Role of IL-22– and TNF-α–Producing Th22 Cells in Uveitis Patients with Behçet's Disease

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Role of IL-22– and TNF-α–Producing Th22 Cells in Uveitis Patients with Behçet’s Disease

Sunao Sugita,*† Yuko Kawazoe,* Ayano Imai,* Tatsushi Kawaguchi,* Shintaro Horie,* Hiroshi Keino,‡ Masayo Takahashi,† and Manabu Mochizuki*

Behçet’s disease is a systemic inflammatory disorder with recurrent episodes of oral ulceration, skin lesions, genital ulceration, and intraocular inflammation (uveitis). The intraocular inflammation is strictly associated with Th effector cells. IL-22 is a member of the IL-10 cytokine family that is involved in inflammatory processes. Recently, Th22 cells were identified as a T cell population that produces IL-22 and TNF-α and are distinct from Th1, Th2, and Th17 cells. In this study, we established Th22-type T cell clones from ocular samples taken from Behçet’s disease patients with active uveitis. These clones produced large amounts of IL-22 and TNF-α but not the Th1 cytokine IFN-γ and the Th17 cytokine IL-17. CD4+ T cells from the peripheral blood of Behçet’s disease patients differentiated into Th22 cells in the presence of IL-6 and TNF-α in vitro. The polarized Th22 cell lines produced large amounts of IL-22, and the polarized Th1 and Th17 cells also produced IL-22. In the presence of anti–TNF-α- and anti–IL-6–blocking Abs, Behçet’s disease Th22-type T cells failed to produce IL-22. In addition, infliximab-pretreated Th22 cells and Th22-type ocular T cells produced less IL-22 and TNF-α. Moreover, IL-22–producing T cells were isolated from mice with experimental autoimmune uveitis, an animal model of Behçet’s disease, and the intraocular T cells from uveitis models produced large amounts of IL-22 in the presence of retinal Ags. Our results suggest that inflammatory cytokines IL-22 and TNF-α may play a key role in the ocular immune response in Behçet’s disease. The Journal of Immunology, 2013, 190: 000–000.

Inflammatory cell infiltration in the eye and secretion of inflammatory cytokines lead to intraocular inflammation that can ultimately cause blindness. During inflammatory conditions, immune tolerance in the eye is not maintained, and inflammatory cytokine-secreting immune cells infiltrate the eye. Inflammatory cytokines are expressed in inflamed eyes and play a significant role in the pathological immune response.

Behçet’s disease, an ocular inflammatory disease, is a serious sight-threatening clinical entity of uveitis that can be accompanied by recurrent oral aphthous ulcers, genital ulcers, and skin lesions. Previous studies suggested that Behçet’s disease is predominated by a Th1 and Th17 immune response (1–6). Increased levels of Th1-associated cytokines, such as IFN-γ, IL-12, and TNF-α, have been found in patients with Behçet’s disease (1, 2). Active Behçet’s disease was characterized by increased levels of IL-17 compared with the disease in remission or healthy controls (3–6). Moreover, genetic surveys, including genome-wide association studies, identified IL23R-IL12RB2 and IL10 as Behçet’s disease susceptibility loci (7, 8). These recent reports suggest that Th1/Th17-type immune responses play a critical role in Behçet’s disease. Therefore, Th1 and Th17 cells should be instrumental in the pathogenesis of Behçet’s disease and uveitis.

Increased levels of IL-22 gene expression were found in patients with autoimmune noninfectious uveitis by gene analysis (9). Th22 cells are a subset of CD4+ effector T cells that primarily secrete IL-22 and TNF-α. Similar to Th17 cells, Th22 cells express IL-22, CCR4, CCR6, and CCR10. In addition, they do not express IL-17 (Th17 marker), IL-4 (Th2 marker), or IFN-γ (Th1 marker) (10, 11). Thus, these characteristics distinguish Th22 cells as a novel Th cell lineage that is distinct from the Th17, Th2, and Th1 subtypes. The expansion of Th22 cells seems to be regulated by the aryl hydrocarbon receptor transcription factor11, although additional intracellular molecules involved in Th22 differentiation are still being investigated. Activated naïve CD4+ T cells differentiate into Th22 cells in the presence of IL-6 and TNF-α. Thus, the proinflammatory cytokines TNF-α and IL-22 may play a key role in the Th22 immune response.

