CpG ODN ligation of TLR-9 attenuates TNF-α production in untreated active SLE patients

Jeng-Ting Tsao1,2, Chia-Li Yu3, Song-Chou Hsieh3, Tsui-Wen She3, Chia-Chen Kuo1, Shih-Chang Lin1,4

From the Division of Allergy and Immunology, Department of Internal Medicine, Cathay General Hospital, Taipei, Taiwan

1Division of Allergy and Immunology, Department of Internal Medicine, Cathay General Hospital, Taipei, Taiwan

2Division of Rheumatology and Immunology, Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

3Nursing department, Cathay General Hospital, Taipei, Taiwan

4The Laboratory of Allergy and Immunology, Cathay Medical Research Institute, and School of Medicine, Department of Medicine, Fu Jen Catholic University, Taipei, Taiwan

Objective: In the present study, we determined the immunomodulatory effect of toll-like receptor-9 (TLR-9) ligation with hypomethylated CpG oligodeoxynucleotides (CpG ODNs) on TNF-α release in untreated systemic lupus erythematosus (SLE) patients and in a cohort of sex- and age-matched healthy individuals.

Methods: Peripheral blood mononuclear cells (PBMCs) from 13 SLE patients and 8 healthy individuals were stimulated with CpG-ODNs in the presence of phorbol myristate acetate and ionomycin. RT-PCR and ELISA assays were used for analysis of TNF-α mRNA expression and protein secretion.

Results: CpG ODNs increased TLR9-mediated TNF-α protein release in supernatant of PBMC culture in SLE and healthy individuals although there were no significant differences of TNF-α mRNA expression between these two groups. The level of TNF-α protein production was significantly lower in SLE patients than in normal controls (592.5 ± 202.8 ng/mL vs. 1369.8 ± 277.3 ng/mL, p =0.033).

Conclusion: The lower TNF-α production by PBMCs after TLR-9 ligation in untreated SLE patients reflected the dysregulation of this important proinflammatory cytokine. This dysregulation may be involved in the pathogenesis of attenuated innate immunity when patients are exposed to bacteria or viruses with CpG-rich DNA.

Key words: Systemic lupus erythematosus, tumor necrosis factor-α, toll like receptor-9, CpG-ODN

Introduction

Toll-like receptors (TLRs) have been demonstrated to be a key component of the innate immune system. At least 11 TLRs have been identified in mammals, and each one recognizes unique molecular patterns associated with different classes of pathogens. TLR-9 recognizes hypomethylated CpG-rich sequences of DNA present in many bacteria and viral species [1,2]. Although it is
known that TLR-9 plays a key role in the production of pathogenic autoantibodies and in the development of clinical features of systemic lupus erythematosus (SLE), the mechanism by which it recognizes self-antigens and produces autoantibodies is unclear. Two recent studies provided contradictory findings. In one study, anti-dsDNA antibody production was impaired in TLR-9 gene-knockout lupus-prone mice [3], whereas in the other study there was no difference in production between TLR-9 gene-knockout mice and mice with an intact TLR-9 gene [4]. In an animal study on the effectiveness of TLR9, Marc et al. found that TLR9-deficient autoreactive B cells do not undergo class switching to the pathogenic immunoglobulin isotypes IgG2a and IgG2b [5]. Some studies have assessed the efficacy of using inhibitory oligodeoxynucleotides (ODNs) to block the activation of TLR-9 in lupus mice. Notably, administration of ODNs was shown to significantly reduce the severity of clinical features of SLE in one study and was associated with delayed onset of renal disease and prolonged survival in another study [6,7]. It is known that CpG ODNs are endocytosed by B cells, plasma dendritic cells (pDCs), and monocytes, and are subsequently recognized by TLR-9. TLR-9 then recruits the adaptor protein myeloid differentiation factor 88 (MyD88). This recruitment of MyD88 initiates a signaling pathway and finally induces the expression of various oncogenes and proinflammatory cytokines, such as IL-1, IL-6, and TNF-α. The link between TLR-9, CpG ODNs, and TNF-α has been further established by in vitro studies. DNA-containing immune complexes stimulate mouse bone-marrow derived DCs to produce large amounts of TNF-α, and this response was found to be blocked by inhibitors of TLR9 and to be markedly reduced in cells from TLR9-deficient mice [8]. One in vitro study showed that DNA-containing immune complexes from the sera of SLE patients can stimulate the production of TNF-α mRNA in human embryonic dendritic cells [9]. Moreover, CpG-stimulated macrophages can also secrete TNF-α and various cytokines involved in immune reactions [10,11].

