Do Immunologic Events in Degenerative Disc Tissue Alter the Peripheral Immune Tolerance?

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ABSTRACT

AIM: The objective of our research was to determine whether the changes in Treg and IL-17 levels have an effect on peripheral self-tolerance in patients with degenerative disc disease (DDD).

MATERIAL and METHODS: 26 patients with lumbar DDD and 14 autopsy cases as controls were included. From each patient, disc tissues and peripheral blood samples were collected during the surgery. For the controls, disc samples and plasma were obtained during autopsy. The following methods were used: flow cytometry for determining the percentages of lymphocyte subsets as CD4 (Th), CD25 (IL-2R), CD4+CD25+Foxp3+ (Tregs), Foxp3+; ELISA for IL-17, TGF-β, IL-6 and Luminex XMAP for IFN-γ. The results were compared between each group.

RESULTS: Patients demonstrated that CD4 (Th), CD25 (IL-2R), CD4+CD25+Foxp3+ (Tregs), Foxp3+, IL-17, TGF-β, IL-6, IFN-γ were significantly higher in plasma. Tissue levels of CD4+CD25+Foxp3+ (Tregs), IL-17, TGF-β, IL-6, Foxp3+ were significantly higher in the patients. Comparison between the plasma and disc samples in the patients showed significantly higher plasma levels of CD4+CD25+Foxp3+ (Tregs) and CD4. Positive correlations were shown between CD25/CD4, TGF-β/IL-17, CD4+CD25+Foxp3+/Foxp3+ in the patients.

CONCLUSIONS: It would be interesting to investigate in further detail whether the systemic immune reaction associated with Tregs cells and IL-17 has changed.

KEY WORDS: Degenerative disc disease, Immune reactions, Immune tolerance, Lumbar disc herniation, Lymphocytes

INTRODUCTION

The CD4+ T lymphocytes play a pivotal role in orchestrating the physiopathological immune reactions that underlie the pathogenesis of intervertebral disease via the production of distinctive sets of proinflammatory cytokines such as IFN-γ (4, 11). It has been clearly demonstrated that these cytokines effect the activity of Th1 lymphocytes that have traditionally been linked to the induction and progression of tissue damage in degenerative disc diseases (DDD) (4, 7, 11).

TH1 cells produce IFN-γ and regulate antigen presentation and immunity against intracellular pathogens, whereas TH2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 and mediate humoral responses. IFN-γ and IL-4 are not only the key effectors but also mediate the differentiation of TH1 and TH2 cells, respectively (7). Recently, TH17, a third subset of TH cells, has been identified. IL-17 belongs to a recently discovered family of cytokines that contribute to the crosstalk between adaptive and innate immunity (16). TH17 produces IL-17, IL-17F and IL-22 and regulate inflammatory responses by tissue cells (3, 5, 12, 17). TH17 differentiation, at least in the mouse, is initiated by TGF-β and IL-6 (2, 10). On the other hand, TGF-β signaling has also been clearly demonstrated to mediate TH17 differentiation.
in vivo (2, 10), and activated T cells may serve as an important sources of TGF-β for this regulation (9). These immune responses are regulated by a highly complex and intricate network of control elements. Prominent among these regulatory components are the anti-inflammatory cytokines and specific cytokine inhibitors.

Under the physiological conditions, these cytokine inhibitors serve as immunomodulatory elements that limit the potentially injurious effects of sustained or excess inflammatory reactions. We had shown in our previous study how this balance in the immune response changes between cytokine and immune cells DDD (1). The aim of this study was to determine whether the changes in Treg and IL-17 levels have an effect on peripheral self-tolerance.

**MATERIALS and METHODS**

**Subjects**

This work was conducted at the Neurosurgery and Physiology Departments of Cerrahpasa Medical Faculty, Istanbul University and at the Medical Institute of the Turkish Ministry of Justice. The subjects were divided into two groups. Group I included a total of 26 patients who had undergone surgery due to DDD following obtaining the informed consents. This group included 16 males and 10 females with a mean age of 47.46 ± 11.25 years. Group II, also referred to as the controls, included 14 autopsy cases. Peripheral blood samples for plasma were collected during the surgery in group I and blood for plasma was obtained by intracardiac puncture during autopsy in group II. Tissue samples during surgery and autopsy were also obtained from group I and II, respectively.