However, it is unknown whether Th22 cells affect intraocular inflammation in uveitis, and there have been no reports that IL-22 and Th22 cells are involved in the pathogenesis of Behçet’s disease. Therefore, we conducted experiments to determine whether Th22 cells and the cytokines that they produce are involved in the immunopathogenesis of inflammation in the eye.

Materials and Methods

Subjects

Subjects were uveitis patients with Behçet’s disease at Tokyo Medical and Dental University Hospital between 2010 and 2012. The research followed the tenets of the Declaration of Helsinki, and the study was approved by the Institutional Ethics Committee of Tokyo Medical and Dental University.

After informed consent was obtained, samples of aqueous humor were collected from patients with uveitis associated with Behçet’s disease. At the time of sampling, the patients had active intraocular inflammation, but they were not being treated with systemic therapies, such as corticosteroids.
cyclosporine, and infliximab. We also collected aqueous humor samples from patients with active uveitis caused by Vogt-Koyanagi-Harada (VKH) disease and patients with HLA-B27* acute anterior uveitis (AAU). PBMCs were also obtained from the Behçet’s disease patients and healthy donors. The healthy control subjects had no clinical history of uveitis or systemic diseases.

**Establishment of T cell clones and T cell lines**

T cell clones (TCCs) were established by the limiting dilution method, as previously described (13, 14). The cells were all CD4+ T cells obtained from patients with uveitis who had Behçet’s disease (B2-3, B2-25, B2-16, B2-31, B2-48, B2-50, B2-6, B2-6-5), VKH disease (VKH37-1, VKH37-4), or HLA-B27* AAU (AAU4-3, AAU4-6). PBMCs from patients with Behçet’s disease or healthy donors were used to establish CD4+ T cell lines by culturing with anti-human CD3 Ab (2 µg/ml), anti-human CD28 Ab (2 µg/ml), and recombinant human IL-2 (100 U/ml; all from BD Pharmingen) for 5 d. Freshly purified T cells were enriched for CD4+ cells using T cell isolation kits (MACS; Miltenyi Biotec, >94% CD4+) and then used for in vitro assays or flow cytometric analysis.

**Induction of Th22/Tc17 cell lines**

For the induction of human Th22 cells, purified CD4+ T cells from Behçet’s disease patients or healthy donors were cocultured with anti-human CD3 Ab (2 µg/ml), anti-human CD28 Ab (2 µg/ml), anti-human IFN-γ Ab (5 µg/ml; R&D Systems), anti-human IL-4 Ab (5 µg/ml; R&D Systems), and recombinant human proteins, such as TNF-α (50 ng/ml; R&D Systems), IL-6 (20 ng/ml; R&D Systems), and IL-2 (100 U/ml; from BD Pharmingen) for 5 d. Freshly purified T cells were enriched for CD4+ cells using T cell isolation kits (MACS; Miltenyi Biotec, >94% CD4+) and then used for in vitro assays or flow cytometric analysis.

**Flow cytometry**

Flow cytometric analysis of Th22 cell lines and Th22-type TCCs derived from Behçet’s disease patients was performed using PE-labeled anti-human IL-22 mAbs (R&D Systems). T cells were cultured with Golgi-Plug (BD Pharmingen), monensin (0.1 µg/ml; Sigma Chemical), and PMA (40 ng/ml; Merck Chemical, Darmstadt, Germany) for 5 h before intracellular staining. After permeabilization, Th22 cells were stained with PE-labeled anti-human IL-22 Abs and FITC-labeled anti-human CD4 Abs. PE-conjugated mouse IgG (R&D Systems) was used as the isotype control. Cells (1 × 10^6) were stained for 30 min at room temperature in the dark. These T cells, Th22 cells, and infliximab-exposed Th22 cells were also stained with PE-labeled anti-human IL-22 Abs and FITC-labeled anti-human CD4 Abs (Cells, 1 × 10^6) were stained for 30 min at room temperature in the dark.

To stain CCR10 molecules, we prepared fresh CD4+ T cells from a patient with active Behçet’s disease, fresh CD4+ T cells from a healthy donor, Behçet’s disease TCCs (Th22-type), Th22 cell lines from Behçet’s disease patients, and Th22 cell lines from a healthy donor. These T cells were stained with PE-labeled anti-human CCR10 Abs (R&D Systems) and FITC-labeled anti-human CD4 Abs. PE-conjugated rat IgG2a was used as the isotype control. Cells (1 × 10^6) were stained for 30 min at 4°C in the dark. To perform three-color staining for CCR10, TNF-α, and IL-22 in CD4+ TCCs from Behçet’s disease patients, we used PE-labeled anti-human IL-22 Ab, FITC-labeled anti-human TNF-α Ab (BioLegend), and allophycocyanin-labeled anti-human CCR10 Ab (BioLegend).

