Cytokine mRNA repertoire of peripheral blood mononuclear cells in Takayasu’s arteritis

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SUMMARY

We have investigated constitutive and phytohaemagglutinin (PHA) + phorbol 12-myristate 13-acetate (PMA)-induced gene expression of tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-10, IL-12 and granulocyte macrophage colony-stimulating factor (GM-CSF) in peripheral blood mononuclear cells (PBMCs) of 10 patients with Takayasu’s arteritis (TA) and 10 healthy controls by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). The constitutive mRNA expression of TNF-α (69.0 ± 4.0% versus 27.5 ± 18.0%; P = 0.001) and IL-4 (60.0 ± 100.0%; versus 0%; P = 0.001) was significantly higher in patients than controls; that of IL-3 was comparable in both groups (38.0 ± 60.0% versus 32.0 ± 5.0%; P = 0.651) while no constitutive mRNA expression was observed for the other cytokines studied. The stimulated PBMCs of patients, as compared with the controls, had higher mRNA gene expression of TNF-α (127.0 ± 160.0% versus 54.0 ± 60.0%; P = 0.001), IFN-γ (93.0 ± 13.0% versus 57.0 ± 5.0%; P = 0.032), IL-2 (109.0 ± 13.0% versus 68.0 ± 60.0%; P = 0.015), IL-3 (60.0 ± 8.0% versus 21.2 ± 3.0%; P = 0.045) and IL-4 (68.0 ± 7.0% versus 27.0 ± 7.2%; P = 0.001) The mRNA expression of IL-10 was lower in patients than controls (35.0 ± 8.0% versus 75.0 ± 12.0%; P = 0.022). The GM-CSF mRNA was similar (102.0 ± 60.0% versus 89.0 ± 5.0%; P = 0.475) in both groups. Stimulation of cells with PHA + PMA showed no IL-12 expression but stimulation with lipopolysaccharide induced higher IL-12 mRNA in patients than controls (83.0 ± 140.0% versus 33.0 ± 4.0%; P = 0.005). Our data suggest that an inflammatory cytokine signature exists in TA with a key role for TNF-α, IL-4, IL-10 and IL-12 in different pathological processes of the disease.

Keywords IL-4 IL-10 IL-12 mRNA peripheral blood mononuclear cells Takayasu’s arteritis TNF-α

INTRODUCTION

Takayasu’s arteritis (TA) is a chronic granulomatous vasculitis characterized by stenosis, occlusion or sometimes aneurysm of large elastic arteries, mainly the aorta and its major branches including pulmonary and coronary arteries. It is an autoimmune disease and different cellular and humoral immune mechanisms are involved in its pathogenesis [1,2].

Granulomatous inflammation, vascular infiltration of mainly T cells and monocyte/macrophages [3], increased numbers of HLA-DR+ T cells in the circulation [4] and increased serum levels of interleukin (IL)-6 in TA [5] together suggest a primary involvement of T cells and monocytes/macrophages in the disease. The systemic and local production of different cytokines by these immune effector cells may have a crucial role in mediating the infiltration of circulating inflammatory cells into the vascular wall by activating vascular endothelium and in inducing histological damage through their direct cytotoxic effect on vasculature or indirectly by activating cytotoxic effector functions of recruited inflammatory cells [6-8]. Some of these cytokines, such as IL-3 and IL-4, also induce proliferation and migration of smooth muscle cells and/or fibroblasts and thus may be involved in intimal hyperplasia and fibrosis that lead eventually to stenosis or ischaemia of the diseased vessels [9,10]. However, data on cytokines in TA are very limited and the simultaneous detection of a wide repertoire of these mediators has not yet been carried out.

