Pulmonary Inflammation Induced by *Pseudomonas aeruginosa* Lipopolysaccharide, Phospholipase C, and Exotoxin A: Role of Interferon Regulatory Factor 1

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Chronic pulmonary infection with *Pseudomonas aeruginosa* is common in cystic fibrosis (CF) patients. *P. aeruginosa* lipopolysaccharide (LPS), phosholipase C (PLC), and exotoxin A (ETA) were evaluated for their ability to induce pulmonary inflammation in mice following intranasal inoculation. Both LPS and PLC induced high levels of tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), IL-6, gamma interferon (IFN-γ), MIP-1α and MIP-2 in the lungs but did not affect IL-18 levels. ETA did not induce TNF-α and was a weak inducer of IL-1β, IL-6, macrophage inflammatory protein 1α (MIP-1α), and MIP-2. Remarkably, ETA reduced constitutive lung IL-18 levels. LPS was the only factor inducing IFN-γ. LPS, PLC, and ETA all induced cell infiltration in the lungs. The role of interferon regulatory factor-1 (IRF-1) in pulmonary inflammation induced by LPS, PLC, and ETA was evaluated. When inoculated with LPS, IRF-1 gene knockout (IRF-1 KO) mice produced lower levels of TNF-α, IL-1β, and IFN-γ than did wild-type (WT) mice. Similarly, a milder effect of ETA on IL-1β and IL-18 was observed for IRF-1 KO than for WT mice. In contrast, the cytokine response to PLC did not differ between WT and IRF-1 KO mice. Accordingly, LPS and ETA, but not PLC, induced expression of IRF-1 mRNA. IRF-1 deficiency had no effect on MIP-1α and MIP-2 levels and on cell infiltration induced by LPS, PLC, or ETA. Flow cytometric evaluation of lung mononuclear cells revealed strongly reduced percentages of CD8+ and NK cells in IRF-1 KO mice compared to percentages observed for WT mice. These data indicate that different virulence factors from *P. aeruginosa* induce pulmonary inflammation in vivo and that IRF-1 is involved in some of the cytokine responses to LPS and ETA.

Chronic *Pseudomonas aeruginosa* infection accounts for most of the morbidity and mortality in cystic fibrosis (CF) patients. *P. aeruginosa* infection in the lungs of CF patients is associated with a pronounced antibody response against several bacterial antigens and by a massive neutrophilic infiltrate, which eventually leads to tissue damage. *P. aeruginosa*, a gram-negative bacterium, produces several extracellular factors which participate in the pathogenesis of infections caused by this organism (22). Although endotoxin (lipopolysaccharide [LPS]) from *P. aeruginosa* is not as toxic as that from enterobacteria (22), it is nonetheless able to induce a pronounced inflammatory response (13, 14). *P. aeruginosa* produces two known phospholipases C (PLC), one hemolytic and one nonhemolytic (33). Whereas the nonhemolytic PLC has no demonstrated pathogenic activities, the hemolytic form causes increased vascular permeability, end organ damage, and death when injected into mice (2, 27). Therefore, it is likely that hemolytic PLC acts as an important virulent factor during *P. aeruginosa* infection. A third virulence factor produced by *P. aeruginosa* is exotoxin A (ETA), a protein toxin that inhibits polypeptide synthesis through ADP ribosylation of elongation factor 2 (34). ETA is detectable in sputum samples of patients infected with *P. aeruginosa* (13, 15) and displays proinflamma-

tory activities when administered intravenously at high doses (5). Therefore, ETA could also contribute to modulate the host response to *P. aeruginosa* infection.

Primary alterations in the inflammatory response have been implicated as possible factors involved in the increased susceptibility of CF patients to pulmonary infection (1). Recently, Kelley and Elmer reported a reduced expression of interferon regulatory factor 1 (IRF-1) in the nasal and intestinal epithelia of mice with CF, suggesting that a dysregulation of this transcription factor may influence the development of the inflammatory and antibacterial response in patients with CF (18). IRF-1 regulates the expression of several genes involved in immunity and inflammation. Genes regulated by IRF-1 include, among others, the cytokines alpha/beta interferon (IFN-α/β), IFN-γ, interleukin 12 (IL-12), and IL-15, as well as the intracellular cysteine protease caspase 1, inducible nitric oxide synthase, major histocompatibility complex class I molecules, and β₂-microglobulin (8, 10, 17, 21, 23, 24, 31, 37, 38). On the other hand, IRF-1 is induced by IFN-α/β, IFN-γ, and IL-12, therefore generating a positive loop that amplifies IFN effects (11, 12, 28). IRF-1 seems to be positioned at the intersection of different pathways leading to a Th1 response and to host defense against microorganisms.

In the present report, using a model of intranasal inoculation, the proinflammatory activities of *P. aeruginosa* LPS, PLC, and ETA were compared. Production of cytokines and chemokines and cellular infiltration in the lungs were evaluated. Because low levels of IRF-1 are possibly involved in the altered

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inflammatory response typical of CF (18), IRF-1-deficient (IRF-1 gene knockout [IRF-1 KO]) mice were used to investigate a possible role for this transcription factor in pulmonary inflammation induced by *P. aeruginosa* LPS, PLC, and ETA.

MATERIALS AND METHODS

Materials. *P. aeruginosa* LPS (serotype 10) was from Sigma (St. Louis, Mo.), ETA was from List Biological Laboratories (Campbell, Calif.), and PLC was purified from *P. aeruginosa* PAO1 overexpressing the plcHR operon as previously described (39).

Animals and treatments. Animal protocols were approved by the Animal Studies Committee of the University of Colorado Health Sciences Center. Mice homozygous for the IRF-1 gene were previously described (24). Male and female wild-type (WT) and IRF-1 KO mice between 8 and 10 weeks of age and of C57BL/6 background were used. Mice were lightly anesthetized with isoflurane (Abbott, North Chicago, Ill.) and 50 μl of the experimental agent was administered intranasally. Control mice were inoculated with 50 μl of pyrogen-free phosphate-buffered saline (PBS). After 2, 6, 12, 24 or 48 h, mice were anesthetized with isoflurane and bled from the retro-orbital plexus. The whole lungs were excised, weighed, and homogenized in sterile PBS containing 0.01% Tween 20 (1:4, wt/vol). Samples were centrifuged at 3,000 × *g* for 10 min at 4°C, and the supernatant was collected and stored at −70°C until cytokine measurement.

BAL. To evaluate cellular content in the bronchoalveolar lavage (BAL) specimen, the trachea was exposed through a midline incision and cannulated with a sterile, 23-gauge needle. The lavage was performed by instilling 1 ml of ice-cold sterile saline and collecting the fluid by aspiration. BAL fluid was centrifuged (3,000 × *g*, 10 min), and cell counts were performed with a hemocytometer.

RNase protection assay. Mice were sacrificed 1 h after intranasal inoculation of either LPS, PLC, or ETA. The lungs were removed, and total RNA was extracted after homogenization with the RNA Stat-60 solution (Tel-Test, Friendswood, Tex.) according to the manufacturer's instructions. Extracted RNA was quantitated by spectrophotometry. For RNase protection assay, antisense riboprobes were prepared by transcription of cloned DNA templates with the T3 RNA polymerase (Ambion, Austin, Tex.), labeled with [α-^{32}P]UTP, and purified by polyacrylamide gel electrophoresis and elution in ammonium acetate buffer containing EDTA and sodium dodecyl sulfate. Five micrograms of RNA was hybridized overnight at 55°C with approximately 10^5 cpm of each labeled riboprobe. Unhybridized RNA was digested with RNases A and T1 (Ambion) for 1.5 h at 37°C. Hybridized and RNase-protected RNA was precipitated, washed, and electrophoresed on polyacrylamide gels. Hybrids containing the mRNA of interest were electrophoresed with hybrids containing the housekeeping gene RNA cyclophilin. The radioactivity of the riboprobes in the hybrids was quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Cytokine measurement. Tumor necrosis factor alpha (TNF-α), IL-1β, macrophage inflammatory protein 1α (MIP-1α), MIP-2, and IL-18 were measured by electrochemiluminescence assays. The TNF-α, MIP-1α, and IL-18 assays have been previously described (6, 7, 9). For measurement of IL-1β and MIP-2 a similar procedure was employed, using antibodies and recombinant proteins obtained from Pierce Endogen (Rockford, Ill.) for IL-1β and from R&D Systems (Minneapolis, Minn.) for MIP-2. IFN-γ and IL-6 were measured with enzyme-linked immunosorbent assay kits from BD PharMingen (San Diego, Calif.). The IL-18 assay detects both precursor and mature IL-18, whereas the IL-1β assay is specific for mature IL-1β.

MPO assay. Myeloperoxidase (MPO) activity was measured in lung homogenates by the kinetic assay as previously described (25).

LMC isolation. For lung mononuclear cell (LMC) isolation, lungs were excised and minced into small pieces. The tissue was incubated in digestion buffer consisting of 20 ml of Hanks' balanced salt solution (Invitrogen Inc., Carlsbad, Calif.) supplemented with 1% streptomycin and penicillin, 20% fetal calf serum (Invitrogen), dispase II (2.4 U/ml), collagenase II (14.3 U/ml), and collagenase IV (25.5 U/ml; all from Sigma) for 60 min while shaking at 37°C. After two washing steps with Hanks' balanced salt solution, cells were separated from tissue debris by filtration through a 100-μm-pore-size cell strainer followed by purification through a discontinuous 40 to 80% Percoll gradient (Sigma) for 20 min at 600 × *g*. Cells were used for flow cytometry as indicated below.

