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Original Article

Toluene Exposure Leads to a Change in Expression Patterns of β Defensins in the Mouse Tracheal Epithelium

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Abstract: Defensins are generally implicated in the quick resistance of epithelial surfaces to microbials; however, recent reports have indicated that defensins also have unknown purposes in relation to noninfectious diseases. In this study, the localization patterns of anti-microbial peptides, β defensins (BDs), in the tracheal epithelium of male C3H mice under exposure to toluene were analyzed by immunohistochemistry. Mice were exposed one to ten times to toluene for 30 min by nose-only inhalation. Expression of BDs was revealed by immunohistochemistry in serial sections of trachea after the final exposure. Expression of BD-1 was usually observed at almost the same levels in all exposure groups, and expression of BD-2 was observed in the control group; however, the signals for BD-2 decreased gradually with frequency of exposure. In the group exposed ten times, expression of BD-2 decreased to far lower than that of the control group. No expression of BD-3 was detected in any groups. Interestingly, expression of BD-4 increased to the maximum in the group exposed four times and decreased to a level lower than that of the control in the group exposed ten times. The results of the present study indicated that toluene gas might change the expression pattern of BDs in the tracheal epithelial cells. The oscillation of expression of BD-4 was quite characteristic and might contribute to morphological damage in on the epithelial cells. (DOI: 10.1293/tox.26.35; J Toxicol Pathol 2013; 26: 35–40)

Key words: β defensins, mouse, sick building syndrome, toluene, tracheal epithelium

Introduction

Sick building syndrome (SBS) is a health hazard caused by living or staying in certain buildings. There is no single definition or criterion for SBS; however, the term usually refers to a higher than normal prevalence of people displaying symptoms that affect the eyes, head, upper respiratory tract and skin in a particular building [1,2]. Sick building syndrome is generally caused by dampness, chemicals or biological factors. Increased dampness causes higher density levels of microbial agents, eumycetes and molds that produce chemicals that can cause infection and allergy[3]. Volatile organic compounds (VOC) such as toluene or aldehydes are considered the main cause of SBS, as they are likely to irritate the skin, eyes, and mucosal membrane. The mechanisms of a cutaneous reaction induced by long-term VOC exposure have been investigated[4], as have the effects of low level toluene exposure on the airway — which induces inflammatory responses and neurotrophin production in mice[5–8].

The mechanochemical systems of biophylaxis are vital for pathogen resistance in animals, and are able to react prior to the immunoreaction, with defensins being cationic antimicrobial peptides that are the biophylaxis factors. Defensins are grouped into α defensins (NPs) and β defensins (BDs)[9], with epithelial cells producing BDs predominantly[10]. Following microbial invasion into the epithelium, epithelial cells generate higher than usual levels of BDs, which then adhere to the epithelial surface and adversely change the surface charge of invading microbial agents they come into contact with, puncturing them and leading to cell death[11]. They are grouped into subgroups (BD1, BD2, BD3 and BD4)[12], which all have similar activities but different sizes, gene sequences, strengths of effect and localities. Various activities of BDs have previously been reported, some of

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which include antifungal and cytotoxic activity in oral carcinomas and the saliva of patients with oral carcinomas[13–15] and an increase in expression of BD-2 in oral candidiasis[16,17], and recently Kaneda et al. reported expression patterns of BDs in Sjögren's syndrome[18]. In that study, the expressions of BDs were different in the salivary glands of patients with Sjögren's syndrome when compared with normal salivary glands. Interestingly, expressions of BDs are generally enhanced by infection, however, the report indicated that the expression pattern also differed in noninfectious diseases such as Sjögren's syndrome.

Toll-like-receptors (TLRs) are expressed as receptor proteins in human tracheal epithelial cells, and are broadly conserved from drosophila to humans, with structural and functional similarities[19]. It is the recognition of microbial ligands by TLRs that initiates the innate immune system. TLRs binding their ligand mediate the production of cytokines necessary for the initialization of effective immunity. Various TLRs exhibit different patterns of expression[20] and are grouped into subtypes, ten of which have been identified in humans, TLR1 to TLR10. TLR2, as an example, is located on the plasma membrane and senses components of bacteria, mycoplasma, fungi and viruses. It also joins with TLR1 or TLR6 to recognize lipoprotein components and induces the production of various pro-inflammatory cytokines in macrophages and dendritic cells[21].