We thus undertook this study to investigate the mRNA expression of different cytokines including tumour necrosis factor (TNF)-α, interferon (IFN)-γ, IL-2, IL-3, IL-4, IL-10, IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in peripheral blood mononuclear cells (PBMCs) of patients with TA and healthy controls.
SUBJECTS AND METHODS

Subjects
Ten patients with TA (seven females, three males; mean age 26·8 ± 9·3 years; range: 18–42 years) and an equal number of age- and sex-matched healthy controls were enrolled in the study after obtaining their informed consent. This study was approved by the Ethics Committee of Sanjay Gandhi Post-Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, India. All patients included in the study fulfilled at least three of the classification and diagnostic criteria of the American College of Rheumatology, 1990 for TA [11] and had an angiographically proven disease. They were on a maintenance dose of prednisolone (5–10 mg/day) and azathioprine (2 mg/kg) and their disease was in remission as per criteria of the disease activity described previously [12]. The clinical features of the study are given in Table 1.

Isolation and activation of PBMCs
Fifteen ml of venous blood was obtained from each subject in a sterile glass tube containing 150 IU of preservative-free heparin (Sigma, St Louis MO, USA). PBMCs were isolated by the standard Ficoll-Hypaque (Histopaque, Sigma) density centrifugation method. Their viability as assessed by trypan blue exclusion test was always >95%. The preparation of PBMCs was >99% pure and consisted of 65–69% of lymphocytes, 30–35% of monocytes and <1% of polymorphs as determined by routine haematological staining of cytospin smears of the cells. One aliquot of 5 × 10⁶ cells was used immediately for RNA extraction and the remaining (usually ≥10 × 10⁶) cells were used for activation to detect constitutive and induced cytokine gene expression, respectively.

To induce cytokine gene transcription, the cells were stimulated with phytohaemagglutinin (PHA) + phorbol 12-myristate 13-acetate (PMA). The combination of PHA and PMA is a potent stimulus for PBMCs. PHA induces polyclonal activation of T cells via binding directly to T cell receptors or associated molecules as well as binding to carbohydrate residues in the glycoprotein of the cell membrane and PMA acts as an analogue of diacylglycerol and induces stimulation of lymphocytes and monocytes by activating the protein kinase signalling pathway [13]. Briefly, the isolated cells were suspended in complete medium consisting of RPMI-1640 supplemented with 3 mM of L-glutamine, 10 mM of HEPES buffer, 1 mM of sodium pyruvate and 10% of heat-inactivated fetal bovine serum (all from Gibco BRL, Grand Island, NY, USA). A total of 5 × 10⁶ cells/well in a final volume of 2 ml of complete medium were dispensed in a six-well tissue culture plate (Nunclon, Roskilde, Denmark) and incubated for 4 h under standard tissue culture conditions in the presence of 2 μg/ml of PHA (Sigma) plus 25 ng/ml of PMA (Sigma). After incubation cells were harvested and used for RNA extraction.

Because lipopolysaccharide (LPS) is a potent stimulator of mononuclear as well as B cells, which are the main producers of IL-12, and PMA down-regulates the production of this cytokine [14] in an additional activation protocol, approximately 5 × 10⁶ PBMCs in a final volume of 1 ml of complete medium were stimulated by incubating with 10 μg/ml of LPS (Sigma) for 12 h, as described above, to detect IL-12 gene expression in the cells [15].

RNA extraction
Total cellular RNA from unactivated and activated PBMCs was extracted by the modified guanidinium thiocyanate–phenol–chloroform method [16]. The RNA pellet obtained was dissolved in 25–50 μl of RNA storage solution (Ambion, Austin, TX, USA) and stored at −80°C until required. Each sample had approximately 5 μg of total RNA as determined by spectrophotometry.