TNF-α has been found to be a potent immunoregulatory cytokine and has been implicated in autoimmunity of SLE. The relationship between TNF-α and SLE is controversial. Elevated levels of TNF-α have been observed in SLE patients in a limited number of studies, while in other studies the results were inconsistent [12-15]. It is more obvious now that TNF inhibitor on a theoretical level applied in humans with autoimmune disease may lead to the presence of anti-dsDNA antibody, a serological marker of SLE, and even clinical manifestations of SLE [16]. It has been shown that anti-TNF-α antibodies decrease production of immunoglobulins by cultured peripheral blood mononuclear cells (PBMCs) in patients with SLE [17]. The results from some animal studies suggest a local pathogenic role of TNF-α in lupus glomerulonephritis rather than systemic dysregulation [18]. Details regarding the effects of TLR-9 ligation on TNF-α are still not clear. To identify the relationship between TNF-α and TLR-9 in SLE patients, this study explored the change in TNF-α expression at the mRNA and protein levels in PBMCs subjected to TLR-9 ligation with CpG ODNs in untreated SLE patients.

**Materials and Methods**

**Patients and healthy controls**

We enrolled 13 active untreated SLE patients from the Cathay General Hospital and National Taiwan University Hospital, Taipei, Taiwan during the period March 2007 to June 2008. All study subjects provided informed consent. The diagnosis of SLE was based on the classification criteria of the American College of Rheumatology [19]. Sex- and age-matched healthy volunteers were recruited as the normal controls. Demographic characteristics of blood donors are shown in Table 1. Disease activity of SLE in each group was determined using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scoring method [20].

**Preparation of blood samples and cell culture**

Approximately 20 mL of heparinized whole blood was obtained from each study individual and blood samples were subjected to analysis within 4 hours after collection. Plasma was isolated by centrifugation and stored at –80°C. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. PBMCs were plated at a density of 1 × 10^6 cells/mL in 12-well cell culture plates with RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) and 100 µg/mL streptomycin. Cells were stimulated with CpG ODN (1 µM) in the presence of PMA (100 ng/mL) and ionomycin (500 ng/mL) to promote cell-cell interaction. Culture plates were then incubated in a 5% CO2 incubator at 37°C for 24 hours. After culture, total RNA was extracted from PBMCs. Supernatants of cell cultures were collected and stored at –80°C.
Reagents for cell culture and final concentrations

The CpG-ODNs sequences used in this study were selected according to published reports and were commercially synthesized. The final concentration was 1 µM. Phorbol myristate acetate (PMA) was purchased from Sigma-Aldrich, St. Louis, MO, USA. The final concentration was 100 ng/mL. Ionomycin was purchased from Sigma-Aldrich, St. Louis, MO, USA. The final concentration was 500 ng/mL.

CpG-ODNs sequence

The sequence of CpG-ODNs was TCGTCGTTTTGTCGTT.

TNF-α primer pairs

The sequences of primers specific for TNF-α gene were 5'-TGAGCACTGAAAGCATGATCC-3' and 5'-GGTTTGCTACAACA TGGGCTA-3'. The expected product size was 335 base pairs.

Total RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR)

Total mRNA in PBMCs from SLE patients and healthy individuals was extracted using an RNA extraction kit (SuperScript™ II Rnase H – reverse transcriptase, Invitrogen) according to the manufacturer’s instructions. The target gene was TNF-α. For reverse transcription, 1 µg of total RNA was used to synthesize cDNA. Then, 1 µL of RT product was used for the subsequent PCR experiments. The amplification of GAPD mRNA was used as an internal control. PCR was programmed for optimal cycles with denaturation for 10 seconds at 95ºC, annealing for 50 seconds at 59ºC, and extension for 45 seconds at 72ºC. The DNA fragments were separated on 2% agarose gels and visualized by ethidium bromide staining. The quantity of DNA fragments was determined using the ChemiGenius2 imaging system and Gene Tools image analysis computer software (Syngene, Frederick, MD). A standard DNA sample with a known concentration was used to calculate the absolute concentration of DNA fragments in PCR products. The number of transcripts was normalized using GAPD transcript levels as an internal control, and was computed using the following formula:

\[
\text{Level of expression} = \frac{\text{Intensity of each gene}}{\text{Intensity of GAPD gene}} \times 100\%
\]

Enzyme-linked immunosorbent assay (ELISA)

Cytokines in plasma and cell culture supernatants were quantified using a “sandwich-type” ELISA technique. The matched antibody pairs, consisting of unlabelled capture antibody and biotinylated detection antibody, and recombinant protein standards for TNF-α were purchased from Bender MedSystems (Vienna, Austria). The detectable range of TNF-α is 310-20,000 ng/mL.

Statistical analysis

Non-parametric Mann-Whitney rank-sum test was used to compare differences in TNF-α at the transcript and protein levels between SLE patients and normal controls. All tests were two-tailed. A p-value <0.05 was considered significant.

