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G551D Cystic Fibrosis Mice Exhibit Abnormal Regulation of Inflammation in Lungs and Macrophages

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The major cause of death in cystic fibrosis (CF) is chronic lung disease associated with persistent infection by the bacterium, Pseudomonas aeruginosa. S100A8, an S-100 calcium-binding protein with chemotactic activity, is constitutively expressed in the lungs and serum of CF patients. Levels of S100A8 mRNA were found to be three to four times higher in the lungs of mice carrying the G551D mutation in CF transmembrane conductance regulator compared with littermate controls. Intravenous injection of bacterial LPS induced S100A8 mRNA in the lung to a greater extent in G551D mice than in wild-type littermates. Localization of S100A8 mRNA and protein in the lung indicate that it is a marker for neutrophil accumulation. Bone marrow-derived macrophages from G551D mice were shown to also exhibit hypersensitivity to LPS, measured by induction of TNF-α. These results provide evidence that the pathology of CF relates to abnormal regulation of the immune system. The Journal of Immunology, 2000, 164: 3870–3877.

Patients with cystic fibrosis (CF) are highly susceptible to chronic infections, particularly Pseudomonas aeruginosa, which usually leads to ongoing inflammation of the lung and subsequent tissue damage and death. Persistence of P. aeruginosa infection has historically been attributed to the thick mucus in the airways due to abnormal hydration resulting from improper chloride transport. Other possible explanations for the chronic infections include impaired bacterial uptake by airway epithelial cells (1–3), abnormal epithelial cell surface modifications allowing for stronger adherence of the bacteria (4–6), the impairment of peptide antibiotics by high salt concentrations in the airway surface fluid of the lung (7, 8), or improper hydration of airway surface fluid causing poor mucociliary clearance (9).

There is a growing body of evidence which suggests that dysregulation of the inflammatory response to the persistent bacterial infection is also a major feature of CF. Cytokines such as TNF-α and IL-8 are elevated in CF airways (10–12) and inflammation in CF infant lung occurs before overt bacterial infection (13, 14). In a mouse CF model, instillation of P. aeruginosa embedded in agar beads results in increased production of inflammatory cytokines in CF animals compared with similarly treated wild-type controls (15). Recent studies have demonstrated that CF epithelia are not responsible for the overexpression of these proinflammatory cytokines (16).

One indicator of the chronic inflammatory status of CF patients is the presence in the serum of two S-100 calcium-binding proteins, S100A8 and S100A9, also known commonly as myeloid-related protein (MRP)-8/CP-10 and MRP-14 (17). S100A8 was identified independently in mouse as a powerful proinflammatory chemotactic factor (18, 19) while both S100 proteins also exhibit antimicrobial activity (20). The expression of S100A8 mRNA in murine macrophages is inducible by bacterial LPS (21). Both of these S100 proteins have been commonly used as markers of myeloid cell infiltration in inflammation in humans (20, 22–24). In this study, we show that constitutive expression of S100A8/ S100A9 in CF patients is an indicator of underlying hypersensitivity to bacterial challenge.

Materials and Methods

Animal resources

This study utilized the CF model strain carrying the G551D mutation in CF transmembrane conductance regulator (25). All mice were maintained in specific pathogen-free conditions before experimentation. G551D animals and littermate controls were housed together under identical conditions. Samples of lung tissues were plated on Luria-Bertani agar, and no evidence of bacterial infection was detected.

RNA isolation, Northern blotting, and analysis

Total RNA was isolated from tissues and cultured cells using Trizol reagent (Life Technologies, Rockville, MD). Northern blotting onto nylon membranes was conducted by standard methods (26). Northern blot hybridization was conducted by standard methods using radiactively labeled probes. RNA loading variation was corrected by use of a control probe (18S rRNA). Blots were analyzed with a Bio-Rad GS-363 molecular imager (Richmond, CA).

LPS injections

Tail veins of mice were injected with 500 ng of Escherichia coli LPS (serotype 0111:B4; Sigma, St. Louis, MO) diluted in PBS. After 4 h, the mice were sacrificed by cervical dislocation and their lungs were extracted for RNA preparation.

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3 Abbreviations used in this paper: CF, cystic fibrosis; MRP, myeloid-related protein; BMM, bone marrow-derived macrophage; CFTR, CF transmembrane conductance regulator.
In situ hybridization and immunofluorescence

Tissues were fixed by whole-body perfusion with 4% paraformaldehyde and the lungs were excised and embedded in OCT (Tissue-Tek; Sakura, Torrance, CA). In situ hybridization was conducted on 10-μm cryosections with ³⁵S-labeled sense and antisense riboprobes of S100A8 using previously described methods (27). Additional cryosections were subjected to standard immunofluorescence with rabbit-anti-S100A8 Ab (a gift from C. Sorg Institute for Experimental Dermatology, Munster, Germany) and goat anti-rabbit -Cy3 conjugate (Molecular Probes, Eugene, OR).