Reverse transcriptase-polymerase chain reaction (RT-PCR)
Cytokine gene expression was detected by a single-step RT-PCR method that includes reverse transcription of mRNA into complementary DNA (cDNA) and its subsequent PCR amplification by a continuous thermal cycling programme, using the Robust RT-PCR kit (Finnzymes, OY, Finland) according to the manufacturer’s instructions. Briefly, for each RT-PCR reaction 50 ng of total RNA was mixed to a standard reaction mixture consisting of 1 × reaction buffer, 1·5 mM MgCl₂, 200 μM of each dNTP, 10 pmol each oligonucleotide primer, 5 units of avian myeloblastosis virus reverse transcriptase (AMV-RT), 2 units of DyNAzyme EXT DNA polymerase and diethyl pyrocarbonate (DEPC) (Sigma)-treated sterile water up to a final volume of 50 μl in a sterile 250 μl PCR tube (Genei, Bangalore, India). The reaction was carried out in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA) and consisted of the following steps: (a) an initial cDNA synthesis at 48°C for 45 min followed by inactivation of AMV-RT and denaturation of cDNA/RNA hybrid at 94°C for 2 min; (b) PCR amplification by 35 sequential cycles of denaturation (94°C for 45 s), annealing (at cytokine specific temperature for 45 s) and primer extension (72°C for 60 s); and (c) a final extension at 72°C for 7 min followed by cooling to 12°C as end of the reaction.

<table>
<thead>
<tr>
<th>TA patient</th>
<th>Age/sex</th>
<th>Type of disease</th>
<th>ESR (mm/h)</th>
<th>CRP (mg/dl)</th>
<th>Treatment</th>
<th>Disease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>23/F</td>
<td>III</td>
<td>38</td>
<td>&lt;0.5</td>
<td>Prednisolone (5 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
<tr>
<td>2.</td>
<td>40/F</td>
<td>III</td>
<td>22</td>
<td>0·698</td>
<td>Prednisolone (5 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
<tr>
<td>3.</td>
<td>20/M</td>
<td>III</td>
<td>12</td>
<td>&lt;0.5</td>
<td>Prednisolone (10 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
<tr>
<td>4.</td>
<td>42/F</td>
<td>I</td>
<td>20</td>
<td>&lt;0.5</td>
<td>Prednisolone (5 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
<tr>
<td>5.</td>
<td>20/F</td>
<td>III</td>
<td>11</td>
<td>&lt;0.5</td>
<td>Prednisolone (10 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
<tr>
<td>6.</td>
<td>18/F</td>
<td>III</td>
<td>22</td>
<td>0·25</td>
<td>Prednisolone (10 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
<tr>
<td>7.</td>
<td>30/F</td>
<td>III</td>
<td>15</td>
<td>0·65</td>
<td>Prednisolone (10 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
<tr>
<td>8.</td>
<td>22/M</td>
<td>III</td>
<td>25</td>
<td>&lt;0.5</td>
<td>Prednisolone (5 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
<tr>
<td>9.</td>
<td>35/M</td>
<td>III</td>
<td>18</td>
<td>&lt;0.5</td>
<td>Prednisolone (5 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
<tr>
<td>10.</td>
<td>18/F</td>
<td>III</td>
<td>25</td>
<td>&lt;0.5</td>
<td>Prednisolone (10 mg/day), azathioprine (2 mg/kg)</td>
<td>Inactive</td>
</tr>
</tbody>
</table>
Amplification of RNA for the housekeeping gene β-actin was used as the internal quality control. The sequences of the primers (Oligo, Hilden, Germany), optimal PCR annealing temperatures (Ta) and size of the amplified PCR product are given in Table 2. A negative control consisting of a reaction mixture with no AMV-RT was included in each set of experiments to rule out amplification of contaminating genomic DNA, if any. A positive control for each cytokine consisted of RNA derived from PHA + PMA or LPS-stimulated PBMCs of a healthy individual.

Statistical analysis
The intensity of a given cytokine and β-actin density in each sample was compared using the Mann–Whitney U-test. Results were expressed as mean ± standard deviation (s.d.). A P-value <0.05 was considered to be statistically significant.

RESULTS
Constitutive expression of cytokine genes
There was a constitutive gene expression of TNF-α, IL-3 and IL-4 but not of the other cytokines studied. The mRNA expression of TNF-α was significantly higher in patients compared to controls (69.0 ± 4.0% versus 27.5 ± 18.0%; P = 0.001), that of IL-4 was present only in patients but not in controls (60.0 ± 10.0% versus 0%; P = 0.001) while that of IL-3 was comparable in both groups (38.0 ± 6.0% versus 32.0 ± 5.0%; P = 0.651) (Fig. 1a).