This study analyzed the expression patterns of both BDs and TLR2 in mouse tracheal epithelium that had been treated with toluene. Toluene treatment induces similar characteristics to SBS, and it was our aim to identify certain criteria that would aid in quick diagnosis of SBS.

Materials and Methods

Animals

All experiments were undertaken in accordance with the Guidelines and Regulations for Animal Experimentation of Okayama University and were conducted with approval from the National Institute for Environmental Studies Ethics Committee for Experimental Animals. Nine-week-old male C3H/HeN mice (n=24) were used and were given access to food and water ad libitum. All mice were sacrificed under ether anesthesia after the study period. Mice were dissected, and their tracheas were removed.

Toluene exposure

Toluene was generated in an organic solvent gas generator (Sibata Scientific Technology Ltd., Saitama, Japan), diluted with clean, filtered air to achieve the desired gas concentrations, and then introduced into an acrylic chamber. The atmospheric concentration of toluene in the chamber was measured by gas chromatography mass spectrometry (INFICON, Syracuse NY, USA). The average level of control toluene was 0.01 ± 0.001 ppm, and 9 ppm toluene was 9.1 ± 0.1 ppm. The airflow rate through the chamber housing the experimental animals was 20 L × min^{-1}. The concentrations of toluene were constant, irrespective of the sampling port location. Groups of mice (n=6) were exposed to various concentrations of toluene for 30 minutes by nose-only inhalation on days 0, 1, 2, 7, 14, 21, 28, 35, 42 and 49 of the study period (Fig. 1). Ovalbumin (OVA) was administered to one mouse of each group. Ovalbumin injection was performed intraperitoneally with 10 μg Bovine Serum Albumin Standards (Thermo Fisher Scientific, Rochester, NY, USA) plus 2 mg alum, and each of these mice was then challenged with nebulized OVA as a booster once every 2 weeks (weeks 2, 4 and 6) during the exposure period.

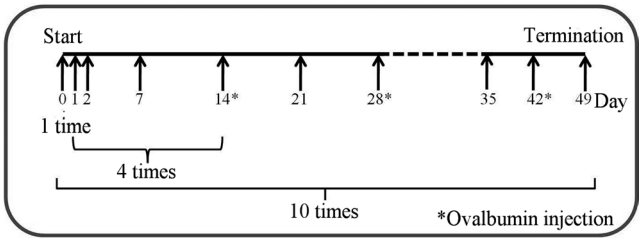

Fig. 1. Exposure schedule. Mice were exposed to various concentrations of toluene for 30 minutes by nose-only inhalation on days 0, 1, 2, 7, 14, 21, 28, 35, 42 and 49 of the study period. The one time (1T) group was exposed to toluene on day 0. The four time (4T) group was exposed to toluene on days 1, 2, 7 and 14. The ten time (10T) group was exposed to toluene on days 0, 1, 2, 7, 14, 21, 28, 35, 42 and 49. Ovalbumin injection was performed intraperitoneally, and each of these mice was then challenged with nebulized OVA as a booster once every 2 weeks (weeks 2, 4 and 6) during the exposure period.

Immunohistochemistry

Removed tracheas were fixed with 4% paraformaldehyde for 12 hours at 4°C and dehydrated using a graded ethanol series before embedding in Paraplast Plus (Sigma, St Louis, MO, USA). Serial sections were cut at a thickness of 7 μm and mounted on silane-coated slides. The sections were deparaffinized and soaked in methanol containing 1% hydrogen peroxide to block endogenous peroxidase activity, and then washed in phosphate-buffered saline. Rabbit antibodies directed against BD-1 (sc-20797, 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), BD-2 (sc-10856, 1:200, Santa Cruz Biotechnology, Inc.), BD3 (AB3478, 1:200, Chemicon International, Billerica, MA, USA), BD-4 (sc-30117, 1:200, Santa Cruz Biotechnology, Inc.) and TLR2 (ab9100, 1:200, Abcam, Cambridge, MA, USA) and mouse antibodies directed against NP-3 (T-1034, 1:200, BMA Biomedicals AG, Augst, Switzerland) were used, the specificity of these antibodies already being confirmed. Their product documents showed their cross-reaction with mouse antigen. Immunoreaction was performed with Vectastain ABC Elite Kits (Vector Laboratories, Burlingame, CA, USA), and sections were visualized with 3,3'-diaminobenzidine (DAB) solution. To reduce any nonspecific background, sections were treated with normal serum containing 0.7% sodium azide. Following immunoreaction at room temperature, sections were counterstained with methyl green.