Isolation of bone marrow-derived macrophages (BMMs) and LPS treatment

Three mice of each genotype were sacrificed by cervical dislocation and large leg bones were extracted. The bones were flushed with RPMI 1640 media (BioWhittaker, Walkersville, MD) containing 10% FCS and plated onto bacteriological plates with 10⁴ U/ml human recombinant macrophage CSF-1 (Chiron Therapeutics, Emeryville, CA). After 5 days, the cells were given fresh media and CSF-1. The next day, cells were washed from the plates with PBS containing 5 mM EDTA and replated at 10⁷ cells/10-cm plate. After 24 h, the cells were treated with varying amounts of *S. minnesota* Re595 LPS (Sigma) for 1 h or 100 ng/ml of LPS for various time points before isolation of total RNA.

Results

* S100A8 mRNA is expressed at higher levels in G551D mouse lungs

S100A8 is present at high levels in the serum of patients with CF and other inflammatory diseases (20). To determine whether this proinflammatory agent was also elevated in our G551D mutant mice, we extracted lung RNA from a number of animals and examined the expression of S100A8 mRNA (Fig. 1, A and B). The G551D mice expressed the transcript at higher levels than heterozygous and wild-type littermates, showing an overall 4-fold higher expression whereas no difference was detectable between the heterozygous and wild-type animals (Student’s two-tailed *p* values < 0.05). To confirm this observation, we isolated and analyzed RNA from an additional 19 pairs of G551D and wild-type mice (Fig. 1C). Overall, the G551D animals show a 4-fold higher level of S100A8 mRNA expression than non-CF littermates.

To determine the source of the high levels of S100A8 mRNA expression in response to LPS, in situ hybridization was conducted

* S100A8 expression is a marker of neutrophil accumulation in the lungs

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with riboprobes derived from S100A8. Fig. 3 compares the distribution of S100A8 mRNA between wild-type and G551D mice before and after LPS injection. In sections from the lungs of untreated wild-type mice, few cells were detected that express S100A8 mRNA (Fig. 2A), whereas in the CF lung, all sections contained numerous strongly positive cells (Fig. 2C). LPS treatment increased the number of S100A8-positive cells in control animals (Fig. 2E), and the number of positive cells was dramatically increased in the G551D lung (Fig. 2G). No signal was detectable from the cells lining the larger airways, indicating that the epithelial contribution to S100A8 expression is minor.

Immunofluorescence was conducted on the lung sections using a rabbit polyclonal Ab directed against S100A8 (Fig. 4). In untreated control and CF mice, the expression of S100A8 protein was restricted to cells with similar abundance and distribution to those observed in the mRNA in situ (Fig. 4, A and C). In both wild-type and CF mice, the number of positive cells was increased in response to LPS, but the signal detected in each cell was not as intense as in untreated sections (Fig. 4, E and G). The decreased signal per cell could indicate that S100A8 is being actively secreted from the cells. In support of this view, we have demonstrated high levels of S100A8 monomer and an oxidized dimeric

**FIGURE 3.** In situ localization of S100A8 in mouse lung. Lung cryosections from untreated wild-type (A and B), untreated G551D (C and D), LPS-treated wild-type (E and F), and LPS-treated G551D (G and H) hybridized with S100A8 riboprobes from the antisense (A, C, E, and G) and sense (B, D, F, and H) strands.
form in the bronchoalveolar lavage fluid of mice treated intranasally with LPS (28).

Where nuclear morphology was not obscured by silver grains, most labeled cells expressing S100A8 mRNA by in situ hybridization were polymorphonuclear in appearance, although expression by monocytes and macrophages cannot be excluded. Similarly, at high power, many cells expressing S100A8 protein were clearly neutrophils, segmented nuclear morphology being evident due to reduced staining intensity in the nucleus. Neither of these patterns could be reproduced photographically but the observation is not surprising since both neutrophils and macrophages are known to express abundant S100A8 and A9 mRNA and protein (29). The lung sections were subjected to hematoxylin and eosin staining for histological examination (Figs. 5 and 6). Untreated G551D mice and wild-type controls both exhibit normal lung structure with few detectable neutrophils (Figs. 5 and 6, A and B). LPS-treated animals show increased cellularity in the lung (Figs. 5 and 6, C and D), including numerous neutrophils. The lungs of CF mice clearly showed a greater increase in cellularity and neutrophil influx upon LPS injection than the wild-type animals.