Flow cytometry. The isolated LMC were washed twice in staining buffer consisting of PBS supplemented with 1% fetal calf serum and 0.1% azide. Flow cytometry followed routine procedures using 10^5 LMC per sample. To measure the expression of CD3ε (clone 145-2C11), CD4 (clone L3T4), CD8α (clone 53-6.7), F4/80 (clone MCA497F), and NK1.1 (clone PK136), cells were labeled with either a fluorescein isothiocyanate- or phycoerythrin-labeled antibody (BD

PharMingen; Serotec for F4/80). Flow cytometric analysis was conducted on a FACSCalibur (BD PharMingen) and analyzed by using the CELLQUEST analysis program (BD PharMingen).

Statistical analysis. Data are expressed as means ± standard errors of the means (SEM). The statistical significance of differences between treatment and control groups was determined by factorial analysis of variance (ANOVA) and a Bonferroni-Dunn procedure as post-hoc test. Differences were considered statistically significant for *P* values of <0.05. Statistical analyses were performed using Stat-View 4.51 software (Abacus Concepts, Calabasas, Calif.).

RESULTS

Pattern of cytokine production following intranasal inoculation of *P. aeruginosa* LPS, PLC, or ETA. To characterize the in vivo proinflammatory activity of *P. aeruginosa* LPS, PLC, and ETA, we evaluated the kinetics of cytokine production in the lungs of WT mice inoculated with these virulence factors. Preliminary dose-response experiments were performed to identify the optimal doses of LPS, PLC, and ETA (not shown). For each virulence factor, a dose that induced lung cytokine production without resulting in lethality during the first 48 h following inoculation was selected. The selected doses were 10 μg/mouse for LPS, 0.5 μg/mouse for PLC, and 2 μg/mouse for ETA. These doses were used throughout the course of the subsequent studies.

As expected, high cytokine levels were observed in the lung homogenate of mice inoculated with LPS (Table 1). The kinetics of cytokine production indicates early (2 to 6 h) increases in the levels of the proinflammatory cytokines TNF-α, IL-1β, IL-6 and the chemokines MIP-1α and MIP-2. On the other hand, IFN-γ levels were significantly increased only 24 to 48 h following inoculation of LPS. No major changes in lung-associated IL-18 levels were observed after administration of LPS. Inoculation of PLC induced a pattern of cytokine production similar to the one observed with LPS, although TNF-α levels were lower in PLC-inoculated mice than in mice exposed to LPS (Table 1). PLC did not result in significant alterations in lung IL-18 or IFN-γ levels at any of the time points evaluated. Administration of ETA did not induce TNF-α or IFN-γ (Table 1). However, levels of IL-1β, IL-6, MIP-1α, and MIP-2 were all significantly elevated in the lungs of ETA-inoculated mice. The kinetics of cytokine production was delayed, and the maximal levels were lower in mice receiving ETA than in groups inoculated with LPS or PLC. Of interest, expression of IL-18 was significantly reduced in the lungs of mice receiving ETA; this effect was already evident 2 h following inoculation and continued until 48 h. Doses of ETA as low as 20 ng/mouse were effective in significantly reducing lung IL-18 levels (Table 2). No alterations in serum or liver-associated IL-18 levels were observed following intranasal inoculation of ETA, indicating a local effect in the lung (Table 2). Similarly, intranasal inoculation of either LPS, PLC, or ETA did not alter serum or liver-associated levels of any of the cytokines measured (data not shown).

Lung inflammation following administration of LPS, PLC, or ETA. Figure 1 shows the time course of relative lung wet weight in WT mice receiving intranasal inoculation of either LPS, PLC, or ETA. As shown, each of the three virulence factors significantly increased lung wet weight. ETA had a delayed effect compared to LPS or PLC.

Pulmonary cellular infiltration in WT mice receiving LPS, PLC,

TABLE 1. Cytokine levels in lung homogenates of mice inoculated with LPS, PLC, or ETA[a]