Results

Demographic characteristics of study populations

There was no significant difference in male to female ratio between the SLE group and control group. The SLEDAI score for SLE patients ranged from 6 to 17. One of the SLE patients had central nervous system involvement and 11 SLE patients had lupus nephritis. All 13 SLE patients had positive antinuclear and anti-dsDNA antibodies. Demographic characteristics of active SLE patients and normal individuals were shown in Table 1.

mRNA of cytokine expression in PBMCs

To investigate the effect of microbial agents on TNF-α release from PBMCs, PBMCs obtained from active lupus patients and normal individuals were stimulated with the TLR-9 ligand CpG ODN for 24 hours.

<table>
<thead>
<tr>
<th>Normal individuals</th>
<th>Active SLE patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case numbers</td>
<td>8</td>
</tr>
<tr>
<td>Female:Male (% female)</td>
<td>6:2 (75%)</td>
</tr>
<tr>
<td>Age (years, mean ± standard error)</td>
<td>31.4 ± 3.3</td>
</tr>
<tr>
<td>Disease status</td>
<td>-</td>
</tr>
<tr>
<td>SLEDAI score</td>
<td>-</td>
</tr>
</tbody>
</table>

*Mean ± standard error
CpG ligation of TLR-9 and TNF-α in active SLE

Table 2. Cytokine mRNA levels of PBMCs activated via toll-like receptor (TLR) ligation

<table>
<thead>
<tr>
<th>cytokine receptor ligand</th>
<th>Responders (ng/mL)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR-9 (CpG ODN)</td>
<td>26.6 ± 4.4</td>
<td>0.56</td>
</tr>
<tr>
<td>Un-treated</td>
<td>35.6 ± 11.1</td>
<td>0.56</td>
</tr>
</tbody>
</table>

*Data are represented as mean ± standard error.

Dysregulation of cytokine production in PBMCs from SLE patients

TNF-α protein concentrations in the supernatant of PBMC culture after ligation of TLR-9 with CpG ODNs and in plasma of active SLE patients and normal individuals were determined by ELISA. The TNF-α protein levels in plasma did not differ significantly between the two groups (SLE: 13.8 ± 6.6 ng/mL; normal: 241.9 ± 211.2 ng/mL; p=0.56). As for CpG ODN-treated PBMCs, TNF-α RNA expression levels were 26.6 ± 4.4 ng/mL in lupus patients and 22.0 ± 7.3 ng/mL in normal individuals (p=0.56). There were no significant differences in TNF-α transcript levels between these two groups (Table 2).

Discussion

TLR-9 has been shown to play an important role in the pathogenesis of SLE. Previous studies have shown that active SLE patients have increased proportions of mature B cells, plasma cells, and monocytes that express TLR-9, and that the expansion of TLR-9-expressing B cells correlates with anti-dsDNA antibody production [19]. The relevant point is that TLR-9 exerts powerful synergistic effect in determining class-switch recombination and maturation of B cells to become autoantibody-secreting cells, along with B cell receptor ligation and T cell help. Our study investigated the effect of TLR-9 ligation with CpG ODN on proinflammatory cytokine production from PBMCs. Of many cytokines involved in pathogenesis of SLE, TNF-α has been chosen, because the level of TNF-α may reflect the level of inflammation in patients with SLE from previous studies, and that lupus patients are characterized by a higher level of TNF-α compared to a healthy population [20]. In a human SLE study, TNF-α concentration is higher in the glomerular tissue of SLE patients, while systemic TNF-α concentration is not increased [21]. Our findings, however, showed that there were no significant differences in TNF-α mRNA expression between SLE patients and normal individuals though the TNF-α protein expression was less potentiated in SLE groups after TLR-9 ligation with CpG ODN on PBMCs (Table 2 and Table 3). These findings suggested a local pathogenic effect of TNF-α rather than systemic dysregulation of this cytokine in SLE patients from baseline conditions. Furthermore, the up-regulation of TNF-α was indeed insufficient when SLE patients encountered invasion of pathogen containing CpG ODN.

The reason why PBMCs instead of a subpopulation of B cells or pDCs were used in this study was to investigate the effect of TLR-9 ligation with CpG ODN on cell-cell communication. There is growing evidence that TLR-9 expression and/or function can be upregulated in B cells [22] and other cell types, such as macrophages/monocytes, cDCs, granulocytes and even epithelial cells. These observations underscore that those various cell types, other than B cells and pDCs, potentially play TLR9-mediated protective or pathogenic roles in infection and other immunological disorders. A combination of PMA (a phorbol ester / PKC activator) and ionomycin (a calcium ionophore) were used in cell culture to stimulate T cell activation and subsequent interaction to other cell populations.

