Stimulation of bacterial biofilms on Th17 immune cells

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ABSTRACT. We investigated the role of bacterial biofilms in stimulating T helper 17 (Th17) cells in infected organisms. The formation of bacterial biofilms isolated from clinical lavage fluid samples was measured. Th17 cells and interleukin 17 (IL-17) levels in the peripheral blood of healthy individuals, people infected by biofilm bacteria, people infected by non-biofilm bacteria, and in the lavage fluid from people infected by bacteria were determined. Differences in those data were tested using the SPSS 17.0 statistical software. Th17 cells and IL-17 levels in the peripheral blood of biofilm bacteria-infected people, non-biofilm bacteria-infected people, and healthy controls were 0.59 ± 0.18% and 108.8 ± 20.5 pg/mL; 0.58 ± 0.18% and 100.1 ± 20.7 pg/mL; and 0.55 ± 0.17% and 100.0 ± 21.4 pg/mL, respectively; there were no statistically significant differences (P > 0.05). Th17 cells and IL-17 levels in the lavage fluid of biofilm bacteria-infected people and non-biofilm bacteria-infected people were 1.37 ± 0.34% and 157.4 ± 30.8 pg/mL; and 1.11 ± 0.21% and 136.2 ± 24.3 mg/mL, respectively; the differences were statistically significant (P < 0.05). Bacterial biofilms can increase the expression...
levels of Th17 cells and IL-17 in local infections; this may be the mechanism by which chronic injuries are caused by biofilm infections.

**Key words:** Biofilm; Bacteria; T helper 17 cells; Interleukin 17

**INTRODUCTION**

Bacterial biofilm is the most common bacterial structure and is formed in a variety of external environments. Biofilm formation is a reaction to the pressure of the external environment, and the formation of biofilms provides bacterial with protection from adverse environmental factors, including antibiotics and host immune systems (Sadowska et al., 2013). There is a broad consensus that chronic infections are caused by the formation of bacterial biofilms (Prabhakara et al., 2011b; Moutsopoulos et al., 2012; Peyyala et al., 2012; Cantero et al., 2013a). There have been a few reports on immune responses caused by bacterial biofilm infections (Sun et al., 2012; Granslo et al., 2013), and some reports have shown that bacterial biofilm infection stimulates T helper 17 (Th17) cell proliferation and interleukin 17 (IL-17) expression (Ohlrich et al., 2009; Prabhakara et al., 2011a; Snowden et al., 2012; Cheng et al., 2014), while some reports have shown that biofilms do not cause a significant immune response (Wood et al., 2012). In view of this, the purpose of this study was to examine the relationship between the ability for biofilm formation and Th17 cells.

**MATERIAL AND METHODS**

**Materials**

**Bacterial strains used in the experiment**

*Pseudomonas aeruginosa* and *Acinetobacter baumannii* used in the experiment were isolated from clinical lavage fluid samples (bacteria were isolated from lavage fluid) in the First Affiliated Hospital of Xinxiang Medical Collage.

**Instruments and reagents**

A FACSCanto analyzer was purchased from BD in the US, and the automated enzyme immunoassay workstation was provided by Yantai Addcare Bio-Tech Co. Ltd. The Th17 flow detection kit from the BioLegend Corporation included fluorescein isothiocyanate (FITC)-labeled anti-human CD3, phycoerythrin (PE)-labeled anti-human CD4, Alexa Fluor 647-labeled anti-human IL-7, a matched rat IgGl as isotype control, fixative, and rupture wash. The CD4 + CD25 + Foxp3 + Treg-step flow cytometry kit from the BioLegend Corporation included: FITC-labeled anti-human CD4, PE-labeled anti-human CD25, Alexa Fluor 647-labeled Foxp3, a matched murine IgGl as isotype control, Foxp + 3 fixative/rupture reagent, and rupture buffer. The phorbol 12-myristate 13-acetate and ionomycin were purchased from Sigma; monensin was purchased from the BioLegend Corporation; an IL-17 enzyme-linked immunosorbent assay (ELISA) kit was provided by the BioSource Corporation; and we synthesized the crystal violet dye.
**Experimental methods**

*Detection of IL-17*

Clinical lavage fluid collected aseptically using four layers of sterile gauze filter was injected into 10-mL sterile tubes and centrifuged at 2000 rpm for 5 min. The supernatant was used for detection of IL-17 strictly according to manufacturer instructions, and centrifugal precipitation was used for detecting Th17 cells and bacterial culture. Lavage fluid samples (253) were collected between January 2013 and December 2013. In addition, IL-17 levels in the peripheral serum of patients and healthy controls were detected.

*Bacterial culture and identification*

After centrifugation, the supernatant from the lavage fluid of some samples was removed for bacterial culture and identification under sterile conditions. The remaining specimens were used for the detection of Th17 cells; precipitation samples were inoculated on blood agar plates and MacConkey culture plates, and single colonies were used for identification of bacteria on the following day.

*Determination of formation ability of bacterial biofilms*

The biofilm formation capacities of *A. baumannii* and *P. aeruginosa* were tested by crystal violet staining (Yang et al., 2009). The specific method was as follows: *A. baumannii* and *P. aeruginosa* broths were adjusted to 0.5 McFarland turbidity using a bacterial standard turbidity tube, and the broth (10 μL) was incubated on a 96-well flat-bottomed culture plate (each well containing 200 μL 1:50 diluted lysogeny broth). Each strain was placed in three wells; a blank control well contained culture medium alone. The strains were cultured statically at 37°C for 48 h; 150 μL 0.25 g/L crystal violet dye was added to the triplicate wells for each strain at room temperature for 20 min. The cultures were then washed five times with saline before drying. Ethanol (300 μL, 95%) was added to each well and bleaching at room temperature took place for 10 min. Absorbance at 570 nm (A570) of the bleaching solution in each well was detected using a UV-visible spectrophotometer. The average value of A570 of each bacterium equaled the difference between the mean of the A570 values of the three wells and that of the blank well.