Cytokine	Inoculum	Mean ± SEM of cytokine level (pg/ml) at					
		Control	2	6	12	24	48
TNF-α	LPS	<15	517.7 ± 94.7***	171.7 ± 12.3*	146.1 ± 23.6*	32.2 ± 17.1	<15
	PLC	<15	93.7 ± 4.6***	41.5 ± 20.8**	<15	<15	<15
	ETA	<15	<15	<15	<15	<15	<15
IL-1β	LPS	<10	670.7 ± 70.9***	678.9 ± 173.5***	710.0 ± 133.0***	631.0 ± 125.2**	240.0 ± 53.5
	PLC	<10	458.8 ± 27.8***	744.5 ± 55.5***	237.0 ± 2.9*	90.3 ± 3.3	<10
	ETA	<10	<10	<10	18.6 ± 6.7*	17.5 ± 1.9*	11.3 ± 1.4
IL-6	LPS	<20	4,408 ± 1899*	6,234 ± 1187**	4,702 ± 1087*	768 ± 294.4*	5,367 ± 976.0*
	PLC	<20	7,588 ± 1826*	6,265 ± 1346**	1,015 ± 658.2*	2,413 ± 1317*	<20
	ETA	<20	<20	83.3 ± 68.0	503.5 ± 396.3*	<20	<20
IL-18	LPS	741 ± 271	714 ± 276	797 ± 185	775 ± 86.8	937 ± 67	618 ± 51.9
	PLC	700 ± 54.2	494 ± 51.6	509 ± 26.0	564 ± 59.2	510 ± 52.8	594 ± 113.9
	ETA	825 ± 80.1	660 ± 50.3*	355 ± 60.7***	335 ± 22.3***	210 ± 12.6***	125 ± 5.9***
IFN-γ	LPS	55.9 ± 2.1	35.3 ± 2.9	51.5 ± 4.6	63.0 ± 4.8	266.1 ± 72.9**	244.9 ± 11.1**
	PLC	44.4 ± 7.4	42.8 ± 3.5	53.7 ± 7.2	68.2 ± 4.7	56.4 ± 6.3	81.6 ± 9.3
	ETA	74.4 ± 13.3	58.6 ± 18.8	76.4 ± 16.2	63.6 ± 4.6	90.9 ± 36.2	75.2 ± 8.3
MIP-1α	LPS	<15	349.0 ± 67.7***	400.3 ± 56.4***	284.1 ± 23.0***	235.8 ± 27.0***	189.6 ± 19.5**
	PLC	<15	275.1 ± 27.8***	259.1 ± 55.5***	30.0 ± 2.9	18.8 ± 3.3	<15
	ETA	<15	<15	<15	19.0 ± 6.7*	16.8 ± 3.2*	<15
MIP-2	LPS	<15	3,543 ± 1194***	1,657 ± 166*	1,169 ± 89.8*	870.5 ± 116.2*	541.6 ± 20.9
	PLC	<15	1,875 ± 77.5***	1,014 ± 272.4***	400.5 ± 38.4	270.1 ± 57.2	215.0 ± 35.9
	ETA	<15	<15	<15	163.3 ± 28.5*	172.3 ± 11.1***	145.1 ± 11.6***

[a] Groups of three to five mice were inoculated with either LPS (10 µg/mouse), PLC (0.5 µg/mouse), or ETA (2µg/mouse). Control mice received vehicle. Lungs were removed at the indicated time points (in hours) and cytokine levels were measured in lung homogenates.
[b] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus respective control by factorial ANOVA.

or ETA was evaluated by measuring lung MPO activity and by counting the number of cells in the BAL fluid. As shown in Fig. 2, inoculation of each of the three virulence factors significantly increased lung MPO activity and BAL cellular content.

IRF-1 induction by LPS, PLC, or ETA. We next investigated whether intranasal inoculation of LPS, PLC, and ETA was able to induce expression of IRF-1 in the lungs of WT mice. Results shown in Fig. 3 indicate a significant increase in IRF-1 mRNA levels in the lungs of mice inoculated with LPS or ETA, whereas the increase observed in PLC-inoculated mice did not reach statistical significance. However, since we measured IRF-1 mRNA levels only 1 h following inoculation, the possibility remains that PLC might induce IRF-1 expression at a later or earlier time point.

TABLE 2. Inhibition of lung IL-18 levels by ETA

Dose of ETA (ng/mouse)[a]	Mean ± SEM of IL-18 (pg/ml) in indicated tissue[b]		
	Lung[c]	Liver	Serum
0 (control)	838.5 ± 59.8	1,480 ± 140	55.3 ± 8.6
20	683.2 ± 40.1*	1,540 ± 120	79.7 ± 7.3
200	449.7 ± 40.7***	1,780 ± 70	63.7 ± 23.4
2,000	286.1 ± 28.5***	1,580 ± 60	83.3 ± 18.2

[a] Groups of three to five mice were inoculated with the indicated doses of ETA. Control mice received vehicle.
[b] Lungs and liver were removed 24 h following inoculation, and IL-18 levels were measured in the homogenates. Blood was collected 24 h following inoculation, and serum was prepared.
[c] *, $P < 0.05$; **, $P < 0.001$ versus respective control by factorial ANOVA.

Cytokine production in the lungs of IRF-1 KO mice. The role of IRF-1 in mediating cytokine production following administration of LPS, PLC, and ETA was evaluated by comparing the responses of WT and IRF-1 KO mice. The time points for measurement of TNF-α, IL-1β, IL-18, and IFN-γ were selected based on results of the time course experiments presented in Table 1. As shown in Fig. 4, markedly reduced levels of TNF-α, IL-1β, and IFN-γ were observed for IRF-1 KO compared to WT mice receiving LPS. In contrast to what was observed following administration of LPS, similar levels of TNF-α and IL-1β were induced in WT and IRF-1 KO mice by administration of PLC. On the other hand, significantly reduced levels of IL-1β were present in IRF-1 KO compared to WT mice inoculated with ETA. In addition, the inhibition of lung-associated IL-18 levels was less marked in IRF-1 KO than in WT mice. In fact, a 77% reduction in lung IL-18 levels was observed for WT mice, whereas the reduction was 54% for IRF-1 KO mice. These data indicate that modulation of cytokine production by LPS and ETA is partially IRF-1 dependent, whereas the effect of PLC is not.