Our results showed that the production of TNF-α protein by PBMCs was increased in both SLE patients and normal controls after TLR-9 ligation with CpG ODN. However, the range of TNF-α levels was significantly lower in the SLE group. In 2006, a vitro study published by Liu et al. demonstrated that pretreatment with low-dose CpG ODN suppresses TNF-α release in response to a subsequent challenge
with high-dose CpG ODN [23]. These data provide a possible explanation for our hypothesis that, with defective clearance of apoptotic cells, SLE patients with preceding exposure to endogenously derived CpG ODN may demonstrate suppressed TNF-α release in response to subsequent challenge of CpG ODN. The resulting “tolerant state” of TLR-9 ligation with CpG ODN in SLE may be associated with TLR signal tolerance, which has been proved in a hypersensitive state of macrophages as a marked decrease in proinflammatory cytokine production following secondary challenge with microbial ligands, such as lipopolysaccharide, bacterial lipoprotein, and CpG ODN [24-26]. Our finding that TLR-9 ligation less effective in potentiating TNF-α production by PBMCs in SLE patients may also provide a mechanism to explain the impaired immune response against micro-organisms in patients with SLE. With regard to the inconsistent results between TNF-α protein and mRNA expression in our study, we believe that the diluted effect of pooled PBMCs in cell culture in vitro may have been a contributing factor. In other words, the increased level of TNF-α transcript in monocytes could have been masked by other cell populations. There has been another possibility that TNF-α is a proinflammatory cytokine whose production is usually reflected very quickly to infectious circumstances. The increase of TNF-α mRNA expression could happen earlier than 24 hours. It needs further study to investigate whether TNF-α mRNA expression will synchronized increase as protein production within two to eight hours after TLR-9 of PBMCs ligation with CpG ODN. Although there has been doubt that PMA or ionomycin can stimulate T cells to produce many cytokines, including TNF-α, the influence on results can be overlooked since the same stimulants were added in cell cultures of both studied groups.

**Conclusion**

TLR-9 ligation with CpG ODN was less effective in potentiating TNF-α production by PBMCs in SLE patients with active disease than in healthy individuals. This suppressed TNF-α production from PBMCs in SLE may be associated with TLR-9 tolerance and might partly explain the pathogenesis of disease flare and the development of further immunocompromised status when SLE patients encounter CpG ODN-containing pathogens. It remains to be seen whether TLR-9 ligation suppresses TNF-α expression in different immune cell subpopulations in order to identify the specific dysregulated immune cells in SLE patients.

**Acknowledgement**

Sponsor by grants from Cathay Medical Research Institute, Hsichih, Taipei Shien, Taiwan.

**Reference**

12. Svenungsson E, Gunnarsson I, Fei GZ, Lundberg IE,


在未經治療活動性全身性紅斑性狼瘡病患以CpG去氧核糖寡核甘酸連結類鐸蛋白受體-9會減弱腫瘤壞死因子-α的產生

曹正婷1,2 余家利2  謝松洲2  徐翠文3  郭家禎1  林世昌1

1國泰綜合醫院 過敏免疫科  
2台灣大學醫學院 附設醫院 風濕免疫科  
3國泰綜合醫院 護理部

目的：本研究探討在未經過治療且處於疾病活動期的全身性紅斑性狼瘡患者，與性別、年齡對應的健康受試者以CpG去氧核糖寡核甘酸連結類鐸蛋白受體9對於腫瘤壞死因子-α（TNF-α）的產生，是否有差異。材料與方法：13位全身性紅斑性狼瘡患者與8位健康受試者的周邊血液單核球，以CpG去氧核糖寡核甘酸和phorbol myristate acetate及ionomycin刺激，分別以反轉錄聚合酶反應和酵素免疫法分析TNF-α的mRNA表達量和蛋白的產量。

結果：全身性紅斑性狼瘡患者和健康受試者，以CpG去氧核糖寡核甘酸連結類鐸蛋白受體-9，都會增加週邊血液單核球產生TNF-α，但是在全身性紅斑性狼瘡患者產生的TNF-α的量比健康受試者低，並且達統計上顯著差異，對週邊血液單核球TNF-α核糖核酸的表達量在這兩組則未達顯著差異。

結論：未經過治療且處於疾病活動期的全身性紅斑性狼瘡患者，其周邊血液單核球在類鐸蛋白受體-9被CpG去氧核糖寡核甘酸連結後減弱了TNF-α的產生，此一結果反應出這個重要的前驅發炎激素在全身性紅斑性狼瘡患者失去正常調節的現象，而這個現象可能和全身性紅斑性狼瘡患者感染到帶有CpG序列的細菌或病毒時其內生性免疫力會減弱有關。

關鍵詞：全身性紅斑性狼瘡，腫瘤壞死因子，類鐸蛋白受體-9，CpG去氧核糖寡核甘酸