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Molecular mechanisms of epithelial host defense in the airways

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CHAPTER 4

PSEUDOMONAS AERUGINOSA RAPIDLY INDUCES A TRANSIENT INCREASE IN EXPRESSION OF S100A8 AND S100A9 IN BRONCHIAL EPITHELIAL CELLS

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ABSTRACT

Using high-throughput gene expression profiling technology we have recently identified the calcium-binding proteins S100A8 and S100A9 as the most transcribed differentially expressed genes by bronchial epithelial cells in the early response to *P. aeruginosa*. The heterodimeric protein complex of S100A8 and S100A9 is a potentially important effector molecule exerting various functions related to host defense including antimicrobial activity, chemoattraction and enhancing transendothelial migration of leukocytes. Expression has been demonstrated in various cell types in response to microorganisms, cytokines and physical damage. We investigated the expression of S100A8 and S100A9 and formation of the protein complex in subcultures of primary bronchial epithelial cells that were exposed to a microbial, an inflammatory and an oxidative stimulus. Our results demonstrated that *P. aeruginosa*, a mixture of IL-1β and TNFα, and cigarette smoke condensate not only increases the expression of the S100A8 and S100A9 genes but also enhanced the release of the S100A8/A9 protein complex, implicating functionality of S100 proteins in host defense and in the response to oxidative stress.

INTRODUCTION

The airways epithelium represents the body's largest surface contacting the external environment, and yet the lungs generally succeed in remaining free of infection. The epithelium contributes to host defense by producing effector molecules such as antimicrobial peptides, proteinase inhibitors, cytokines and chemokines [1]. Exposure to microbes may activate epithelial host defense directly through pattern recognition receptors present on epithelial cells or indirectly by cytokines released by macrophages such as IL-1β and TNFα[2,3]. To gain insight into epithelial host defense, we recently applied the large scale gene expression profiling technique SAGE to assess the transcriptional response of cultured primary bronchial epithelial cells exposed to heat-inactivated *Pseudomonas aeruginosa* and a mixture of the pro-inflammatory cytokines IL-1β and TNFα [4]. Several members of the S100 calcium-binding proteins were among the differentially expressed genes. S100A9 was the most abundant differentially expressed gene after *P. aeruginosa* and IL-1β/TNFα exposure. S100A8 expression was also affected, albeit that the level of expression was lower as compared to S100A9. Expression of S100A9 and S100A8 has also been demonstrated in various other cell types including myeloid cells [5-7] and endothelial cells [8]. Increased levels of S100A8/A9 in the circulation and mucosal secretions have been observed for a variety of inflammatory disorders, including cystic fibrosis and chronic bronchitis [9]. S100A8/A9 may display activities in both the intra- and extracellular compartments [10-12] that are mostly, if not all, calcium-dependent. Intracellularly, the complex may interact with the cytoskeleton [13,14], serve as antioxidant [15,16] or function as arachidonic acid binding protein [17]. For extracellular functions, the protein complex is actively secreted via a tubulin-dependent pathway [18] which is independent from the classical endoplasmic reticulum-Golgi pathway since localization signals are lacking. The protein complex formed by S100A8 and S100A9, (S100A8/A9; also known as CF antigen and calprotectin) may be of relevance in host defense because of its antimicrobial activity [9] resulting from its zinc-chelating properties [19]. The complex may inhibit bacterial binding to epithelial cells [20] and could also competitively inhibit tissue damage by sequestering iron required for the enzymatic action of matrix metalloproteinases [21]. Finally, the complex may induce expression of IL-8 in lung epithelial cells [22], thus, propagating inflammation. Gene expression for S100A8 and S100A9 is induced not only by inflammatory stimuli [4,23] but also by sources of physical stress [24-26] or exposure to oxidants. Tissue damage caused by smoking may therefore trigger the expression of S100A8 and S100A9. Although the importance of S100 proteins is becoming more apparent, their main physiological function and the regulation mechanisms are incompletely understood.