Induced expression of cytokine genes
There was significantly increased mRNA gene expression of TNF-α (127.0 ± 16.0% versus 54.0 ± 6.0%; P = 0.001), IFN-γ (93.0 ± 13.0% versus 57.0 ± 5.0%; P = 0.032), IL-2 (109.0 ± 13.0% versus 68.0 ± 6.0%; P = 0.015), IL-3 (60.0 ± 8.0% versus 21.2 ± 3.0%; P = 0.045) and IL-4 (68.0 ± 7.0% versus 27.0 ± 7.2%; P = 0.01) in the PHA + PMA-stimulated PBMCs of the patients compared to controls. The induced expression of IL-10 in the patients was significantly lower than controls (35.0 ± 8.0% versus 75.0 ± 12.0%; P = 0.022) and that of GM-CSF was comparable in both groups (102.0 ± 6.0% versus 89.0 ± 5.0%; P = 0.475)

No mRNA expression of IL-12 was detected in PHA + PMA-stimulated PBMCs. However, stimulation of cells with LPS induced a significantly increased expression of cytokine mRNA in patients compared to controls (83.0 ± 14.0% versus 33.0 ± 4.0%; P = 0.005) (Fig. 1b).

DISCUSSION
The salient findings of our study are the presence of an increased constitutive mRNA expression of TNF-α and IL-4 and a decrease in the induced expression of IL-10 mRNA in patients with TA. In addition, the induced mRNA expression of TNF-α, IFN-γ, IL-2, IL-3, IL-4 and IL-12 was significantly higher in patients compared to controls while that of GM-CSF was comparable in both groups. To the best of our knowledge this is the first study detecting simultaneously such a large repertoire of cytokines on mRNA or protein level in TA or other vasculitides.

Some of these cytokines, including TNF-α, IFN-γ, IL-2 and IL-4, have been studied previously in TA but the data available are very limited. The gene transcripts of TNF-α and IFN-γ using RT-PCR have been demonstrated in the aortic tissues of TA patients with TA by Seko et al. [18] and support our finding in the present study of increased mRNA expression of these cytokines. The increased constitutive and induced mRNA expression of TNF-α in TA PBMCs reported here confirms our recent data.
of increased frequency of TNF-α-positive T cells in patients with active TA and increased plasma levels of the cytokine in patients with active and inactive disease (manuscript submitted). The enhanced gene expression and spontaneous production of TNF-α by PBMCs [19] as well as elevated serum levels [20,21] and expression of the cytokine protein in mononuclear cells infiltrating the arterial wall in other vasculitides [22] corroborates well with our findings. In contradiction to our previous observation of increased frequency of TNF-α-positive T cells and normal serum levels of the cytokine (manuscript submitted), in this study we found an increased induced expression of TNF-α mRNA in the patients. An important reason for this difference between our two studies could be the expression of TNF-α by mononuclear cell populations other than T cells, such as natural killer (NK) cells and monocyte/macrophages [23]. Similar to our observation, an increased IFN-γ mRNA expression has been reported in the lesions of giant cell arteritis (GCA) [24] and in the lesions as well as PBMCs of patients with Wegener’s granulomatosis (WG) [25].

The data available on IL-2 in TA is contradictory. Using a bioassay, Alcocer-Varela et al. [26] have shown a decrease in production of IL-2 by PHA-stimulated T cells in active TA. However, Sharma et al. [27], using a similar method, reported an increased production of IL-2 by T cells in the disease. Similarly, the increased IL-2 gene expression TA PBMCs reported here does not corroborate our previous data of a lower frequency of IL-2-producing T cells and a decrease in plasma levels of the cytokine in active TA (manuscript submitted). In the present study all the patients were on immunosuppressive therapy and were in remission; however, in the previous study, although numbers of IL-2-producing T cells and plasma levels of the cytokine were low in active TA, inactive patients had IL-2-positive T cells and plasma levels of the cytokine comparable to controls. The increased IL-2 mRNA expression in patients with inactive disease reported here might be due to differences in stimulation protocol and/or sensitivity of the techniques used.