**G551D macrophages are hypersensitive to LPS**

To determine whether the differences in response to LPS between G551D and wild-type mice could be due to alterations in cytokine responses, we compared induction of TNF-α mRNA in BMMs from wild-type, heterozygous, and G551D mice (Fig. 7). Cells were treated for 1 h with doses of LPS ranging from 0 to 100 ng/ml and levels of TNF-α mRNA were examined (Fig. 7, A and B). Maximum stimulation of transcript was observed at doses of 10–100 ng/ml and were consistently higher in G551D-derived cells.

when compared with wild-type cells. Cells were next treated with 100 ng/ml LPS for various lengths of time and TNF-α transcription was measured. TNF-α mRNA was stimulated to high levels within 1 h of treatment with LPS and returned to basal levels of expression 12–24 h later. In BMMs from G551D animals, LPS induced approximately four times higher levels of TNF-α mRNA compared with wild-type cells.

Discussion

The major cause of mortality in CF patients is lung failure resulting from chronic bacterial infection and the attendant inflammatory response. This inflammation has been considered secondary to the chronic infection, but recent evidence has suggested that CF patients have an innate defect in their inflammatory response which contributes to the cycle of infection and lung damage (10, 12–14, 30–32). Studying the production of cytokines in the lungs of CF patients is complicated by the difficulties of studying individuals before lung infection occurs. To overcome this difficulty, we have studied the cytokine response in G551D mutant mice, which are free of lung infection. We demonstrate that mice carrying the G551D mutation in their CFTR gene have an altered regulation of the inflammatory response to bacterial LPS.

Patients with CF or chronic inflammatory disease have high concentrations of the S-100 protein, S100A8 (MRP-8), and its heterodimer partner, S100A9 (MRP-14), in their serum (20). S100A8 is a potent chemoattractant in recruitment of neutrophils to sites of infection in mice (24, 33), and the human proteins have been shown to be expressed and processed abnormally in CF cells (34, 35). We have demonstrated that S100A8 is expressed constitutively in the lungs of CF mice and is a marker for the early response of the lung to LPS challenge. We have also been able to detect higher levels of S100A8 protein in the serum of G551D mice, and the levels also increase dramatically in response to LPS injection (data not shown). There is no evidence of overt bacterial infection in the lungs of these animals, which are maintained under strict barrier isolation conditions, although this cannot be rigorously excluded. If there is an infection that cannot be detected, the CF animals are exposed to precisely the same environment as their wild-type littersmates. We feel it is more likely that low levels of serum LPS derived from normal gut flora provide a sufficient signal to provoke basal expression of S100A8 in CF lungs and that additional variation observed is due to genetic background effects.

Localization of the mRNA and protein for S100A8 in the lungs of LPS-treated mice suggested that the increased level of expression is in part a surrogate marker for extensive neutrophil infiltration. Contrary to studies on human tracheal epithelial cells (34), no expression of S100A8 was evident in the lung epithelial cells. Examination of the lung sections labeled for S100A8 mRNA expression indicated that many of the labeled cells were neutrophils, but expression of the gene in interstitial or alveolar macrophages and infiltrating monocytes also appears likely. We have demonstrated high levels of S100A8 monomer and an oxidized dimeric form in the bronchoalveolar lavage fluid of mice treated intranasally with LPS, implying that much of the LPS-induced protein is secreted or released from dying cells (28).

The induction of neutrophil infiltration is partly dependent on the expression of TNF-α, a proinflammatory cytokine produced by macrophages (36). We have demonstrated that cultured macrophages from CF animals are hypersensitive to LPS, with a higher maximal induction of TNF-α mRNA, consistent with similar results found in human patients (10) and with the expression of CFTR in macrophages (37), which we have confirmed in the mouse BMMs by RT-PCR (data not shown). Excess induction of TNF-α could contribute directly to lung pathology in CF, as this
molecule acts as a proximal inflammatory mediator which triggers a cascade of other inflammatory factors and leads to the recruit-
ment and activation of neutrophils (38–40). Induction of TNF-α
production is a crucial determinant of the protective inflammatory
response to P. aeruginosa challenge in mouse lung (41, 42) and
others have found that CF mouse lung displays a hyperinflamma-
atory response to such a challenge (15).

In view of the known proinflammatory activity (19, 33),
S100A8 protein could be involved directly in the lung pathology of
CF and normal protective immune responses in the lung. We have
produced a targeted disruption of the S100A8 gene in the mouse
germine (43). The gene is expressed specifically in trophoblasts
derived from ectoplacental cone at 7.5–8.5 days postcoitum. The
transcript accumulates to high levels in these cells, but the protein
does not, suggesting active secretion of the product. S100A8 null
embryos are resorbed by the mother at exactly the time the gene is
expressed, indicating that secreted S100A8 may have an immuno-
regulatory function.

The combination of overexpression of a potent recruiter of neu-
trophils (S100A8) and a potent activator of neutrophils (TNF-α)
may be an important clue in explaining the excessive inflammatory
response mounted by CF patients to bacterial infection. Down-
regulation of the anti-inflammatory cytokine IL-10 has been de-
scribed in CF (30, 31, 44). A lack of anti-inflammatory molecules from epithelial cells (IL-10) and an excess of proinflammatory
response mounted by CF patients to bacterial infection. Down-

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