In view of their involvement in host defense and inflammation and possible action as anti-oxidant, we aimed to study the dynamics of expression of S100A8 and S100A9

at the mRNA and protein level in primary bronchial epithelial cells (PBEC) exposed to pro-inflammatory cytokines, *P. aeruginosa*, or cigarette smoke condensate. Cytosolic and extracellular S100A8/A9 complex protein content was assessed to determine changes in release of the complex by bronchial epithelial cells.

MATERIAL AND METHODS

Bronchial epithelial cells

Subcultures of human bronchial epithelial cells were derived from bronchial tissue specimens as described previously [27]. Tissue specimens were found to be normal as determined macroscopically by a pathologist and microscopically at the time of dissection. Cells were grown to near-confluence in 24-well plates pre-coated with a matrix of vitrogen (30 µg/ml; Celtrix Laboratories, Palo Alto, CA), fibronectin (10 µg/ml; isolated from human plasma) and bovine serum albumin (BSA, 10 µg/ml; Boehringer Mannheim, Mannheim, Germany) in serum-free keratinocyte-SFM medium (KSFM, GibcoBRL/Life Technologies, Breda, The Netherlands) supplemented with 0.2 ng/ml epidermal growth factor (EGF; GibcoBRL/Life Technologies), 25 µg/ml bovine pituitary extract (BPE; GibcoBRL/Life Technologies), 1 mM isoproterenol (Sigma Chemicals, St. Louis, MO), 20 U/ml penicillin (Bio Whittaker, Walkersville, MD) and 20 µg/ml streptomycin (Bio Whittaker). After the cells had reached near-confluence, cells were incubated for 36 hours in high calcium medium to allow differentiation as described previously [27]. High calcium medium was composed of KSFM (GibcoBRL/Life Technologies) supplemented with 1 mM $CaCl_2$, 5 nM retinoic acid (Sigma Chemicals), 0.2 ng/ml EGF, 25 µg/ml BPE, 20 U/ml penicillin and 20 µg/ml streptomycin.

Pseudomonas aeruginosa (PA01)

The non-mucoid *P. aeruginosa* strain PA01 (BAA-47, American Type Culture Collection, Rockville, MD, USA) was grown overnight in Brain-Heart Infusion (BHI) medium to stationary phase at an agitation rate of 275 rotations per minute at 37°C. After three washes with PBS, bacteria were resuspended in PBS containing 50% glycerol at a concentration of 10^9 colony forming units (CFU) per ml as determined by optical density. Samples of the bacterial suspension were plated onto blood agar plates to verify the measured CFU of the suspension. The bacteria were heat-inactivated for 45 minutes at 95°C in a water bath and stored in aliquots at –80°C until further use.

Preparation of cigarette smoke condensate

For each experiment, cigarette smoke condensate (CSC) was prepared freshly from filterless cigarettes (Caballero, British American Tobacco Group, London, UK), essentially as

described previously [28]. In short, smoke derived from two cigarettes was drawn into a plastic syringe and subsequently bubbled through 2 ml of PBS at room temperature. Prior to cell stimulation, the CSC was filtered with a 0.2 µm filter (Schleicher & Schuell GmbH, Dassel, Germany) and wrapped in aluminum foil to prevent breakdown of its reactive oxygen intermediates. To enable standardization, the absorbance of the solution was determined by measuring the maximal optical density of a 1:100 diluted sample of the CSC at 270-280nm. To calculate the concentration of CSC in arbitrary units, the following formula was used, CSC (AU/ml) = OD_{max} x 2 x dilution factor. The CSC was further diluted to the required concentration for cell stimulation

Stimulation of PBEC

Cells were stimulated with high calcium medium alone, CSC (2.5 AU/ml), a mixture of recombinant human tumor necrosis factor-α (TNFα; 20 ng/ml; PeproTech, Rocky Hill, NJ, USA) and interleukin-1β (IL-1β; 20 ng/ml; PeproTech), or heat-killed *Pseudomonas aeruginosa* (10^7 CFU/ml). For subsequent mRNA expression experiments cells were stimulated for 12 hours at 3 hour intervals and a final time point at 24 hours. For S100A8/A9 protein analysis, cells were stimulated for 6, 16 and 24 hours. After stimulation, cell-free supernatant was collected and either RNA was isolated or cellular lysates for S100A8/A9 protein analysis was prepared. Stimulations were performed in triplicate.

Quantitative real-time PCR

Expression of S100A8 and S100A9 was assessed by quantitative real-time PCR (qPCR). Total RNA from the cell cultures was extracted using the RNeasy mini kit (Qiagen Benelux BV, Venlo, The Netherlands), combined with on-column DNA digestion with DNase I (Qiagen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized of total RNA using M-MLV Reverse Transcriptase primed with Oligo-dT (both from Invitrogen/Life Technologies, Breda, The Netherlands) in the presence of a RNase inhibitor (RNaseOUT; Invitrogen/Life Technologies) according to the manufacturer's instructions. Gene-specific primers were designed for S100A8 (Forward: 5'-TTTCCATGCCGTCTACAG-3' and Reverse: 5'-ACGCCCATCTTTATCACC-3', annealing temperature 57°C, 3 mM $MgCl_2$) and S100A9 (Forward: 5'-GCTGGAACGCAACATAGAG-3' and Reverse: 5'-GGTCCTCCATGATGTGTTC-3' annealing temperature 59°C, 3.5 mM $MgCl_2$). All primers were synthesized by Isogen (Maarssen, The Netherlands). To correct for differences in cDNA input, lamin A/C (Forward: 5'-GTGGAGGAGGTGGATGAG-3' and Reverse: 5'-ACGGGTAAGTCAGCAAGG-3', annealing temperature 63°C, 3 mM $MgCl_2$) was used as normalization gene as described previously [4]. Unique amplification of the target genes was verified by gel electrophoresis and melting curve analysis. qPCR analysis was performed on an iCycler PCR machine (BioRad, Hercules, CA, USA) using SYBR green I chemistry. Samples were run in triplicate and threshold cycle numbers (C_T) were calculated using

the iCycler v3.0a analysis software (BioRad). C_T values were used to calculate arbitrary mRNA concentrations using the relative standard curve method using a serial dilution of a cDNA sample containing message for the gene of interest. Relative mRNA concentrations for S100A8, S100A9 and LMNA were used to calculate the expression ratios.

Cell lysis

For S100A8/A9 complex detection, cells were first washed with washing buffer (500 µl/well; pH 7.4, 5 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) before placing the plates on ice. Cells were lysed for 30 minutes in 100 µl/well ice-cold lysis buffer (0.5% (vol/vol) Triton X-100, 1 mM Na_3VO_4 and mini-complete protease inhibitor cocktail (Boehringer, Mannheim, Germany) in washing buffer). Lysates were centrifuged for 5 minutes at 10,000 rpm and 4°C. Supernatant containing the cytosolic proteins were collected and stored at –20°C. Protein concentrations of the lysates were measured by the bicinchoninic acid protein assay system (Pierce, Rockford, IL, USA).

IL-8 ELISA

The concentration of IL-8 in the cell-free supernatants was determined by the commercially available human IL-8 enzyme-linked immunosorbent assay (Biosource human IL-8 cytoset; Biosource Europe S.A., Nivelles, Belgium) according to the manufacturer's instructions. Fold change in IL-8 release was determined at each time point by dividing measured IL-8 levels of stimulated cells by measured IL-8 levels of control cells. Differences in IL-8 release by primary bronchial epithelial cells were statistically tested using a 'two tailed' Student's t-test ($P<0.05$).

S100A8/A9 ELISA

S100A8/A9 complex ELISAs were performed according to the procedure described by Ryckman *et al.* [29]. In brief, Costar high-binding 96-well plates (Corning, NY) were coated with 100 µl/well of 1 µg/ml anti-human S100A8/A9-specific monoclonal antibody 5.5 diluted in 0.1 M carbonate buffer (pH 9.6) for overnight incubation at 4°C and blocked with 100 µl/well PBS/0.1% Tween-20/2% bovine serum albumin (BSA) for 30 minutes at room temperature. Samples were diluted in blocking buffer and incubated for 40 minutes at room temperature, washed for three times with PBS/0.1% Tween-20 and incubated with 100 µl/well S100A9 polyclonal antibody (Rab1-14, diluted 1/10,000 in PBS/0.1% Tween-20/2% BSA) for 40 min at room temperature. Peroxidase-labelled donkey anti-rabbit IgG was used as conjugate and was added at 100 µl/well at a dilution of 1/7,500 in blocking buffer for 40 min at room temperature and developed with 100 µl TMB-S according to the manufacturer's instructions. Substrate conversion was determined at 500 nm. S100A8/A9 release was determined by dividing the concentration of extracellular S100A8/A9 by the intracellular concentration. At each time point, the calculated ratio in the control

group was set on 1. Treatment groups were normalized to the control group. Changes in S100A8/A9 complex are presented as fold change. Differences in S100A8/A9 complex release by primary bronchial epithelial cells were statistically tested using a 'two tailed' Student's t-test ($P<0.05$).

RESULTS

Pseudomonas aeruginosa, IL-1β/TNFα but not cigarette smoke, induce the release of IL-8 by human primary bronchial epithelial cells

To assess treatment efficacy of *P. aeruginosa* and the mixture of IL-1β/TNFα, IL-8 protein levels were measured in cell-free supernatants of PBEC. Both heat-inactivated *P. aeruginosa* and the cytokine mixture resulted in enhanced IL-8 release by PBEC, whereas cigarette smoke condensate did not result in increased IL-8 release by these cells ***(Figure 1)***.

Pseudomonas aeruginosa, IL-1β/TNFα and cigarette smoke transiently increase gene expression of S100A8 and S100A9 in human primary bronchial epithelial cells

To study the expression kinetics of S100A8 and S100A9, we next explored the temporal transcription from 3 to 24 hours. Enhanced expression for both S100A8 and S100A9 was found for all stimuli as compared to control values. Differences were observed in both the time of induction and duration of enhanced expression of these genes ***(Figure 2)***. Exposure of bronchial epithelial cells to cigarette smoke condensate resulted in a rapid increase in gene expression after three hours of stimulation. Increased expression of

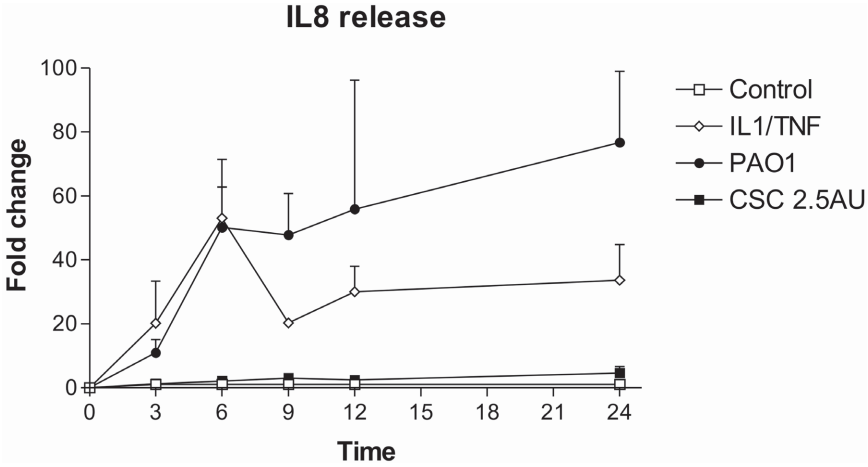

Figure 1: Inflammatory stimuli result in enhanced IL8 release by human primary bronchial epithelial cells. IL8 levels in supernatants of bronchial epithelial cells exposed for 3, 6, 9, 12 and 24 hours to *P. aeruginosa* (10^7 CFU/ml), IL1β/TNFα (20 ng/ml each) or cigarette smoke condensate (2.5 AU/ml) were determined by ELISA. Shown data is +SEM of 3 experiments using three different donors.