*Th17 cell detection*

Using the cultivated lavage fluid samples containing *P. aeruginosa* and *A. baumannii* to determine biofilm infection, peripheral blood from the 28 infected patients and the 24 healthy controls was investigated for the presence of Th17 cells.

*Statistics*

Data were analyzed using the statistical software SPSS 17.0; all means are reported as means ± standard deviation (means ± SD); the mean difference between the groups was tested using independent samples *t*-test and *t*-test for paired data; and P-values < 0.05 were considered to be statistically significant.
RESULTS

Results of bacterial culture and identification

Fifty-nine cases of *P. aeruginosa* and 54 cases of *A. baumannii* were isolated, totaling 113 cases.

Biofilm generation capacity of bacteria

The maximum distribution range of A570 of the 113 bacterial samples in the biofilm-forming ability test was 0.15-2.12; 24 samples had an A570 value of 0.5 or less and 28 samples had an A570 value of more than 1.5. In this study, the 24 bacterial samples with A570 values less than 0.5 were deemed to have weak biofilm formation capacity, and patients infected with these bacteria were designated as the non-biofilm infection group. The 28 samples with A570 values of more than 1.5 were deemed to have strong biofilm forming capacity, and patients infected with these bacteria were designated as the biofilm infection group.

Th17 cells and IL-17 levels in the biofilm infection and non-biofilm infection groups

Th17 cell percentages (detected by flow cytometry) and serum IL-17 levels in the peripheral blood of the different experimental groups (means ± SD) are shown in Table 1. Th17 cell percentages and IL-17 levels in the bronchoalveolar lavage fluid of the different experimental groups (means ± SD) are shown in Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>Th17 cells (%)</th>
<th>IL-17 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofilm infection</td>
<td>28</td>
<td>0.59 ± 0.18</td>
<td>108.8 ± 20.5</td>
</tr>
<tr>
<td>Non-biofilm infection</td>
<td>24</td>
<td>0.58 ± 0.18</td>
<td>100.1 ± 20.7</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>24</td>
<td>0.55 ± 0.17</td>
<td>100.0 ± 21.4</td>
</tr>
</tbody>
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Table 1. Comparison of T helper 17 (Th17) cell percentages and serum interleukin 17 (IL-17) levels in the peripheral blood of the different experimental groups (means ± SD).

There was no significant difference in Th17 cells among the three groups (P > 0.05); P values were respectively 0.409, 0.779, and 0.598 between the biofilm infection and non-biofilm infection groups, the biofilm infection and healthy control groups, and between the non-biofilm infection and healthy control groups. There was also no significant difference in serum IL-17 levels among the three groups (P > 0.05); P values were respectively 0.138, 0.136, and 0.992 between the biofilm infection and non-biofilm infection groups, the biofilm infection and healthy control groups, and between the non-biofilm infection and healthy control groups.

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</tr>
<tr>
<td>P value</td>
<td>-</td>
<td>0.002</td>
<td>0.009</td>
</tr>
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Table 2. Comparison of T helper 17 (Th17) cell percentages and interleukin 17 (IL-17) levels in the bronchoalveolar lavage fluid of the different experimental groups (means ± SD).
DISCUSSION

Bacterial biofilm is a general structure and forms at ambient pressure as an adaptive response to external pressure. The formation of biofilms increases a bacteria’s resistance to the outside world, including drug resistance and immune evasion of antibiotics. Some studies have reported that bacterial biofilms stimulate the body, cause the body’s immune response, and induced infections that cause tissue damage (Prabhakara et al., 2011a), while other studies have reported that bacterial biofilms do not cause the immune response (Wood et al., 2012). Some studies have shown that bacterial biofilms induce an increase of IL-6 and IL-8 (Eberhard et al., 2009; Cantero et al., 2013a,b). Some studies have reported that planktonic bacteria cause the body to produce more IL-6, IL-8, vascular endothelial growth factor, and transforming growth factor beta 1 compared with biofilm bacteria, while biofilm bacteria induce more tumor necrosis factor alpha (Kirker et al., 2012).

Th17 cells belong to the newly discovered T cell line, and play a key role in dealing with planktonic bacterial cell infection. Th17 cells may also play a key role in bronchiectasis or cystic fibrosis in chronic biofilm infections (Aujla et al., 2007). Given the inconsistent results in the reported bacterial biofilm infections, our research focused on the changes in Th17 cells that were caused by bacterial biofilm infections.

Our results showed that biofilm infection mainly caused a change in Th17 cells at localized infection sites, and did not cause systemic immune changes. Th17 cells accumulate locally and secrete chemokines, attracting more neutrophils to the site of infection (Aujla et al., 2007); neutrophils cause damage to body tissue while killing bacteria. However, biofilms lead to multi-drug resistant bacteria (Pradeep et al., 2013) and immunity resistance (Alhede et al., 2014), which may be the mechanism of chronic infection caused by bacterial biofilm formation, leading to chronic damage to the infected organism. Therefore, biofilm bacterial infections that elicit a reduced immune response will help reduce inflammation damage at local infection sites. We have not retrieved any relevant literature on the mechanism by which bacterial biofilms cause Th17 cell proliferation, and our group is continuing to research the mechanism by studying the main components of biological membranes.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES