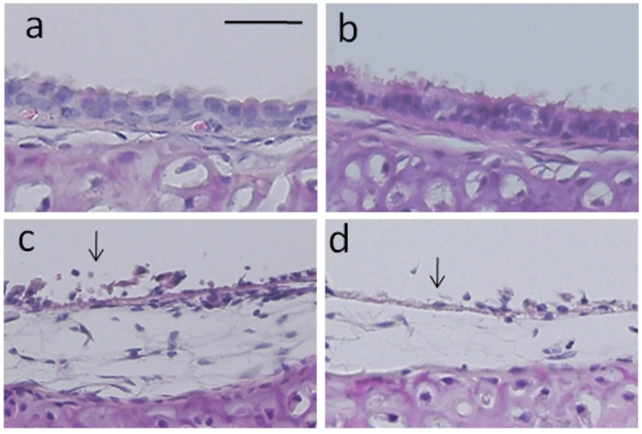

Fig. 2. Mouse tracheal epithelium visualized by HE staining. Damage to the tracheal epithelium was not observed in either the control group (a) or 1T group (b). Morphological damage to the tracheal epithelium was observed in the 4T group (c). More than 50% of tracheal epithelial cells were remarkably damaged in the 10T group (d). The arrowhead indicates the detached and/or fragmented tracheal epithelium. The bar indicates 50 μm.

Statistical analysis

Signal intensities for BDs from sections from all groups were evaluated according to the average number of points score. Four different amounts of points (0 pt, 0.5 pt, 1 pt and 1.5 pt) were used to evaluate signal intensities, and the averages of 100 or more sections from each group were used for statistical analysis. Usually weak signals of BDs are found in normal tracheal epithelial cells, so we considered the average points of the control group to be 1 pt. The points of each group in this study were always calibrated to the points of the control group. If there was no difference in the signal intensity compared with the control group, the signal intensity was scored as 1 pt. The signal intensity of sections with more intensity than that of the control was scored as 1.5 pt. The sections with less intense signals than that of the control group were assigned a score of 0.5 pt. The sections with no signal intensity were assigned a score of 0 pt.

Results

Histological overview and NP-3 expression

No differences in damage to the tracheal epithelial structure between the ovalbumin injection group and the non-injection group were noted. Signals for anti-BDs were observed in the normal tracheal epithelial cells, no relationship was shown with the ovalbumin injection group. Damage to the tracheal epithelial structure was first observed in the group exposed four times (4T group) by HE staining, including a partially detached tracheal epithelium (Fig. 2). In the group exposed ten times (10T group), more than half of the epithelial cells in the trachea were remarkably damaged, with the tracheal epithelium becoming detached or fragmented (Fig. 2). Dark brown signals for anti-BD-1, anti-BD-2 and anti-BD-4 antibodies appeared at the luminal side of the tracheal epithelial cells. No neutrophils or macrophages had migrated into the lamina propria in any group, nor was expression of an NP-3 signal detected (Fig. 4). No dendritic cells appeared in the tracheal epithelium.

Expression of BD-1

Expression of BD-1 was detected at nearly the same levels in all groups. The signal intensity for anti-BD-1 was observed uniformly at the luminal side of the tracheal epithelium in all groups. There was no difference in signal intensity between the control and any exposure groups (Fig. 3). The average points of the group exposed one time (1T), 4T group and 10T group were 1 pt, 1.05 pt and 1.04 pt, respectively (Fig. 5).

Expression of BD-2

Expression of BD-2 was generally observed at the luminal side of the tracheal epithelium in all groups (Fig. 3). No difference between the control and 1T groups was observed. The average signal intensity score of the 1T group was 1 pt. However, expression of BD-2 decreased significantly in the 4T group when compared with the control. The average signal intensity score of the 4T group was 0.88 pt. In the 10T group, signal intensity for anti-BD-2 was remarkably decreased compared with that of the other groups. The average signal intensity score of the 10T group was 0.56 pt (Fig. 5).

Expression of BD-3

No significant signal intensity for anti-BD-3 was observed in either the control or any exposure groups (Fig. 3). The average signal intensity score of all groups was 0 pt (Fig. 5).