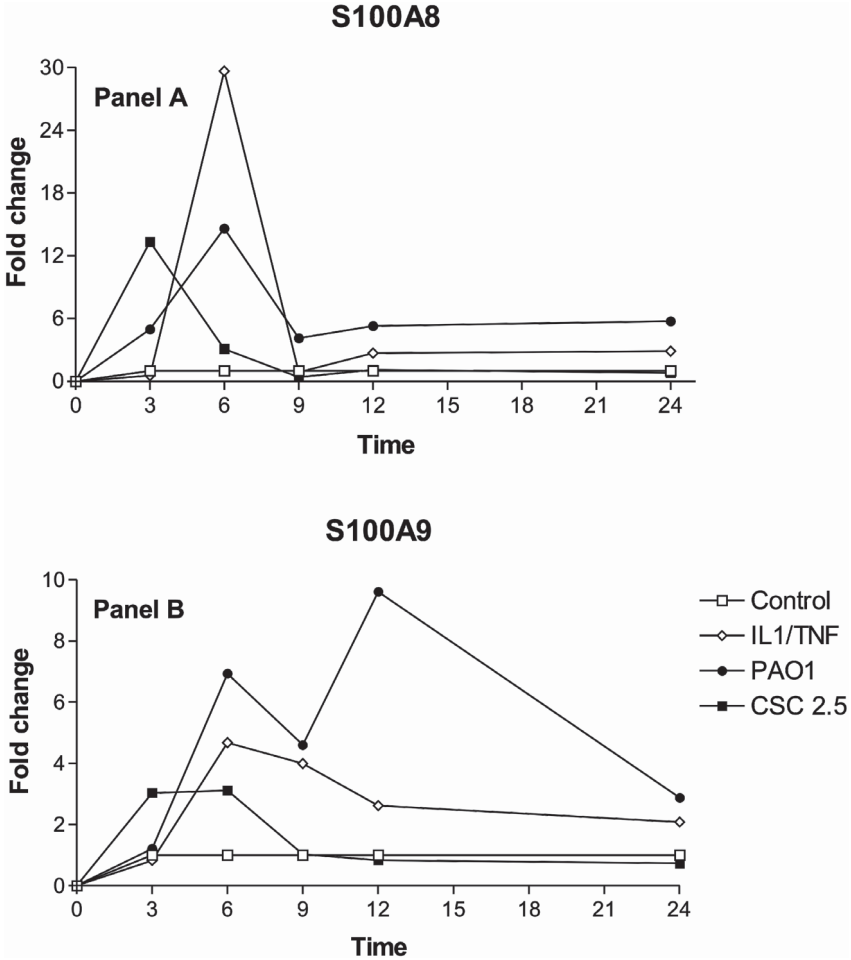

Figure 2: Quantitative realtime PCR analysis of S100A8 and S100A9 mRNA expression by human primary bronchial epithelial cells exposed for 3, 6, 9, 12 and 24 hours to P. aeruginosa (10^7 CFU/ml), IL1β/TNFα (20 ng/ml each) or cigarette smoke condensate (2.5 AU/ml). Panel A shows the expression data for S100A8 and panel B reflects the expression data for S100A9. The expression patterns shown are mean values of three experiments using three donors and are representative for three experiments.

S100A8 was transient and declined to baseline levels after 6-9 hours, whereas expression of S100A9 was sustained up to 6 hours and returned to baseline levels after 9-12 hours. Exposure of cells to heat-inactivated *P. aeruginosa* resulted in a pronounced induction of S100A8 at six hours after stimulation and declined to baseline levels rapidly thereafter. A similar pattern of expression kinetics was observed for S100A9 after *P. aeruginosa* stimulation. The mixture of IL-1β/TNFα resulted in increased expression of S100A8 and S100A9 at 6 hours after stimulation. In addition, for S100A9, a second peak in expression was observed after 12 hours of stimulation, whereas this effect was less apparent for S100A8. Similarly, a weak biphasic response was also seen in IL-8 release after the cytokine stimulation.

Pseudomonas aeruginosa, IL-1β/TNFα and cigarette smoke induce the protein release of S100A8/A9 complex by human primary bronchial epithelial cells

We analyzed whether the observed transcriptional changes were reflected at the protein level. Intracellular and extracellular S100A8/A9 complex was measured 6, 16 and 24 hours after stimulation ***(Figure 3)***. By determining the ratio between intracellular and extracellular S100A8/A9, complex changes in release of the protein complex were assessed. Extracellular complex concentrations ranged from 12-343 ng/ml, whereas intracellular concentrations varied between 637-17,296 ng/ml indicating that the majority of the complex remains inside the cell. Heat-inactivated *P. aeruginosa*, the mixture of IL-1β/TNFα and cigarette smoke condensate all rapidly enhanced the release of S100A8/A9 protein complex by primary bronchial epithelial cells after 6 hours of stimulation. Rate of protein release of the S100A8/A9 complex was reduced after 16 hours with all stimulations. Upon 24 hours stimulation with *P. aeruginosa* and cigarette smoke condensate, the percentage of released complex has decreased to baseline levels. Similar to the observed transcriptional changes in S100A9 and IL-8 release, IL-1β/TNFα stimulation resulted in a second peak of protein complex release after 24 hours.

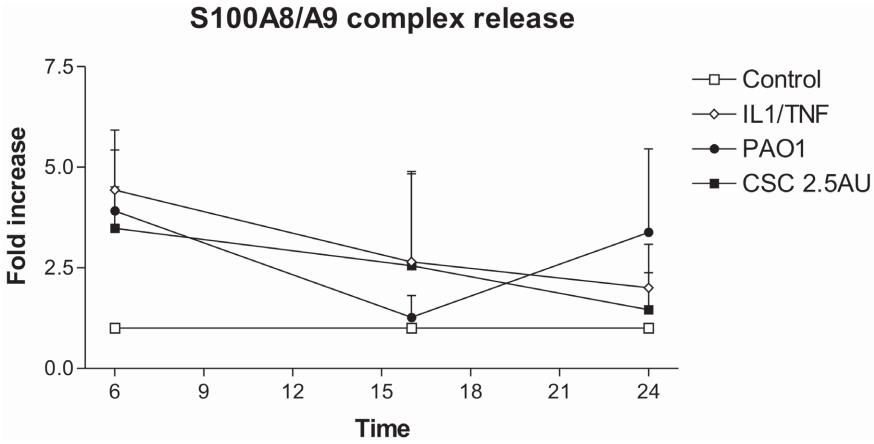

Figure 3: Release of S100A8/A9 upon stimulation of human bronchial epithelial cells by P. aeruginosa, IL1β/TNFα , or cigarette smoke condensate. S100A8/A9-specific ELISA was used to quantify the amount of S100A8/A9 complex in supernatants and cytosolic extracts of human primary bronchial epithelial cells exposed for 6, 16 and 24 hours to P. aeruginosa (10^7 CFU/ml), ***)***, IL1β/TNFα (20 ng/ml each) or cigarette smoke condensate (2.5 AU/ml). Protein ratios of cytosolic and extracellular S100A8/A9 were determined to quantify the change in release of the protein complex by epithelial cells. Shown values are +SEM of 3 experiments using three different donors.

DISCUSSION

In the present study, we investigated the expression dynamics of S100A8 and S100A9 at the mRNA and protein level. We show that mRNA expression of S100A9 and S100A8 is transiently enhanced upon exposure to *P. aeruginosa*, IL-1β/TNFα and cigarette smoke

condensate. Along with increased mRNA expression, active secretion of the S100A8/A9 protein complex by bronchial epithelial cells was transiently elevated.

Despite increasing evidence of involvement of S100A8 and S100A9 in inflammation, still little is known about the physiological importance of these genes. Elevated serum levels of S100A8/A9 in patients with inflammatory diseases suggest an eminent role for these proteins in inflammation [30], but also in wound repair [26,31] and the response to stress [24]. Expression of S100A8 and S100A9 in epithelial tissues has been predominantly associated with acute and chronic inflammation affecting these tissues [32,33]. Others have shown that S100A8/A9 protein complex is present in monocytes, neutrophils and activated macrophages in both mice and humans. In the various cell types different functions have been ascribed to the complex. Whereas the S100A8/A9 in monocytes is associated with early stages of differentiation and infiltration during the inflammatory response [34], the protein complex forms ~40% of the cytosolic protein content in neutrophils which upon release may function as antimicrobial agent [35] and may facilitate transendothelial migration by binding of the S100A8/A9 to microvascular endothelial cells [36]. Investigations into the functional significance of S100A8/A9 in epithelial tissues are limited and have been mainly focussed on the localization of these proteins in these tissues.