There has been only a single previous study on IL-4 in TA by Sharma et al. [27]. Using a bioassay, these authors have shown normal production of IL-4 by PHA-stimulated purified T cells of patients with TA. Our results, including a constitutive mRNA expression of IL-4 in patients but not in controls and its increased expression in PHA + PMA-stimulated TA PBMC compared to controls, do not support this study. An important reason for the failure to detect increased T cell production of IL-4 by Sharma et al. [27] may be the low sensitivity of the bioassay compared to RT-PCR and/or loss of IL-4-producing T cell subsets during the T cell purification procedure. Previous reports of different vasculopathies, including circulating levels of IL-4 in Kawasaki disease [28], IL-4 mRNA expression by peripheral blood T cells as well as by T cell clones derived from diseased tissue in WG [25] and the increased expression of the cytokine mRNA by PBMCs as well as plasma levels of the cytokine in scleroderma, a microvascular fibrotic disease [29], are consistent with our findings of IL-4 in TA.

Other cytokines, including IL-3, IL-10, IL-12 and GM-CSF, either at mRNA or protein levels have not been studied previously in TA. We have observed increased expression of IL-3 mRNA occurring only after induction in the patients. Among inflammatory vasculopathies, increased IL-3 gene expression has been demonstrated previously in T cells infiltrating the atherosclerotic lesions [7] and our present study constitutes the first report suggesting the role of IL-3 in a vasculitic entity. We also studied GM-CSF, but its expression was not different to controls. The IL-10 mRNA was not detected under constitutive conditions as well as PBMCs of patients with giant cell arteritis (GCA) [24] and in the lesions as well as PBMCs of patients with Wegener’s granulomatosis (WG) [25].

Table 2. Oligonucleotide primers and related information

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence:</th>
<th>Accession no.</th>
<th>Ta° (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Forward: 5′-3′ (bp numbering)</td>
<td>M10988</td>
<td>66</td>
<td>444</td>
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<tr>
<td>IFN-γ</td>
<td>Reverse: 5′-3′ (bp numbering)</td>
<td>AY255837</td>
<td>55</td>
<td>321</td>
</tr>
<tr>
<td>IL-2</td>
<td>CATCAGAATGTCCTTGCTTC (230–250)</td>
<td>BC066254</td>
<td>55</td>
<td>457</td>
</tr>
<tr>
<td>IL-3</td>
<td>TCCAAGACCTGCCGCTGCC (40–60)</td>
<td>BC066276</td>
<td>60</td>
<td>211</td>
</tr>
<tr>
<td>IL-4</td>
<td>GCCTGTGTCTCCGGCTAGCA (93–113)</td>
<td>BC067515</td>
<td>58</td>
<td>300</td>
</tr>
<tr>
<td>IL-10</td>
<td>ACAGCTCACACTGCTCGT (64–83)</td>
<td>NM000572</td>
<td>60</td>
<td>327</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>AGTGTCCTAAAGAAGCTGAGGG (505–524)</td>
<td>NM002187</td>
<td>60</td>
<td>363</td>
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<tr>
<td>GM-CSF</td>
<td>AACGCATGATGCTCAGGAG (849–867)</td>
<td>M10663</td>
<td>60</td>
<td>390</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GACATCTCGTCAATCATTGTCTC (1148–1169)</td>
<td>NM001101</td>
<td>62</td>
<td>311</td>
</tr>
</tbody>
</table>

*Ta° = annealing temperature of primers.
compared to healthy controls and a reciprocal relationship exists between IL-10 and IL-4 mRNA expression in these patients [25]. This study in WG suggests that the low IL-10 mRNA expression observed by us in TA could be due to an increased constitutive and induced transcription of IL-4 in the patients.