Chemokine levels and MPO activity in WT and IRF-1 KO mice. We next evaluated whether IRF-1 plays any role in the induction of the chemokines MIP-1α and MIP-2 and in the neutrophilic infiltration following administration of LPS, PLC, or ETA. As shown in Fig. 5, no significant differences were observed between WT and IRF-1 KO mice in terms of chemokine production. In addition, a small but significant increase in MPO activity was observed for IRF-1 KO compared to WT
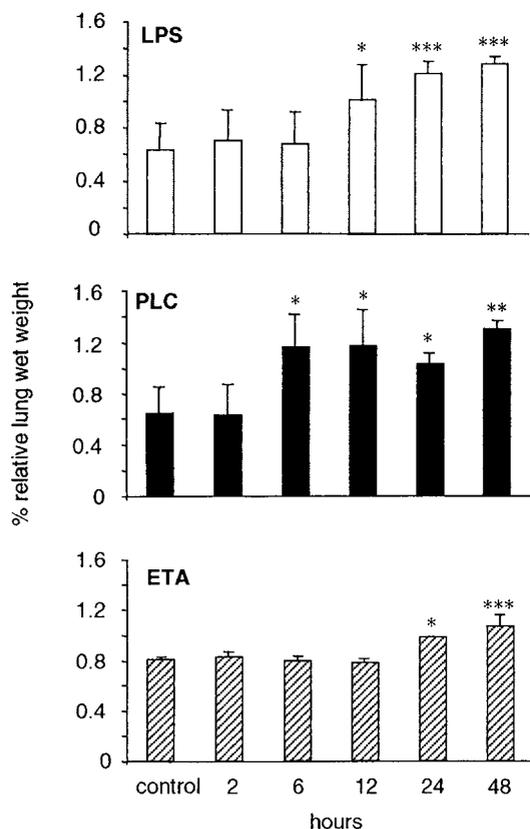
FIG. 1. Increase in relative lung wet weight following administration of LPS, PLC, or ETA. Mice were inoculated with either LPS (10 μg/mouse), PLC (0.5 μg/mouse), or ETA (2 μg/mouse). Control mice received vehicle. At the indicated time points mice were bled and sacrificed, and body weight was recorded. Lungs were removed and weighed. Data are means ± SEM of five mice per group and are expressed as percentage of lung weight over body weight. ∗, $P < 0.05$; ∗∗∗, $P < 0.001$ versus respective control by factorial ANOVA.
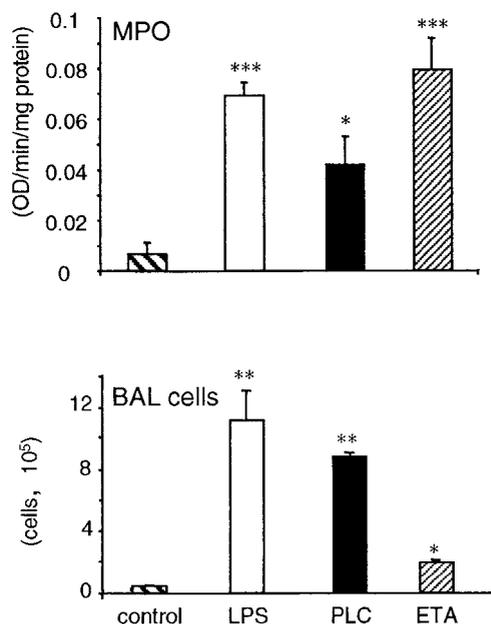

FIG. 2. Cellular infiltrate in the lungs following inoculation of LPS, PLC, or ETA. Mice were inoculated with either LPS (10 μg/mouse), PLC (0.5 μg/mouse), or ETA (2 μg/mouse). Control mice received vehicle. (Top) Mice were sacrificed either 6 h (LPS and PLC) or 24 h (ETA) following inoculation. Lungs were removed, and MPO levels were evaluated as described in Materials and Methods. (Bottom) Mice were sacrificed 24 h following inoculation, and BAL was performed as described in Materials and Methods. Data are means ± SEM of five mice per group. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ versus respective control by factorial ANOVA.

DISCUSSION

In the present report we compared the pulmonary proinflammatory activities of three *P. aeruginosa* virulence factors and evaluated the role played by the transcription factor IRF-1 in modulating these responses.