**Flow cytometry**

The flow cytometric method was used for determining the percentages of lymphocyte subsets as: CD4 (Th), CD25 (IL-2R), CD4+CD25+Foxp3+ (Tregs), Foxp3+. Enzyme-linked immunosorbent assay (ELISA) was used for obtaining levels of IL-17, TGF-β, IL-6. Multiple analyte platform (XMAP, luminex) was used for obtaining levels of IFN-γ. The results were compared between the groups.

The following antibodies were used for evaluation of immunophenotyping: CD4 (Th) FITC, CD25 (IL-2R) (PE), CD4+CD25+Foxp3+ (Tregs) (PE), Foxp3+ (PE) (all from BD Biosciences, USA). Blood samples obtained from group I were placed into EDTA tubes, and then with 20 μL of monoclonal antibody was incubated at room temperature for 20 minutes. Erythrocytes were separated by adding 2 ml of lysing solution x 1 (BD Biosciences, USA) and incubated for 10 minutes at room temperature. The solution was twice washed with phosphate-buffered saline (PBS) and centrifuged at 400 g. The pellets were then suspended in 300μL of PBS and analyzed with FACS Calibur instrument (Becton Dickinson Immunocytometry Systems, USA) using Cell Quest software (BD Biosciences, USA). A total of 10000 cells were counted in the lymphocyte gate.

**ELISA**

A competitive ELISA kit (all from Biovendor, Czech) was used for obtaining levels of IL-17, TGF-β, and IFN-γ. 50µL quantities of all standards and samples were added in duplicate to the wells of a micro plate which were pre-coated with antibody. 100 μL of conjugate was added into each well and incubated for 1 hour at 37°C. The plates were then washed and dried with distilled water five times. Then, 100 μL substrate solutions was added to each well, which was covered and incubated for 15 minutes at 37 °C, after which 50 μL of stop solution was added. After mixing, the optical density (OD) at 450 nm was promptly read using a micro plate reader.

**XMAP, Luminex**

Twenty-five μl of standards and samples were pipette and incubated on the plate shaker with at 500 RPM in room temperature for 30 minutes. After plate contents were vacuumed 150 μl of washing solution and 25 μl of detection antibody were added and incubated on the plate shaker at 500 RPM in room temperature for 30 minutes. Every sample was washed three times and again 50 μl of streptavidin-PE was added and incubated at 500 RPM in room temperature for 5 minutes. The washing procedure was then performed again. 120 μl of reading buffer was added and on the plate shaker and they were incubated for 5 minutes. The data was then read on luminex.

**Statistical Analysis**

All data collected from each sample were organized in a database (Excel, Microsoft Corp.). Numeric variables were provided as the mean ± SD. For statistical analysis, we used the non-parametric Mann-Whitney U test and chi-square test for categorical variables. For correlation analysis, the Pearson correlation was used.
and a probability value less than 0.05 was considered statistically significant. All statistical calculations were performed using commercially available software (SPSS version 12.0, SPSS Inc.).

RESULTS

The results were summarized in Table 1 and Figures 1 and 2. Comparing the two groups regarding the plasma demonstrated that CD4 (Th), CD25 (IL-2R), CD4+CD25+Foxp3+ (Tregs), Foxp3+, IL-17, TGF-β, IL-6, IFN-γ were significantly higher in the group I those of the group II (p < 0.05). We determined that CD4+CD25+Foxp3+ (Tregs), IL-17, TGF-β, IL-6, Foxp3+ in disc samples of the group I showed significant differences compared with the group II. The comparison between the plasma and disc samples in the group I, plasma showed significantly higher levels of CD4+CD25+Foxp3+ (Tregs) and CD4. Positive correlations were shown between CD25/CD4, TGF-β/IL-17, CD4+CD25+Foxp3+ / Foxp3+ in the group I.

Table 1: Summary of statistical comparisons of the changes in cytokine levels of plasma in both groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group I (n=26)</th>
<th>Group II (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 (%)</td>
<td>64.23±1 4.24</td>
<td>15.9± 3.0</td>
</tr>
<tr>
<td>CD25 (%)</td>
<td>6.57 ± 0.5</td>
<td>3.4 ± 1.3</td>
</tr>
<tr>
<td>CD4+CD25+Foxp3 (%)</td>
<td>3.7± 1.1</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>Foxp3+ (%)</td>
<td>1.2± 0.3</td>
<td>0.6 ± 0.05</td>
</tr>
<tr>
<td>IL-17 (pg/ml)</td>
<td>24.4 ± 3.2</td>
<td>12.8 ± 4.5</td>
</tr>
<tr>
<td>TGF-β (pg/ml)</td>
<td>34.5± 6.4</td>
<td>10.7 ± 2.5</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>20.2 ± 1.9</td>
<td>16.6 ± 1.7</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>8.94 ± 3.1</td>
<td>4.62 ± 1.19</td>
</tr>
</tbody>
</table>

(*P<0.05, **P<0.01, ***P<0.001).

DISCUSSION

This study demonstrated that plasma levels of IL-17, TGF-β, IL-6, and IFN-γ in patients showed significant increase compared to the controls. These results are comparable to our previous study which had shown significant increase except IFN-γ in disc samples of the patients with DDD (12). IL-6 orchestrates a series of ‘downstream’ cytokine-dependent signaling pathways that, in concert with TGF-β, amplify differentiation of Th17 cells (18). The differentiation of Th17 cells requires the coordinate and specific action of the proinflammatory cytokine, IL-6, and the immunosuppressive cytokine, TGF-β (15, 19). Under inflammatory conditions and constant surveillance by regulatory mechanisms, IL-6 and TGF-β are key cytokines for the differentiation of producing IL-17 and designated Th17. IL-17 stimulates proinflammatory cytokine production, neutrophil activation and tissue inflammation. Our results have shown that changes in the levels of IL-17, TGF-β, IL-6,
IFN-γ in degenerative disc samples have been similarly reflected in the peripheral blood. However, this reflection has not been found to be as strong as it was in tissue (8).

Recently, a subset of interleukin IL-17-producing T (Th17) cells distinct from Th1 or Th2 cells has been described and shown to have a crucial role in the induction of tissue degeneration. In contrast, CD4+CD25+Foxp3+ regulatory T (T(reg)) cells protect against tissue degeneration (1, 10). Research has shown that functional disorders in CD4+ CD25+ Foxp3+ T cells or intervention in Foxp3 result in numerous conditions. It is believed that Foxp3 is the main regulator in CD25+ Treg cell development (13, 14).

In our study when the plasma of the patients were compared with controls, it was found that CD4 (Th), CD25 (IL-2R), CD4+CD25+Foxp3+ (Tregs), Foxp3+ increased. In our former study, different results were obtained when comparing the tissue samples of the patients with the controls (1). In that study it was found that CD4 (Th), CD25 (IL-2R) did not show significant changes and also CD4+CD25+Foxp3+ (Tregs), Foxp3+ increased significantly.

When CD25 separates from CD4+CD25+Tregs cells, multiorgan autoimmunity and degeneration emerges. Therefore CD25 cells have an important role in preventing self-reactivity and in maintaining immune regulation. In our study, plasma levels of CD25 in patients showed significant increase compared with the controls. On comparing the immune changes in the plasma of the patients with those in the disc samples, plasma showed significantly higher levels of CD4+CD25+Foxp3+ (Tregs) and CD4. On the other hand, other parameters were found to be significantly higher in the disc samples.

The suppressive effects of Treg Cells which have an immune response regulatory role are quite different. Additionally, they prevent chronically damaging immunopathology by inhibiting longer immune responses without preventing the stimulation of CD4. In our study, the meaningfully high levels of Treg cells and CD4 in the plasma could be an indicator that immune tolerance is developing in the peripheral circulation in patients with DDD.

CONCLUSION

An imbalance in cytokine production profiles has been demonstrated in several clinical conditions and in pathophysiological mechanism in patients with DDD. The release of proinflammatory cytokines by peripheral immune cells during inflammation may limit the disease progression. It is reasonable to suggest that the situation may affect peripheral immune tolerance in degenerative disc disease; however global evaluation of the immune system should be considered to define appropriate medical treatment.

REFERENCES


