. Macrophages were then cultured at 37°C under a 10% (v/v) CO₂ atmosphere for 5 to 7 days with feeding after 5 days.

Determination of MIP-2 and TNF- α Protein Levels in Culture Supernatants

ELISAs were used to determine the concentration of MIP-2 and TNF- α in cell culture supernatants of bone marrow-derived macrophages. The ELISA assays were performed according to the manufacturer's instructions. The sensitivity of both assays was <10 pg/ml. All ELISA data were normalized to cell count obtained using a Coulter Counter Model ZM from Coulter Corporate Communications (Hialeah, FL).

Reverse Transcription (RT) and Quantitative Real-Time PCR for CFTR

Total RNA was isolated from bone marrow-derived macrophages, whole kidney, whole lung, and bronchoalveolar lavage cells using the TRIzol reagent (Life Technologies, Grand Island, NY). RT was performed on 1 μ g of total RNA with random hexamers in a 50- μ l reaction using the TaqMan RT reagents (Applied Biosystems, Foster City, CA). Real-time PCR was performed on the ABI Prism

Table 1. Primers and Probes for Detection of CFTR by Real-Time PCR

Primers	
Exon 5	5'-TGGACAACCTGTTAGTCTTCTTTCCA-3'
Exon 6	5'-CCATCAGAAGAGTCACCTTGTAAGGA-3'
Exon 11	5'-GGATTATGCCGGGTACTATCAAAG-3'
Exon 12	5'-CTCCAAGAAGTGTGTGTCTGTTC-3'
Exon 19	5'-TACTGAGGGCCTACTTCCTTCATAC-3'
Exon 20	5'-GTCCAGAGTCCTTTTAAGCTGTCA-3'
Probes	
Exon 5/6	5'-FAM-TGTGCCAAGGCAAGTCCTTCATCAAATTT-TAMRA-3'
Exon 11/12	5'-FAM-TGCCAACTACAGCAGGACATCACCAAGTT-TAMRA-3'
Exon 19/20	5'-FAM-TGAAAATTGGACTCCTTCAGATTCC-TAMRA-3'

7700 sequence detection system (Applied Biosystems, Foster City, CA). The 50- μ l PCR reactions for CFTR contained either 60 or 100 ng of cDNA, 200 nmol/L fluorogenic probe, 500 nmol/L of each primer, and the other components within the TaqMan Universal PCR Master Mix. These probe and primer concentrations were determined following a rigorous optimization protocol. Three divergent regions of the *cftr* gene were examined by PCR to detect potential splice variants or differences in RT and/or PCR efficiency. The probes, labeled on the 5'-end with the reporter dye 6-carboxyfluorescein and on the 3'-end with the quencher dye 6-carboxytetramethylrhodamine, all spanned an exon-exon junction. The corresponding primers were thus in separate but adjacent exons. Table 1 shows the sequence (accession number M60493) and location of the probes and primers used to detect CFTR mRNA. The specificity of PCR was verified by no signal being present in the no-template and no-RT controls. The housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was evaluated using a PCR protocol similar to CFTR with the exceptions being that the probe concentration was 200 nmol/L, the primer concentration was 100 nmol/L, and the 5' label was 6-carboxyrhodamine. The *GAPDH* probe and primer set were the TaqMan Rodent *GAPDH* Control Reagents part number 4308313 (Applied Biosystems). The threshold cycle (C_T) was recorded for each sample. The relative CFTR mRNA expression levels were determined using the comparative C_T method. The ΔC_T (CFTR average C_T - *GAPDH* average C_T) was calculated. Next, the comparative difference in CFTR expression between the kidney, bronchoalveolar lavage cells, or lung and macrophages, $\Delta\Delta C_T$ (ΔC_T sample - ΔC_T macrophage) was calculated. Finally, the relative-fold difference was determined by using the term, $2^{-\Delta\Delta C_T}$.