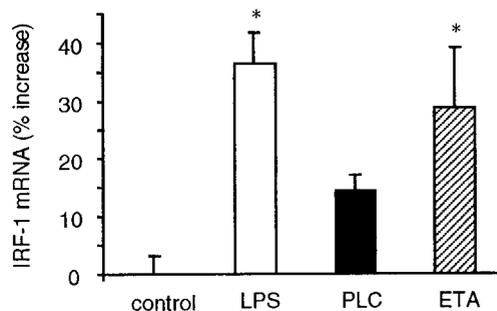Each of the factors examined—LPS, PLC, and ETA—in-

mice receiving LPS, whereas no differences were observed when PLC or ETA was administered.

LMC populations in WT and IRF-1 KO mice. In order to investigate whether differences in LMC populations could account for the observed alterations in cytokine production between WT and IRF-1 KO mice, flow cytometric analysis was performed. As shown in Fig. 6, the percentage of CD8^{+} cells was strongly reduced in IRF-1 KO compared to WT mice (4.7% ± 0.8% versus 16.4% ± 1.4% CD8^{+} cells in IRF-1 KO versus WT mice, respectively; mean ± SEM, $n = 6$, $P = 0.0001$). In addition, as shown in Fig. 7, significantly fewer NK cells were present in the lungs of IRF-1 KO mice than in the lungs of WT mice (2.9% ± 1.2% versus 7.4% ± 0.1% NK1.1^{+} cells in IRF-1 KO versus WT mice, respectively; mean ± SEM, $n = 6$, $P = 0.015$). In contrast with the reduction in CD8^{+} and NK1.1^{+} cells, an increased percentage of macrophages (F4/80^{+} cells) was observed in the lungs of IRF-1 KO compared to WT mice (17.7% ± 0.8% versus 8.6% ± 0.7% F4/80^{+} cells in IRF-1 KO versus WT mice, respectively; mean ± SEM, $n = 6$, $P = 0.0002$) (Fig. 8). No significant differences between the lung B-cell populations (CD45R^{+}/B220 cells) of WT and IRF-1 KO mice were observed (data not shown).

FIG. 3. Induction of IRF-1 mRNA following inoculation of LPS, PLC, or ETA. Mice were inoculated with either LPS (10 μg/mouse), PLC (0.5 μg/mouse), or ETA (2 μg/mouse). Control mice received vehicle. One hour after inoculation, lungs were removed, total RNA was extracted, and RNase protection assay for IRF-1 was performed as described in Materials and Methods. Data are means ± SEM of five mice per group and are expressed as the percent increase of IRF-1 mRNA compared to control mice. ∗, $P < 0.05$ versus control by factorial ANOVA.
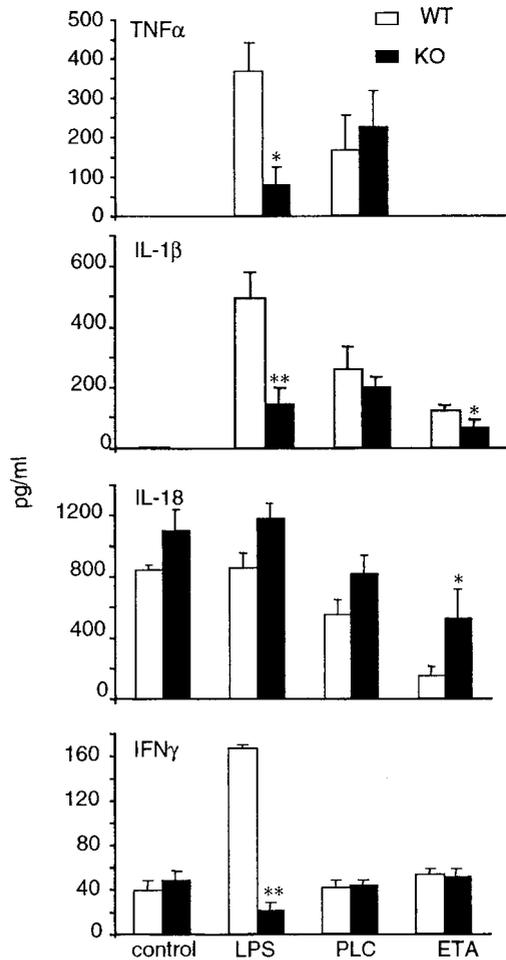
FIG. 4. Cytokine levels in WT and IRF-1 KO mice following inoculation of LPS, PLC, or ETA. WT (open bars) and IRF-1 KO (filled bars) mice were inoculated with either LPS (10 μg/mouse), PLC (0.5 μg/mouse), or ETA (2 μg/mouse). Control mice received vehicle. Mice were sacrificed either 2 h (for TNF-α), 6 h (for IL-1β and IL-18) or 24 h (for IFN-γ) after inoculation, and cytokine levels were measured in the lung homogenates. Data are means ± SEM of five mice per group. *, $P < 0.05$; **, $P < 0.01$ versus respective WT by unpaired Student's t test.
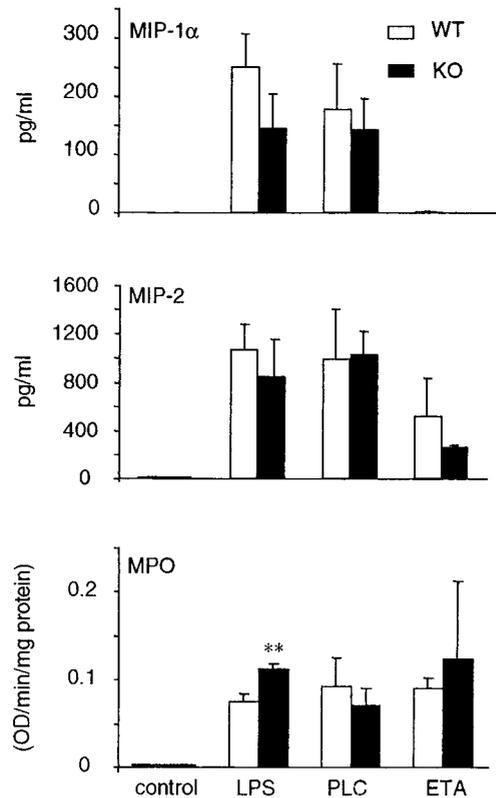