We did not detect IL-12 mRNA in PHA + PMA-stimulated PBMCs in TA, but LPS stimulation of the cells induced significantly increased mRNA expression in patients compared to controls. Increased expression of IL-12 in arterial lesions in GCA [24] and increased intracellular and extracellular production of the cytokine by monocytes in WG [30,31] support our findings of increased IL-12 in TA. The elevated mRNA levels of different inflammatory cytokines observed in the present study further support increased transcription of IL-12 in the disease. We did not observe an increased constitutive mRNA expression of most of the cytokines, including IL-12, because all our patients were under immunosuppressive therapy and their disease was in remission. It is probable that, like TNF-α and IL-4, more cytokines may have a constitutive expression in untreated patients with active TA.

According to the classical Th1 : Th2 paradigm [32], most cytokines, such as TNF-α, IFN-γ, IL-2, IL-3 and IL-12 that have been observed to have increased mRNA expression in the present study, are Th1-type. IL-4, which is viewed generally as a Th2 cytokine, has also been shown recently to promote the production of a Th1 cytokine such as IFN-γ in the presence of IL-2 and IL-12 and induce cellular immunity [33]. Thus increased expression of all these cytokines and reduced expression of IL-10, which is a major Th2 cytokine, together suggest that a Th1-type cytokine profile exists in patients with TA.

The presence of constitutive transcripts of TNF-α and IL-4 in TA PBMCs is one of the important findings of this study. TNF-α, along with IFN-γ, is reported to have multiple activating and cytotoxic effects on vasculature. They activate endothelial cells to express adhesion molecules and chemokines that direct circulating leucocytes into the vessel wall. In addition, local production of these cytokines by the infiltrating cells are potent mediators of granulomatous inflammation and may cause vascular damage either directly or by priming the vascular tissues for T cell-mediated injury [34–36]. Different pathological findings in TA, including granulomatous inflammation, increased expression of intercellular adhesion molecule-1 and MHC-I and MHC-II molecules in the lesion, validate the role of these cytokines in the disease [2]. Furthermore, cytokines such as TNF-α, IFN-γ, IL-4 and IL-12 also promote humoral immunity [37] and together are responsible for the generation of different autoantibodies observed in TA [2,12]. Although IL-4 is considered generally to be an anti-inflammatory cytokine, recent reports suggest that it may be an important inflammatory cytokine in vasculitides. It synergizes with TNF-α in inducing expression of vascular cell adhesion molecule-1 on endothelial cells and causes apoptosis through the caspase-3-dependent pathway [38] and induces Fas-ligand-mediated cytotoxic activity of T cells [39]. IL-4 is also a potent proinflammatory cytokine [40] and may have a role in vessel occlusion, which occurs during the chronic stage of the disease.

Another important finding of our study is the expression of low IL-10 and high IL-12 mRNA in stimulated PBMCs of the patients and may have important implications in TA. IL-10 is a potent anti-inflammatory cytokine that maintains peripheral tolerance by reducing antigen presentation, inhibiting cytokine production by T cells and monocytes/macrophages and executing apoptosis of antigen-activated T cells [41]. However, IL-12 promotes inflammation by inducing inflammatory cytokine production by T cells and monocyte/macrophages [42]. Thus, low IL-10 and high IL-12 mRNA expression in the patients may constitute a crucial factor responsible for the persistence of circulating activated T cells and chronic inflammation in TA.

In conclusion, we have demonstrated an increased constitutive expression of TNF-α and IL-4 mRNA as well as increased induced expression of TNF-α, IFN-γ, IL-2, IL-3, IL-4 and IL-12 mRNA but an impaired induction of IL-10 mRNA in patients with TA, suggesting an important role of these cytokines in different pathological processes and immune dysfunction in the disease. Further studies focusing on trigger(s) that induce such a cytokine profile and molecular characterization of the cells producing TNF-α, IL-4 and IL-10 may provide new insights into the immunopathogenesis of TA and lead to the development of specific biological therapy for the disease.

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