Statistical Analysis

Results are expressed as mean $\pm$ SE. Statistical analyses were performed using InStat version 3.0b for Macintosh (GraphPad, San Diego, CA). In the CF and non-CF mouse studies, statistical analyses were done using SAS version 8 (SAS Institute, Cary, NC). We used a two-way analysis of variance with preplanned contrasts to analyze MIP-2 (normalized to cell count) between CF and control. Before the actual analysis, we transformed MIP-2 normalized to cell count using log base 10 to stabilize the variance as described.¹⁹

Results

NaCl Stimulates MIP-2 Production by Mouse Macrophages

To examine the effect of NaCl on MIP-2 production by macrophages, we incubated monolayers of C57Bl/6 bone marrow-derived macrophages with increasing amounts of NaCl (0 to 100 mmol/L; 315 to 490 mOsm/kg H₂O) for 24 hours and quantified MIP-2 levels in the culture supernatants by ELISA. Addition of NaCl to the culture media yielded osmolalities as shown in Table 2. The range of NaCl concentrations was chosen to span the range of osmolalities and Na⁺ and Cl⁻ concentrations reported in ASL from normal subjects and CF patients.^{6,7,20,21} As shown in Figure 1A, unstimulated macrophages did not produce detectable amounts of MIP-2. Incubation with NaCl resulted in a dose-dependent significant increase ($P < 0.05$) in MIP-2 production beginning at 385 mOsm. The increase in MIP-2 production by NaCl was not due to contaminating LPS as stimulation of

Table 2. Osmolalities and Corresponding Concentrations of Added Osmolites

mOsm/kgH ₂ O	NaCl (mmol/L)	Sorbitol (mmol/L)	Mannitol (mmol/L)	Na ⁺ gluconate (mmol/L)	Choline Cl ⁻ (mmol/L)
315	0 (100)	0	0	0	0
350	20 (120)	40	40	20	20
385	40 (140)	80	80	40	40
420	60 (160)	120	120	60	60
455	80 (180)	160	160	80	80
490	100 (200)	200	200	100	100

Stimuli were prepared by adding the osmolites, at the concentrations shown, to DMEM. In the case of NaCl, the figures in parentheses represent the total concentration of NaCl in DMEM plus the added NaCl. The osmolality of each solution, including untreated DMEM, was measured by osmometry.

Figure 1. NaCl stimulates MIP-2 production by mouse macrophages. **A:** ELISA analysis of MIP-2 production by C57Bl/6 bone marrow-derived macrophages stimulated with increasing concentrations of NaCl, sorbitol, and mannitol. Protein levels of MIP-2 in the culture supernatants were normalized to the number of macrophages present at the beginning of the experiment. Osmolality was verified by measurement with an osmometer. The values represent the mean from three or more independent experiments $\pm$ SE. *, Significantly different ($P < 0.05$) from the 315 mOsm point. **B:** ELISA analysis of MIP-2 production by bone marrow-derived macrophages stimulated with NaCl, Na⁺ gluconate, and choline Cl⁻. The values represent the mean from three or more independent experiments $\pm$ SE. *, Significantly different ($P < 0.05$) from the 315 mOsm point on the Na⁺ gluconate curve; +, significantly different ($P < 0.05$) from the 315 mOsm point on the choline Cl⁻ curve; #, significantly different ($P < 0.05$) using one-way analysis of variance and comparing NaCl to Na⁺ gluconate and choline Cl⁻.

mouse macrophages from LPS-resistant C3H/HeJ mice yielded similar results (data not shown). To determine whether the increased production of MIP-2 was due to a general effect of hyperosmolarity, macrophages were exposed to either sorbitol or mannitol at the same osmolalities achieved with NaCl (Table 2). In contrast to NaCl, sorbitol and mannitol had a negligible effect on MIP-2 production (Figure 1A). These data indicate that the ability of NaCl to increase MIP-2 production by mouse macrophages is selective to NaCl and is not due to a general response to hyperosmolarity.

Next, we determined whether Na⁺, Cl⁻, or both ions were necessary for maximal NaCl-induced MIP-2 production. Macrophages were incubated with increasing concentrations of Na⁺ gluconate or choline Cl⁻ for 24 hours, and MIP-2 levels were measured in the culture supernatants. As can be seen in Figure 1B, at osmolalities below 450 mOsm, Na⁺ gluconate and choline Cl⁻ significantly increased ($P < 0.05$) MIP-2 production to a similar extent as NaCl. At osmolalities above 450 mOsm, the level of MIP-2 produced by each ion was approximately half that seen from when both ions were present. Thus, although Na⁺ or Cl⁻ alone can increase MIP-2, both are required for maximal MIP-2 production.

Costimulation by Pro-Inflammatory Stimuli and NaCl Leads to a Potentiation in MIP-2 Production

While CF patients exhibit basal airway inflammation, infection with common gram-negative bacteria greatly augments the inflammatory response of the airways.² To model the effects of gram-negative bacteria, we determined the effect of NaCl on macrophage MIP-2 production in the presence of LPS. Macrophage monolayers were incubated for 24 hours with increasing amounts of NaCl (0 to 100 mmol/L; 315 to 490 mOsm/kg H₂O) in the presence or absence of LPS (1 ng/ml). Additionally, we determined the effect of NaCl on MIP-2 production in response to TNF- α (10 ng/ml). As shown in Figure 2A, while treatment with either LPS or TNF- α in medium alone (315 mOsm/kg H₂O) induced significant ($P < 0.05$) MIP-2 production by macrophages (LPS: 17.13 ± 2.91 , TNF- α : 2.07 ± 0.25 compared to unstimulated: 0.30 ± 0.11 ng MIP-2/10⁶ cells), NaCl synergistically increased MIP-2 production in response to both stimuli.

To determine whether the synergistic increase in MIP-2 production by NaCl was representative of a more general response to hyperosmolarity, macrophages were exposed to sorbitol at identical osmolalities to that of NaCl either alone or with LPS (1 ng/ml) or TNF- α (10 ng/ml) for 24 hours before measurement of MIP-2 levels in culture supernatants. As shown in Figure 2B, the effect of sorbitol on either LPS or TNF- α -induced MIP-2 production was negligible. Collectively, these data indicate that NaCl greatly augments LPS- and TNF- α -induced MIP-2 production by macrophages and that this effect is not simply due to hyperosmolarity.

NaCl-Induced MIP-2 Production Is Partially Dependent on TNF- α

Many pro-inflammatory cytokines, including MIP-2, have been shown to be induced by TNF- α .^{22,23} For this reason, we investigated the hypothesis that NaCl increases MIP-2 production in a TNF- α -dependent fashion. First, we determined if NaCl increased TNF- α production. Monolayers of C57Bl/6 bone marrow-derived macrophages were incubated with increasing amounts of NaCl (0 to 100 mmol/L; 315 to 490 mOsm/kg H₂O) for 24 hours, and

Figure 2. Costimulation by pro-inflammatory stimuli and NaCl leads to a potentiation in MIP-2 production. **A:** ELISA analysis of C57Bl/6 bone marrow-derived macrophages stimulated with increasing concentrations of NaCl in either the presence or absence of 1 ng/ml LPS or 10 ng/ml TNF-α. Protein levels of MIP-2 in the culture supernatants were normalized to the number of macrophages present at the beginning of the experiment. Osmolality was verified by measurement with an osmometer. The values represent the mean from three or more independent experiments $\pm$ SE. * P < 0.05 when compared to the 315 mOsm point on the NaCl plus TNF-α curve. + P < 0.05 on the NaCl plus LPS curve. # P < 0.05 using one-way analysis of variance analysis comparing NaCl to NaCl plus LPS and NaCl plus TNF-α. **B:** ELISA analysis of C57Bl/6 bone marrow-derived macrophages stimulated with increasing concentrations of sorbitol in either the presence or absence of 1 ng/ml LPS or 10 ng/ml TNF-α. The values represent the mean from three independent experiments $\pm$ SE.

TNF-α levels were measured in culture supernatants by ELISA. As shown in Figure 3A, NaCl significantly (P < 0.05) increased TNF-α production. Further, the increase in TNF-α production qualitatively paralleled that of MIP-2 (Figure 1A).

Second, to determine the role of endogenously produced TNF-α on MIP-2 production, we investigated the effect of NaCl on macrophages from *tnf-α*^{-/-} and *tnf-r1/r2*^{-/-} mice. Macrophages from *tnf-α*^{-/-} and *tnf-r1/r2*^{-/-} mice and their wild-type controls (C57Bl/6 and C57Bl/6 $\times$ 129, respectively) were incubated with increasing amounts of NaCl (0 to 100 mmol/L; 315 to 490 mOsm/kg H₂O) for 24 hours, and the amount of MIP-2 in the culture

Figure 3. NaCl-induced MIP-2 production is partially dependent on TNF-α. **A:** ELISA analysis of TNF-α production by C57Bl/6 bone marrow-derived macrophages stimulated with increasing concentrations of NaCl. Protein levels of TNF-α in the culture supernatants were normalized to the number of macrophages present at the beginning of the experiment. Osmolality was verified by measurement with an osmometer. The values represent the mean from three independent experiments $\pm$ SE. * P < 0.05 compared to the 315 mOsm point. **B:** ELISA analysis of MIP-2 production by C57Bl/6 and *tnf-α*^{-/-} bone marrow-derived macrophages stimulated with increasing concentrations of NaCl. The values represent the mean from three independent experiments $\pm$ SE. # P < 0.05 for differences between the means for C57Bl/6 and *tnf-α*^{-/-} macrophages. **C:** ELISA analysis of MIP-2 production by C57Bl/6 $\times$ 129 and *tnf-r1/r2*^{-/-} bone marrow-derived macrophages stimulated with increasing concentrations of NaCl. The values represent the mean from three independent experiments $\pm$ SE. # P < 0.05 for differences between the means for C57Bl/6 $\times$ 129 and *tnf-r1/r2*^{-/-} macrophages.

supernatants was determined. Both the inability of macrophages to produce TNF- α (Figure 3B) and the inability to respond to TNF- α (Figure 3C) resulted in a significant ($P < 0.05$), though incomplete, inhibition of MIP-2 production in response to NaCl. Similar experiments were performed on C57Bl/6 macrophages in the presence of an anti-TNF- α neutralizing antibody. Here, too, MIP-2 production was significantly ($P < 0.05$) but incompletely inhibited (data not shown). Collectively, these findings suggest that the production of MIP-2 in response to NaCl is partially dependent on endogenously produced TNF- α via signaling through TNF-R1 and/or TNF-R2.

Next, we investigated the role of autocrine signaling by TNF- α on the augmentation of LPS-induced MIP-2 production by NaCl. To determine whether NaCl potentiated TNF- α production by LPS, we exposed C57Bl/6 macrophages to increasing amounts of NaCl (0 to 100 mmol/L; 315 to 490 mOsm/kg H₂O) in the presence of LPS (1 ng/ml) for 24 hours and then measured the amount of TNF- α present in the culture supernatants. As shown in Figure 4A, macrophages produced ~ 1 ng of TNF- α /10⁶ cells following stimulation with LPS alone, whereas incubation with LPS in the presence of NaCl resulted in a significant ($P < 0.05$) potentiation of TNF- α production.

To directly evaluate the role of autocrine-signaling by TNF- α in the synergistic increase of MIP-2 production by NaCl and LPS, macrophage monolayers from *tnf- α ^{-/-}* and *tnf-r1/r2^{-/-}* mice and their wild-type controls (C57Bl/6 and C57Bl/6 $\times$ 129, respectively) were incubated with increasing concentrations of NaCl in the presence of LPS (1 ng/ml) for 24 hours before determination of MIP-2 levels. Compared to wild-type controls, macrophages from *tnf- α ^{-/-}* (Figure 4B) and *tnf-r1/r2^{-/-}* (Figure 4C) produced less MIP-2 in response to LPS alone. Strikingly, the potentiating effect of NaCl on LPS-induced MIP-2 production was significantly ($P < 0.05$) though incompletely reduced in macrophages from both strains of mutant mice. Similar results were obtained with wild-type macrophages when endogenous TNF- α activity was blocked with a neutralizing antibody (data not shown). Thus, autocrine signaling by TNF- α also contributes to the NaCl-induced potentiation of MIP-2 production in response to LPS.