**Induction of experimental autoimmune uveitis**

Mice were immunized s.c. in the neck region with 200 µg IRBP peptide (IRBP1-20; GPTHLQPSVLDMKA) for 4 h. As a control, T cells were prepared in the absence of peptide. Spleen T cells were also collected from EAU mice and a normal nonimmunized mouse for the assay. ELISA was used to measure the IL-22 cytokine concentration in the supernatants of the T cell cultures.

To show that Th22 cells are involved in the pathogenesis of uveitis, we used blocking reagents for IL-22 Ab and TNF-α Ab (both from BioLegend) in the murine uveitis model. We administered these blocking Abs during different phases of EAU in mice. C57BL/6 mice were immunized with IRBP peptides on day 0 and were given 100 µl anti–IL-22 and/or anti–TNF-α Abs on days 0, 1, 3, 5, and 7. Each Ab (5 µg/mouse) was injected i.v. Then, funduscopic examination (clinical score) was performed 14 and 21 d after immunization. Pathological examination (histological score) was also performed after 21 d.

**Assay for neutralizing Ab**

T cells from Behçet’s disease patients (Th22 cell lines and Th22-type TCCs) were cocultured with IL-2 and anti-human CD3/CD28 Abs in the presence of infliximab (10 µg/ml) for 48 h. In other experiments, anti-human TNF-α Abs (10 µg/ml) and anti-human IL-6 mAbs (10 µg/ml; both from R&D Systems) were used.

**Quantitative RT-PCR**

Total RNA was isolated from fresh CD4+ T cells from patients with active uveitis and Behçet’s disease and a healthy donor. Total RNA was also collected from T cells after coculture with supernatants from Th22 cells or Th1 cells derived from Behçet’s disease patients or a healthy donor for 48 h. After cdna synthesis, expression of CCL2 (MCP-1) and CCL5 (RANTES) in triplicate samples was analyzed by quantitative RT-PCR (qRT-PCR) with a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using Roche’s qPCR MasterMix and highly specific Universal ProbeLibrary assays (Roche Diagnostics). The following primers were used: CCL2, 5'-AGTCTCTGGCGCCCTTTCT-3' (forward primer) and 5'-GTGACTG- GGCCATTTATTG-3' (reverse primer), Universal Probe #40; CCL5, 5'-TACACACGTTGGCAAGTCTC-3' (forward primer) and 5'-TCTCT- TTGCCATCTTGCAGC-3' (reverse primer), Universal Probe #3; and GAPDH, 5'-AGCCACATGCTAGCAC-3' (forward primer) and 5'-GCCAATCAGCAAAATC-3' (reverse primer), Universal Probe #60.

The qRT-PCR was performed by denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 1 s. Relative mRNA expression was calculated with Relative Quantification Software (Roche Diagnostics) using an efficiency-corrected algorithm with standard curves and reference gene normalization against GAPDH (the housekeeping gene).

**Statistical evaluation**

All experiments were repeated at least twice with similar results. All statistical analyses were conducted using the Student t test. Results of the EAU experiments were analyzed using the Mann–Whitney U test. Values were considered statistically significant if p < 0.05.
Results
Production of Th22-related cytokines and receptors by T cells from uveitis patients with Behçet’s disease

First, we examined whether CD4+ T cells from Behçet’s disease patients express IL-22. We established TCCs from the aqueous humor of patients with active uveitis and Behçet’s disease. As controls, TCCs from uveitis patients with VKH disease and HLA-B27–associated AAU were also established. The results for cytokine production (Th22-type cytokines IL-22 and TNF-α, Th1-type cytokine IFN-γ, and Th17-type cytokine IL-17) are summarized in Table I. We found that Th22-type TCCs established from ocular samples of patients with active uveitis, including patients with Behçet’s disease, produced large amounts of IL-22 and TNF-α but not IFN-γ or IL-17 (Table I). In contrast, Th1-type TCCs produced large amounts of IFN-γ and TNF-α but not IL-22 or IL-17. We also established T cell lines from the PBMCs of patients with active uveitis and Behçet’s disease. The conventional CD4+ T cell lines produced large amounts of IL-22, as well as other inflammatory cytokines, compared with the control T cell lines from a healthy donor (Table I). These results suggest that T cells established from the inflamed eyes of patients with Behçet’s disease may include Th22-type cells.