Expression of BD-4

Expression of BD-4 was observed to be similar at the luminal side of the tracheal epithelium in the control and 1T groups, and there was no significant difference of signal intensity between them. The average signal intensity score of the 1T group was 1 pt. Expression of BD-4 increased to the maximum level in the 4T group and decreased to a level lower than that of the control group in the 10T group (Fig. 3). The signal intensity score of the 4T group was 1.79 pt. The average signal intensity score of the 10T group was 0.63 pt (Fig. 5).

Expression of TLR2

Expression of TLR2 was detected in all groups. The signal for anti-TLR2 was observed as being localized at the luminal side of the tracheal epithelial cells. The signal intensities of the 4T and 10T groups were slightly higher than that of the control group (Fig. 4). The average of signal intensity score of the 4T group was 1.30 pt. The average signal intensity score of the 10T group was 1.32 pt (Fig. 5).

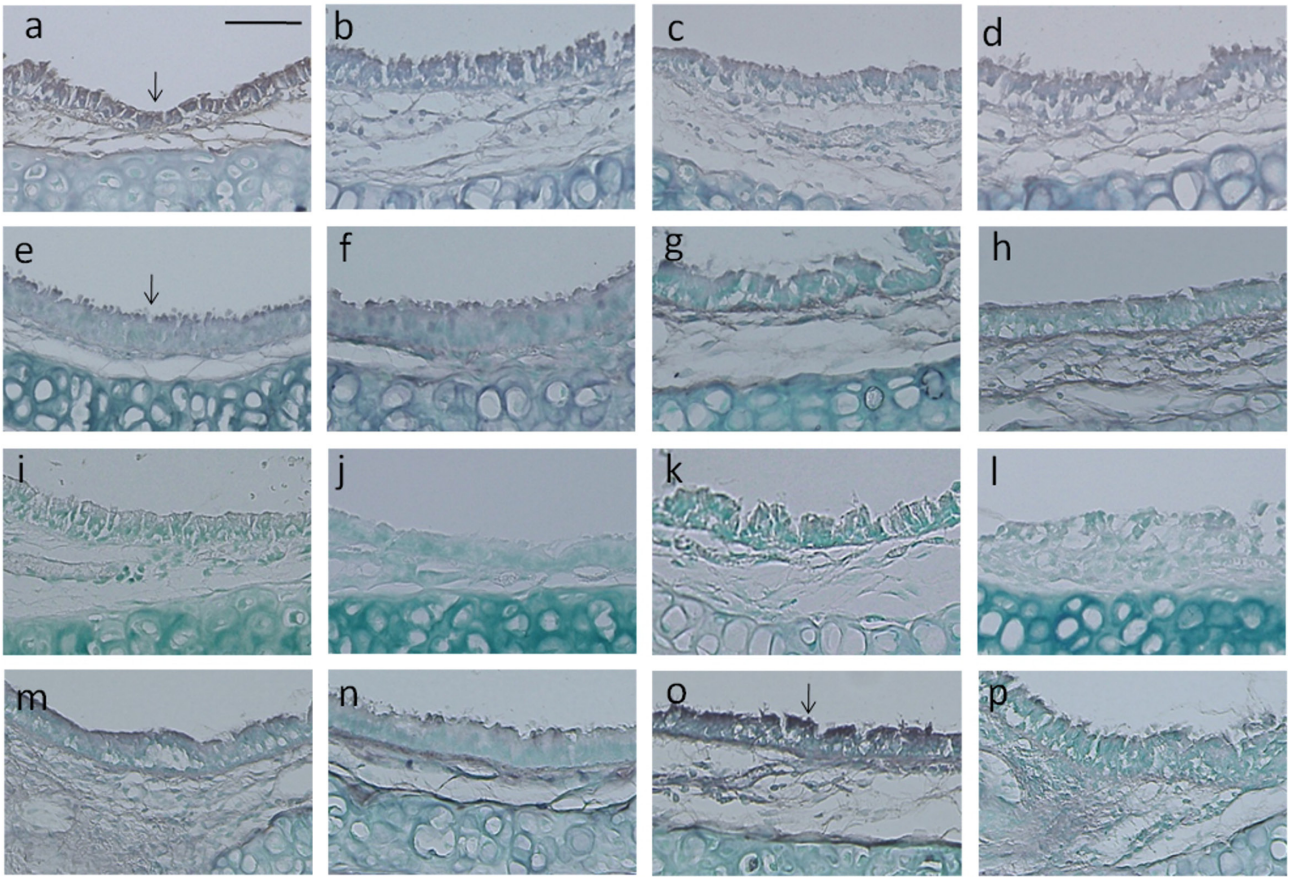

Fig. 3. Immunoreaction for BD-1 (a–d), BD-2 (e–h), BD3 (i–l) and BD-4 (m–p) in the mouse tracheal epithelium. Dark brown signals (arrowheads) for anti-BD-1, anti-BD-2 and anti-BD-4 antibodies appeared at the luminal side of the tracheal epithelial