FIG. 5. Chemokine and MPO levels in WT and IRF-1 KO mice following inoculation of LPS, PLC, or ETA. WT (open bars) and IRF-1 KO (filled bars) mice were inoculated with either LPS (10 μg/mouse), PLC (0.5 μg/mouse), or ETA (2 μg/mouse). Control mice received vehicle. Mice were sacrificed 6 h after inoculation of LPS or PLC and 24 h after administration of ETA. Chemokine and MPO levels were measured in the lung homogenates. Data are means ± SEM of five mice per group. **, $P < 0.01$ versus respective WT by unpaired Student's t test.

of induction similar to those of LPS. A milder cytokine response was induced when ETA was inoculated. These data are in agreement with previous reports indicating that intratracheal administration of ETA does not induce TNF-α produc-
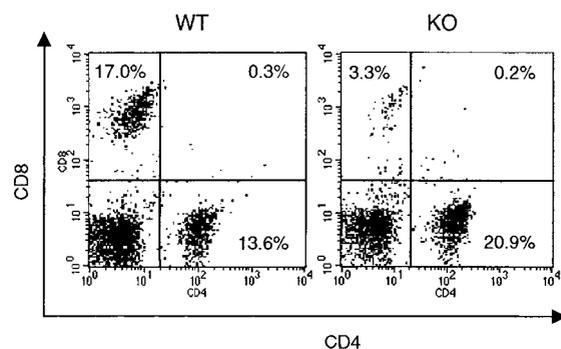
duced a distinctive pattern of cytokine and chemokine production in the lungs. It is important to stress that the method used to administer the three virulence factors, i.e., intranasal inoculation, induced only a local inflammatory response in the lungs, with no apparent systemic effects. Therefore, compared to systemic administration of proinflammatory agents, the model used more closely reflects the pulmonary inflammation observed in CF patients, in which systemic involvement is usually not observed.

Although some in vivo and in vitro proinflammatory activities of PLC have been previously reported (2, 19, 26, 27), to our knowledge this is the first study analyzing a broad range of cytokines induced in vivo by PLC. Furthermore, systemic administration of PLC had previously been employed, whereas local pulmonary inflammation elicited by intranasal administration was used in the present studies. At the dose used, PLC was a strong inflammatory agent, with a potency and a kinetics

FIG. 6. Reduced CD8^{+} cells in the lungs of IRF-1 KO mice. LMC were isolated from untreated WT and IRF-1 KO mice. Cells were evaluated by flow cytometry for expression of CD4 and CD8. Results from one representative mouse per genotype are shown and are representative of four independent analyses performed.
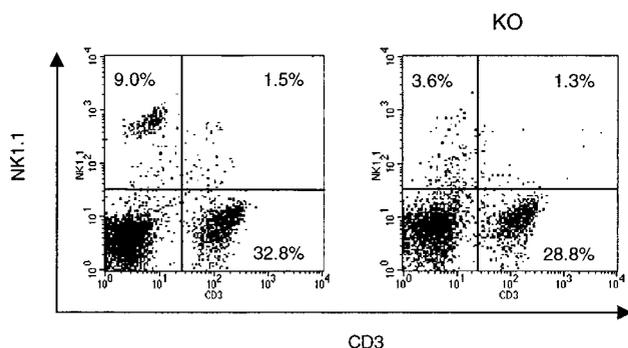
FIG. 7. Reduced NK cells in the lungs of IRF-1 KO mice. LMC were isolated from untreated WT and IRF-1 KO mice. Cells were evaluated by flow cytometry for expression of CD3 and NK1.1. Results from one representative mouse per genotype are shown and are representative of four independent analyses performed.

tion and actually inhibits induction of TNF-α by LPS (14). However, several reports have pointed to a strong proinflammatory effect of ETA when administered intravenously, with induction of TNF-α and IL-18 and consequent liver failure (5, 35). These contrasting data seem to indicate a different target for ETA when local versus systemic administration is used.