As the next step, we performed intracellular staining of T cells by flow cytometric analysis to confirm whether CD4+ T cells from Behçet’s disease patients express Th22-associated cytokines. Fresh CD4+ T cells obtained from a patient with active uveitis and Behçet’s disease expressed large amounts of IL-22 and TNF-α (Fig. 1A). In contrast, fresh T cells from a healthy control expressed lower levels of these cytokines (Fig. 1A). We also evaluated CD4+ T cell lines from the PBMCs of patients with active uveitis and Behçet’s disease. The conventional CD4+ T cell lines produced large amounts of IL-22, as well as other inflammatory cytokines, compared with the control T cell lines from a healthy donor (Table I). These results suggest that T cells established from the inflamed eyes of patients with Behçet’s disease may include Th22-type cells.

We next examined the frequency of Th22 cell clones in aqueous humor–infiltrating cells from eyes with Behçet’s uveitis. As a control, we also examined the aqueous humor–infiltrating cells from VKH disease uveitis patients and PBMCs from healthy donors. The data are shown as the percentage of TCCs from each Behçet’s disease patient (Fig. 1E). Intraocular Th22-type TCCs (CD4+IL-22+TNF-α+IFN-γ−IL-17− cells) were significantly increased in Behçet’s disease compared with VKH disease and healthy donor PBMCs (Fig. 1E). These results suggest the relative predominance of IL-22 production among TCCs from the Behçet’s uveitis anterior chamber compared with other uveitic diseases or a normal control.

Induction of IL-22–producing T cells from uveitis patients with Behçet’s disease

Th22 cells are IL-22–producing CD4+ T cells that are a unique subset of Th cells that develop along a pathway distinct from the Th1-, Th2-, and Th17-differentiation pathways (10, 11). We confirmed that CD4+ T cells from Behçet’s disease patients are converted into Th22 cells after the addition of Th22-differentiation factors. For the assay, we established polarized Th22-type cells by culturing purified CD4+ T cells in the presence of anti-CD3/CD28 Abs, anti–IFN-γ Ab, anti–IL-4 Ab, rIL-6, and rTNF-α. We collected CD4+ T cells from four Behçet’s disease uveitis patients and four healthy controls. Compared with polarized Th22 cells from healthy donors, Th22 cells from Behçet’s disease patients produced large amounts of IL-22, as determined by ELISA (Fig. 2A). Similarly, significant CCR10 expression by Behçet’s disease Th22 cells was shown by flow cytometric analysis (CCR10/CD4+ double-positive cells, 50% and 71%), whereas the expression by control Th22 cells was much lower (5%+, Fig. 2B). These results suggest that IL-22 and Th22 cells may play a role in Behçet’s disease.

Ability of anti–TNF-α mAb to suppress polarized Th22 cells from uveitis patients with Behçet’s disease

A new anti–TNF-α mAb, infliximab, greatly suppresses ocular inflammation in uveitis patients with Behçet’s disease (17–24). We next examined whether infliximab-treated Th22 cells from Behçet’s disease patients express Th22-associated cytokines and receptors. As controls, TCCs from uveitis patients with VKH disease and HLA-B27–associated AAU were also established and used for the assay. Supernatants were measured 48 h after anti-CD3/CD28 stimulation.