cells. Expression of BD-1 was observed in the control group (a), 1T group (b), 4T group (c) and 10T group (d). There was no observable difference in signal intensity between the control and any exposure group. Expression of BD-2 was observed in all groups (e–h). No difference was observed between the control group (e) and 1T group (f). Expression of BD-2 decreased significantly in the 4T group (g) and 10T group (h). No significant signal for anti-BD-3 was observed in the control group (i), 1T group (j), 4T group (k) or 10T group (l). Expression of BD-4 was detected in all groups (m–p). No difference was observed between the control group (m) and 1T group (n). The expression of BD-4 increased to the maximum level in the 4T group (o) and then decreased to a level lower than that of the control group in the 10T group (p). The bar indicates 50 μm.

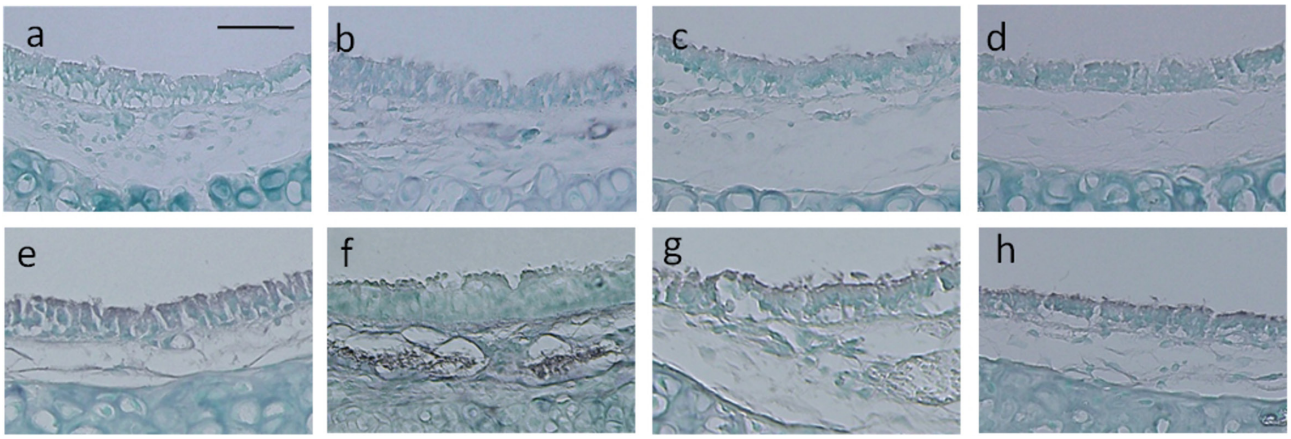

Fig. 4. Immunoreactions for NP-3 and TLR2. No signal intensity for NP-3 was observed in the control group (a), 1T group (b), 4T group (c) or 10T group (d). Expression of TLR2 was observed in the control group (e), 1T group (f), 4T group (g) and 10T group (h). The signal intensities of both the 4T and 10T groups were slightly more than that of the control group. The bar indicates 50 μm.