NaCl- and LPS-Induced MIP-2 Production by Macrophages Occurs Independently of CFTR

Given our findings that macrophages produce increased amounts of MIP-2 in response to NaCl and the reports suggesting that macrophages express CFTR, we next determined if CFTR activity was involved in the regulation of MIP-2 production.¹⁴ To this end we used Δ F508 CF mice as this is the most common mutation in CF, occurring in $\sim 70\%$ of patients,²⁴ to investigate the production of MIP-2 by macrophages. Macrophage monolayers from Δ F508 and C57Bl/6 wild-type littermate control mice were incubated in medium alone, NaCl (450 mOsm), LPS (1 ng/ml), or both LPS and NaCl together for 24 hours before measurement of MIP-2 accumulation in the culture supernatants. Figure 5 shows that macrophages from wild-type

and Δ F508 CF mice produced indistinguishable amounts of MIP-2 in response to all stimuli tested. Analysis of TNF- α production by ELISA assay also demonstrated no difference in the responses of Δ F508 and C57Bl/6 macrophages (data not shown). These findings indicate that the production of MIP-2 induced by NaCl and LPS, as well as the synergistic increase in chemokine production observed following costimulation with LPS and NaCl, occurs independently of CFTR activity in macrophages.

CFTR Is Not Expressed by Macrophages

Based on these findings, we next determined if CFTR mRNA was expressed by mouse macrophages. Kidney and lung mRNAs were used as positive controls for CFTR expression.²⁵ Real-time PCR probes were designed to overlap exon-exon boundaries to negate amplification of genomic DNA. No CFTR was detected in samples that did not receive reverse transcriptase as part of their preparation, indicating a lack of contamination with genomic DNA (data not shown). GAPDH was also amplified to verify the integrity of the RNA/cDNA and to provide a means of controlling for variations in total RNA between samples. The maximum number of amplification cycles used to detect mRNA expression was 40. Therefore, the C_T of any samples in which the mRNA could not be detected was reported as >40 . Table 3 shows that, although CFTR mRNA was readily detected in kidney and lung, it was not detected in macrophages using any of the three primer pairs and probe sets tested. Minimally, kidney cells express 1600- to 3100-fold more CFTR mRNA than macrophages. Lack of detection of CFTR mRNA in macrophages was not a result of degradation of the RNA as GAPDH expression was detected in samples of macrophage RNA and in equivalent levels to that of kidney. We also investigated CFTR mRNA expression in mouse alveolar macrophages. Although CFTR mRNA was detected in RNA isolated from the lungs of BALB/c mice, it was not detected in alveolar macrophages (Table 3).

Discussion

To date, it is still unclear as to how loss of CFTR function in the lung leads to the development of chronic pulmonary inflammation and infection in cystic fibrosis. Based on the hypothesis that loss of CFTR function in pulmonary epithelia leads to elevations in the concentration of NaCl in the ASL of CF patients,⁶ combined with our previous studies showing early pulmonary inflammation in infants in the absence of colonization with common CF pathogens,² we investigated the effects of NaCl on the production of pro-inflammatory chemokines and cytokines by macrophages. The major findings from this study are that elevations in the concentration of NaCl stimulate the production of MIP-2 and amplify MIP-2 production in response to LPS and TNF- α . We also show that these responses are partially dependent on autocrine signaling by TNF- α and occur independently of CFTR expression or function in macrophages.