Table I. Cytokine production by TCCs and T cell lines from Behçet’s disease

<table>
<thead>
<tr>
<th>Clone/Cell Lines</th>
<th>Source</th>
<th>IL-22</th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>IL-17</th>
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<tr>
<td><strong>Th22-type TCC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2-25</td>
<td>Behçet’s disease</td>
<td>528</td>
<td>794</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>B25-48</td>
<td>Behçet’s disease</td>
<td>301</td>
<td>612</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>B25-50</td>
<td>Behçet’s disease</td>
<td>1,649</td>
<td>868</td>
<td>15</td>
<td>&lt;10</td>
</tr>
<tr>
<td>B26-2</td>
<td>Behçet’s disease</td>
<td>773</td>
<td>865</td>
<td>125</td>
<td>&lt;10</td>
</tr>
<tr>
<td>B26-5</td>
<td>Behçet’s disease</td>
<td>2,306</td>
<td>1,436</td>
<td>297</td>
<td>&lt;10</td>
</tr>
<tr>
<td>VKH37-4</td>
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<td>227</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<tr>
<td>AAU4-6</td>
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<td>293</td>
<td>499</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td><strong>Th1-type TCC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2-3</td>
<td>Behçet’s disease</td>
<td>156</td>
<td>657</td>
<td>2,008</td>
<td>&lt;10</td>
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<td>B25-16</td>
<td>Behçet’s disease</td>
<td>75</td>
<td>880</td>
<td>1,887</td>
<td>&lt;10</td>
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<tr>
<td>B25-31</td>
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<td>&lt;10</td>
<td>929</td>
<td>1,580</td>
<td>&lt;10</td>
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<tr>
<td>VKH37-1</td>
<td>VKH</td>
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<td>447</td>
<td>991</td>
<td>&lt;10</td>
</tr>
<tr>
<td>AAU4-3</td>
<td>AAU</td>
<td>44</td>
<td>400</td>
<td>657</td>
<td>&lt;10</td>
</tr>
<tr>
<td><strong>T cell lines</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cell line-1</td>
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<td>14,747</td>
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<tr>
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<td>1,095</td>
<td>10,168</td>
<td>335</td>
</tr>
<tr>
<td>T cell line-3</td>
<td>Healthy donor</td>
<td>359</td>
<td>20</td>
<td>843</td>
<td>&lt;10</td>
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</tbody>
</table>

We established TCCs from aqueous humor of patients with active uveitis and Behçet’s disease. As controls, TCCs from uveitis patients with VKH disease and HLA-B27–associated AAU were also established and used for the assay. Supernatants were measured 48 h after anti-CD3/CD28 stimulation.
FIGURE 1. Expression of Th22-associated cytokines, IL-22 and TNF-α, in T cells from uveitis patients with Behçet’s disease. (A) By flow cytometry, fresh CD4 T cells from a patient with active uveitis and Behçet’s disease (BD: left panels) and a healthy donor (HD: right panels) were stained with anti–IL-22 or anti–TNF-α Abs and anti-CD4 Abs after permeabilization. The percentages of cells that were double-positive for IL-22 or TNF-α/CD4 are shown. (B) Fresh CD4 T cells from a patient with active uveitis and Behçet’s disease (upper panel) or a healthy donor (lower panel) were stained with anti-CCR10 Abs and anti-CD4 Abs. (C) Th22-type TCCs from Behçet’s disease (B25-48 and B25-50) were also stained with anti-CCR10 Abs and anti-CD4 Abs. The percentages of cells that were double positive for CCR10/CD4 are shown. (D) Th22-type TCCs from Behçet’s disease (B26-2) were also stained with anti–IL-22, anti–TNF-α, and anti-CCR10 Abs. (E) Frequency of Th22 cell clones in aqueous humor–infiltrating cells from Behçet’s disease uveitis eyes (n = 3). As a control, aqueous humor–infiltrating cells from VKH uveitis patients (n = 3) and PBMCs from healthy donors (HD; n = 3) were used. The horizontal lines in the graph indicate the mean percentage of Th22 cells in the establishment of T cell clones. *p < 0.05 compared with healthy donor controls.
Behçet’s disease patients can suppress the production of IL-22 cytokines in vitro. We established Th22 cell lines as described above. Th22-type cells from a Behçet’s disease patient and a healthy donor significantly suppressed IL-22 production after exposure to infliximab, particularly the Behçet’s disease Th22 cells (Fig. 3A). Similarly, Behçet’s disease Th22 cells produced large amounts of IL-22 but did not produce large amounts of IL-22 after exposure to infliximab, as determined by flow cytometric analysis (Fig. 3B, upper panels).

We also confirmed whether infliximab-treated intraocular TCCs from Behçet’s disease patients can suppress the production of IL-22 cytokines in vitro. For this assay, we used Th22-type Behçet’s TCCs: B2-25 and B25-48. Th22-type Behçet’s disease TCCs highly expressed IL-22 (IL-22/CD4 double-positive cells = 61%), whereas the expression of infliximab-treated TCC was poor (17%+, Fig. 3B, lower panels), as assessed by flow cytometry.

Next, we examined whether intraocular T cells from EAU mice can produce IL-22 in the presence of IRBP retinal Ags. T cells from EAU mice, particularly intraocular cells, produced large amounts of IL-22 in the presence of IRBP peptide in vitro. However, splenic T cells from normal nonimmunized mice failed to produce IL-22 under the same conditions (Fig. 4B). These results indicate that autoimmune uveitis induced by retinal Ags include Th22-type effector T cells in vivo.