One of the most interesting effects of ETA was the marked inhibition of lung IL-18 levels. Of all the cytokines analyzed, IL-18 is the only one to be constitutively produced (7), and the well-described role of ETA as an inhibitor of polypeptide synthesis (34) likely accounts for the observed effect. However, the implication of IL-18 inhibition by ETA for the response to *P. aeruginosa* infection could be important. In fact, IL-18 acts mainly as a potentiator of Th1 responses (4), whereas in CF patients the response to *P. aeruginosa* infection seems to be shifted towards a Th2 type (30). Inhibition of IL-18 production by ETA is a possible factor contributing to this shift.

Each of the three factors studied induced cellular infiltrate in the lungs, as evaluated by increased MPO activity in lung homogenates and by cell counts in the BAL fluid. Although the strong increase in chemokine production induced by LPS and PLC explains their ability to induce a pulmonary cell infiltrate,

this is not the case for ETA. In fact, although very low levels of MIP-2 and MIP-1α were induced by ETA, the magnitude of MPO induction was not significantly different from the one induced by LPS or ETA. A possible explanation for this phenomenon is the induction of a different set of chemokines or other chemoattractants by ETA.

In agreement with previous data (36), we observed reduced levels of IFN-γ, TNF-α, and IL-1β in IRF-1 KO mice inoculated with LPS. IRF-1 deficiency was also associated with a reduced response to ETA, but not to PLC. These data were in agreement with the relative potency of the three factors in inducing expression of IRF-1 mRNA. Despite the observed alterations on production of proinflammatory cytokines in IRF-1 KO mice, IRF-1 deficiency did not alter induction of chemokines and MPO activity induced by either LPS, PLC, or ETA. A differential role for IRF-1 in chemokine production has been demonstrated. Thus, IRF-1 mediates induction of RANTES but not of IP-10 (3, 20). Our data indicate that IRF-1 is not required for induction of MIP-2 and MIP-1α in vivo and for neutrophilic infiltration in the lungs.

The lack of effect of IRF-1 deficiency on chemokine production occurred despite marked differences in mononuclear cell populations in the lungs of WT versus IRF-1 KO mice. Expanding previous observations by other investigators (24, 31, 32), we demonstrated reduced percentages of CD8^{+} lymphocytes and NK cells in the pulmonary compartment of IRF-1 KO mice. At the same time, the percentage of resident macrophages, as evaluated by F4/80[+] staining, was increased in the lungs of IRF-1 KO compared to WT mice. Therefore, alterations in these immune cell populations are not sufficient to influence the chemokine production and neutrophilic infiltration in response to the three *P. aeruginosa* virulence factors that we studied.

The present report demonstrates that IRF-1 is involved in some inflammatory responses to the *P. aeruginosa* virulence factors LPS and ETA, but not PLC. Furthermore, the absence of IRF-1 did not influence chemokine production induced by any of the agents studied. It thus appears that the *P. aeruginosa* virulence factors studied are still able to induce inflammation even in the absence of IRF-1. IRF-1 is necessary for the generation of a Th1 response (23, 37). Therefore, in the absence of IRF-1, *P. aeruginosa*-induced inflammation develops in the context of reduced Th1 responses. Because Th1 cytokines seem to favor clearance of *P. aeruginosa* (16, 29), the low levels of IRF-1 present in CF patients' epithelia (18) might contribute to the persistence of *P. aeruginosa* infection and the chronic inflammation of the CF patient lung.
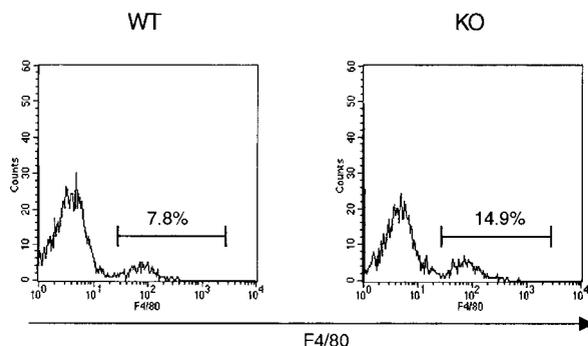
FIG. 8. Increased percentage of macrophages in the lungs of IRF-1 KO mice. LMC were isolated from untreated WT and IRF-1 KO mice. Cells were evaluated by flow cytometry for expression of F4/80. Results from one representative mouse per genotype are shown and are representative of four independent analyses performed.

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