Figure 4. Potentiation of MIP-2 production by NaCl and LPS requires TNF- α signaling. **A:** ELISA analysis of TNF- α production by C57Bl/6 bone marrow-derived macrophages stimulated with increasing concentrations of NaCl in the presence of 1 ng/ml LPS. Protein levels of TNF- α in the culture supernatants were normalized to the number of macrophages present at the beginning of the experiment. Osmolality was verified by measurement with an osmometer. The values represent the mean from three independent experiments $\pm$ SE. * P < 0.05 compared to the 315 mOsm point. **B:** ELISA analysis of MIP-2 production by C57Bl/6 and *tnfr- α* ^{-/-} bone marrow-derived macrophages stimulated with increasing concentrations of NaCl in the presence of 1 ng/ml LPS. The values represent the mean from three or greater independent experiments $\pm$ SE. * P < 0.05 for differences between the means for C57Bl/6 and *tnfr- α* ^{-/-} macrophages. **C:** ELISA analysis of MIP-2 production by C57Bl/6 $\times$ 129 and *tnfr-1/r2*^{-/-} bone marrow-derived macrophages stimulated with increasing concentrations of NaCl in the presence of 1 ng/ml LPS. The values represent the mean from three independent experiments $\pm$ SE. * P < 0.05 for differences between the means for C57Bl/6 $\times$ 129 and *tnfr-1/r2*^{-/-} macrophages.

Figure 5. NaCl- and LPS-induced MIP-2 production by macrophages occurs independently of CFTR. ELISA analysis of MIP-2 production by C57Bl/6 and Δ F508 bone marrow-derived macrophages stimulated with media, 450 mOsm NaCl, 1 ng/ml LPS, or NaCl and LPS together. Protein levels of MIP-2 in the culture supernatants were normalized to the number of macrophages present at the beginning of the experiment. The values represent the mean from six independent experiments $\pm$ SE.

The findings of the present study suggest that the production of MIP-2 in response to NaCl is not solely due to a hyperosmolar response, because stimulation with sorbitol and mannitol did not reproduce the effect of NaCl on MIP-2 production. Our previous studies have shown that sorbitol and mannitol stimulate c-Jun N-terminal kinase and p38 MAPK activation in macrophages,²⁶ confirming that these cells exhibit an appropriate hyperosmolar response to organic osmolytes. In the present study, replacement of Na⁺ or Cl⁻ with choline or gluconate quantitatively reproduced the effect of NaCl on MIP-2 production at lower osmolyte concentrations but not at the highest concentration examined. Taken together, these findings suggest that, although hyperosmolarity per se is ineffective at promoting pro-inflammatory chemokine production by macrophages, hypertonicity is effective. Further, the concentration of Na⁺ and Cl⁻ is important in determining maximal MIP-2 production. Similar conclusions have been drawn in studies of mucociliary transport *in vivo* and in fluid absorption by airway epithelial cells.^{27,28}

Mammalian cells respond to hypertonicity by regulating gene expression. Previous studies have shown that hypertonicity activates NF- κ B in bronchial epithelial cells²⁹ and macrophages, as measured by electrophoretic mobility shift assay analysis (A.G. Kostyk and D.W.H. Riches, unpublished observations). Similarly, hypertonicity has been shown to activate tonicity-responsive enhancer binding protein (TonEBP) in a p38 MAPK-dependent fashion.³⁰ Additionally, TNF- α -induced expression of the aldose reductase gene has been shown to be dependent on NF- κ B binding to tonicity enhancer (TonE) *cis*-elements.³¹ Because 1) the MIP-2 promoter contains *cis*-elements for NF- κ B and AP-1³² as well as three putative TonE *cis*-elements³³ and 2) the maximal production of MIP-2 in response to NaCl alone, as well as the synergistic increase in MIP-2 production seen during costimulation with LPS, is partially