Transcription of S100A8 and S100A9 is rapidly induced by various stimuli in a transient manner, indicating their importance in the early response to acute insults. Similarly, expression of S100A8 is rapidly and transiently induced in murine macrophages exposed to IFNγ or TNFα[5]. The described expression kinetics are comparable with the current results. Peak expression occurred 8 hours after stimulation and declined to baseline with mRNA half live of approximately 8 hours, whereas expression of S100A8 in our bronchial epithelial cells peaked at 6 hours after exposure to *P. aeruginosa* or IL-1β/TNFα with an estimated half life of approximately 8-10 hours. The expression kinetics differed between S100A8 and S100A9, suggesting that these genes are regulated independently. This correlates with the different kinetics of release of S100A8, S100A9 and S100A8/A9 observed in vivo in a murine model of Streptococcal pneumonia (M-A Raquil and P.A. Tessier, unpublished observations). Existence of different promoter elements required for expression of S100A8 and S100A9 [37-39] further supports independent regulation mechanisms for these genes.

Enhancement of S100A8 and S100A9 expression appears to be mediated through different activation routes and is not restricted to inflammatory conditions only. It is not well understood how *P. aeruginosa* modulate the expression of these genes, but it is tempting to speculate that pattern recognition receptors are involved as previously demonstrated in epithelial cells *in vitro* [40-44] as well as *in vivo* [45]. IL-1β and TNFα are known to induce host defense responses in bronchial epithelial cells. The biphasic expression pattern of S100A9, IL-8 and to a lesser extend for the S100A8/A9 protein complex induced by IL-1β and TNFα could be explained by the activation of different downstream

signaling cascades [46]. Evidence that cigarette smoke condensate may boost S100A8 and S100A9 expression by causing oxidative damage is supported by studies demonstrating the increased expression of S100A8 by oxidative metabolites generated by UV irradiated keratinocytes [25] and by increased expression of S100A8 and S100A9 in human skin upon physical stress due to tape stripping or by application of 10% SDS [24]. Although extracellular presence of the protein complex may occur due to necrosis or due to active secretion, no cytotoxicity was observed under any of the experimental conditions as assessed by lactate dehydrogenase (LDH) release (data not shown). This indicates that bronchial epithelial cells are able to actively secrete the S100A8/A9 protein complex.

Enhanced serum levels of the complex have been demonstrated for over 2 decades in patients suffering from inflammatory diseases including cystic fibrosis [47]. Initiation and maintenance of inflammation at epithelial surfaces is regulated locally. Deregulation of inflammation may have considerable impact on the outcome of the initiated immune response. S100A8 and S100A9 may represent potential targets for clinical intervention to control epithelial inflammation [48]. However, it should be noted that these molecules are involved in a multitude of biological functions, complicating clinical intervention without causing adverse side effects. In addition, research into these genes has been hampered due to functional differences between the human and mouse homologs of S100A8 [49]. The functional differences seem to be restricted S100A8 only since Nacken *et al.* provided evidence that the murine and human homologs of S100A9 are equivalent in function [50].

To conclude, we have shown that expression of S100A8 and S100A9 in epithelial cells is induced upon exposure to *P. aeruginosa*, pro-inflammatory cytokines and cigarette smoke condensate. In our experimental model expression of S100A8 and S100A9 is transient at both the mRNA and protein level, indicating a function for these genes in the acute inflammatory response or to acute physical damage. The route by which expression is induced by the applied stimuli is likely to be different for each stimulus and needs further investigation. Taken together, our results indicate that S100A8 and S100A9 are central players in epithelial host defense and epithelial repair.

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