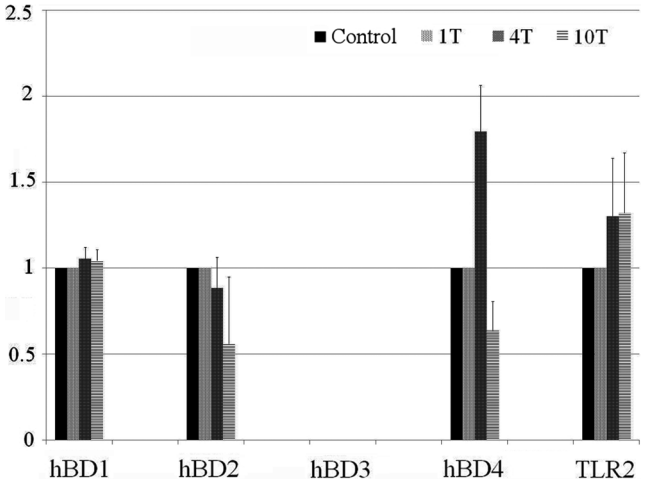

Fig. 5. Bar graph showing the mean values for the signal intensities of BDs in all groups. C, control group; 1T, group exposed one time; 4T, group exposed four times; 10T, group exposed ten times. The mean points for each group are indicated vertically, and each exposure group is indicated horizontally. The control and exposure groups exhibited non-normal distributions. Therefore, the Wilcoxon rank-sum test was used to compare the control group and the exposure groups. No significant difference in signal intensity for BD-1 was observed in any group. However, statistically significant differences in signal intensities for BD-2 were observed between the control group and 4T group ($0.01<P<0.05$) and between the 4T group and 10T group ($0.01<P<0.05$). The mean signal intensity of BD-3 for all groups was 0 pt. The expression pattern of BD-4 was characteristic. The signal intensity reached the maximum in the 4T group and then decreased thereafter. Statistical differences exist between the control group and the 4T group ($P<0.01$) and 10T group ($P<0.01$).

Discussion

This study was the first to attempt to analyze analogous symptoms of SBS using an exposure to toluene with a focus on the localization of BDs in the tracheal epithelium, which may induce something similar to SBS in mice[22]. Furthermore, there have been few reports on the relationship between chemicals and BDs[23,24]. The mouse tracheal epithelium is composed of three layers, the tracheal cartilage, lamina propria, and tracheal epithelium, and morphological changes were observed by HE staining after four exposures to toluene. A possible cause for the changes might be toluene attacking the tracheal epithelium and bringing about fragmentation or exfoliation. No cells positive for anti-NP-3 were observed in any group (Fig. 4), nor were any lymphocytes, which perhaps indicates that the toluene-induced change in the tracheal epithelium is not an adaptive immunoreaction.

Defensins are biophylaxis factors that react quickly even to noninfectious sources[25]. We hypothesized that SBS might induce a decrease in the expression of defensins. Anti-BD-1, anti-BD-2, anti-BD-3 and anti-BD-4 antibodies were used for immunostaining. The expression of BD-1 is generally detected in the epithelia of the airways. The expression of BD-1 was found in tracheal epithelial cells at the same level of signal intensity in all cases. The expression of BD-2 is also generally detected in normal tracheal epithelial cells; however, the expression pattern indicated a decrease after four exposures to toluene and never an increase. It was reported that an infection induces the expression of BD-2[11,16]. On the other hand, this study suggested that toluene exposure reduced the expression of BD-2. It was reported that low doses of residual oil fly ash reduced the gene expression of BD-2[24]. Chemicals might reduce the expression of BD-2.

Expression of β defensin-3 may be a novel strategy to deal with skin and respiratory tract infections[26]. However, the results of this study did not indicate any expression of BD-3 in any of the test groups, which perhaps indicates that BD-3 was not involved in the collapse of the tracheal epithelium caused by toluene exposure.

It is suggested that BD-2 and/or BD-4 might be involved in the morphological changes observed in the tracheal epithelium because toluene exposure changed their signal intensities. A change in expression of BD-4 was observed, with the signal intensity reaching a maximum in the 4T group and diminishing to the lowest levels in the 10T group. Decreased expression of BD-2 and increased expression of BD-4 might be the cause of damage to the tracheal epithelium.

No lymphocyte infiltration was observed, which perhaps indicates that the changes in expression patterns of BDs might not be related to an immunoreaction. No significant expression of BD-3 was detected in any group. The morphological damage to the tracheal epithelial cells observed in the 4T group therefore has no relationship with BD-3. An unbalance caused by an increase in BD-4 and decrease in BD-2 in the 4T group might be related to the damage of the epithelial cells (Fig. 3). It was reported that TLR2 mediated the expression of BD2 in infection[27,28]. However, in spite of an increase in the expression of TLR2, the expression of BD2 decreased in this study. A change in expression of TLR2 was not detected prior to the unbalance of BD-2 and BD-4, therefore, TLR2 might not be related to the unbalance of BD-2 and BD-4. Additionally, with regard to all BDs and TLR2, there was no difference in the expression levels in the 1T group or control group. This suggests that one exposure did not affect the morphology of the tracheal epithelium.

Further research building on the findings of this study and perhaps focusing on the oscillation of the expression of BD-4 may contribute to identifying criteria for the diagnosis for SBS, especially if used in conjunction with a clinical test, such as a sputum smear, which would make the diagnosis for SBS quicker and easier.

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