Table 3. CFTR Expression by Real-Time PCR

	CFTR primer pair and probe set	CFTR average C_T	GAPDH average C_T	Expression level of CFTR relative to macrophage
Macrophage	Exon 5–6	>40	17.00	ND*
	Exon 11–12	>40	17.00	ND
	Exon 19–20	>40	17.00	ND
Kidney	Exon 5–6	29.13	16.85	>1686
	Exon 11–12	28.24	16.85	>3114
	Exon 19–20	28.94	16.85	>1917
Alveolar macrophage	Exon 5–6	>40	23.03	ND
	Exon 11–12	>40	23.03	ND
	Exon 19–20	>40	27.81	ND
Lung	Exon 5–6	30.06	20.42	>161
	Exon 11–12	28.51	20.42	>471
	Exon 19–20	29.99	23.27	>44

CFTR is not expressed by macrophages. Real-time PCR analysis of CFTR expression in C57Bl/6 bone marrow-derived macrophages and kidney and BALB/c alveolar macrophages and lung. Macrophage, kidney, alveolar macrophage, and lung mRNA were isolated, CFTR mRNA was amplified using the primer pair and probe sets shown, and the threshold cycle (C_T) was measured. The C_T for GAPDH was obtained independently and was used to normalize the CFTR expression to the amount of total mRNA in the sample. Relative -fold increase in CFTR expression was determined by the comparative C_T method as stated in the Materials and Methods. A representative experiment is shown.

*ND, not detected.

dependent on endogenously produced TNF- α , we hypothesize that the amplification of MIP-2 production involves cooperativity between NF- κ B, TonEBP, and AP-1 resulting from both NaCl and TNF- α signaling.

Although it is clear that the environment created by the loss of function of CFTR in the lung is of fundamental importance to the development of pulmonary inflammation and colonization with CF pathogens, it is unclear whether or not macrophages themselves are affected in CF, independent of the effects of other cells types. Previous studies have suggested that macrophages from CF patients produce more TNF- α protein and mRNA compared to normal subjects (23). It has also been reported that macrophages from G551D CF mice express more TNF- α mRNA than wild-type macrophages following LPS stimulation.^{15,16} However, we were unable to confirm these findings using macrophages from Δ F508 CF mice. Furthermore, the responses of these mice to NaCl, or to costimulation with NaCl and LPS were indistinguishable from wild-type control macrophages, with regards to MIP-2 production. Although these studies may not be directly comparable and therefore could relate to differences in the genotypes studied or to the stimulation conditions used, we addressed the question of whether or not macrophages express CFTR. Using three different primer pair and probe sets with real-time PCR, we were unable to detect any CFTR expression in bone marrow-derived macrophages or alveolar macrophages. Similar results have recently been reported in transformed macrophage cell lines³⁴ and using electrophysiology in rat alveolar macrophages.³⁵ These findings suggest that, although the expression of chemokines and pro-inflammatory cytokines is increased in CF, it is likely that this represents a response of the macrophages to the abnormal conditions created by loss of function of CFTR in other cells types, especially the airway and submucosal gland epithelium rather than to an intrinsic CFTR-dependent abnormality in macrophage function.

The findings from the present study support two conclusions. First, the abnormal environment created in the

airways of CF patients is an important factor in regulating macrophage pro-inflammatory chemokine and cytokine production. We have shown that elevations in NaCl concentration, as has been reported to occur in the airways of CF patients, stimulate the production of MIP-2. Moreover, although elevations in NaCl in ASL are dependent on loss of function of CFTR in epithelial cells, CFTR is not involved in the responses of macrophages to NaCl or to LPS. This conclusion is consistent with studies reported by Oceandy et al³⁶ in which expression of human CFTR in airway epithelial cells of G551D CF mice rescued the mice from *Pseudomonas aeruginosa*-induced pulmonary inflammation, whereas complementation with CFTR in macrophages was ineffective. Second, we showed that the augmented responses of macrophages to NaCl involved a mechanism that was partially dependent on endogenously produced TNF- α . Because therapy directed against TNF- α has proven highly efficacious in other chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease,^{37,38} future studies could be aimed at evaluating anti-TNF- α therapies in CF and mouse models of CF. However, as with the use of corticosteroids in CF, caution should be exercised to ensure that inhibiting TNF- α does not compromise innate and adaptive immune responses against common CF pathogens.

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