To show that Th22 cells are involved in the pathogenesis of uveitis, we used blocking reagents for anti–IL-22 and anti–TNF-α Abs in the mouse uveitis model and evaluated their effect on the pathogenesis of EAU. We used these blocking Abs during the effenter phase (early stage in EAU mice), as described in Materials and Methods. Administration of both Abs, but not IL-22 Ab alone, significantly suppressed ocular inflammation in EAU models when evaluated on days 14 and 21 (Fig. 4C). In addition, there was no significant effect unless we used both Abs together (Fig. 4C), suggesting that IL-22 together with TNF-α may work as proinflammatory cytokines in ocular inflammation.

Production of IL-22 by polarized Th1 and Th17 cells

We also determined whether Th1 and Th17 cells can produce IL-22, because a recent report showed that these inflammatory T cells, including Th1 and Th17 cells, can produce IL-22-type, but not Th2-type, cells (11). In addition, IFN-γ and IL-17 are inflammatory cytokines that promote inflammatory responses and correlate...
FIGURE 3. Ability of TNF-α blockade to inhibit Th22 cells from uveitis patients with Behçet’s disease. (A) For ELISA analysis, polarized Th22 cell lines from a Behçet’s disease patient (BD; open bar) were cocultured with infliximab (IFX; black bar). As a control, Th22 cell lines from a healthy donor (HD) were also exposed to infliximab. The graph shows the amount of IL-22 determined by ELISA (ng/ml). **p < 0.005, ***p < 0.0005, between two groups. (B) For flow cytometric analysis, Behçet’s disease Th22 cell lines (upper panels) and Behçet’s disease TCCs (B25-48; lower panels) were stained with anti–IL-22 Abs and anti-CD4 Abs after permeabilization. Th22 cells cocultured with infliximab (IFX; right panels) and Th22 cells without IFX (left panels). The percentages of cells that were double positive for IL-22/CD4 are shown. (C) Behçet’s disease TCCs (B2-25) were cocultured with anti-human TNF-α–neutralizing Abs, anti-human IL-6–neutralizing Abs, or both Abs. The bar graph shows the amount of IL-22, as determined by ELISA (pg/ml). **p < 0.005, ***p < 0.0005 compared with control data without Abs (open bar).
with autoimmune disorders, including eye disorders and Behçet’s disease (1–6). We used polarized murine Th1 and Th17 cells induced by Th1- or Th17-differential factors. Compared with conventional CD4+ T cell lines, Th1 cells and Th17 cells produced large amounts of IL-22, as determined by ELISA (Fig. 5A). Similarly, the polarized Th1 and Th17 cells highly expressed IL-22, as assessed by flow cytometry (Fig. 5B).

**Involvement of Th22 cells in the inflammatory response in Behçet’s disease**

As a final step, we examined the involvement of Th22 cells in the inflammatory response in Behçet’s disease. We first determined whether Th22 cells from Behçet’s disease patients can express the inflammatory chemokines MCP-1 (CCL2) and RANTES (CCL5). As a control, Th1 cell lines from a Behçet’s disease patient were

![FIGURE 4. Detection of IL-22-producing cells in EAU donors. Normal mice (n = 30) were immunized with IRBP, and their intraocular T cells and spleen cells were collected on day 14 or 21. (A) By flow cytometric analysis, fresh T cells from EAU eyes (upper panels), fresh cells from EAU spleen (lower left panel), and fresh cells from the spleen of a normal mouse (lower right panel) were stained with anti-mouse IL-22 Abs and anti-mouse CD4 Abs after permeabilization. The percentages of cells that were double positive for IL-22/CD4 are shown. (B) Intraocular T cells were collected from EAU eyes and evaluated using the IRBP retinal Ag-specific assay. The EAU T cells (1 × 10^5/well) were cocultured with APCs (20 Gy x-ray–irradiated spleen cells; 1 × 10^5/well) plus mouse IRBP peptide (10 μg/ml) for 48 h. As controls, spleen T cells (EAU or normal mouse) were prepared in the presence of IRBP peptide. ELISA was used to measure the IL-22 cytokine concentration in the supernatants of the T cell cultures. (C) C57BL/6 mice were immunized with IRBP peptides on day 0 and were given 100 μl of anti–IL-22 and/or anti–TNF-α on days 0, 1, 3, 5, and 7 (5 μg/mouse, i.v. injection). The inflammation after the administration of anti–IL-22 (n = 8), anti–TNF-α (n = 8), anti–IL-22 plus anti–TNF-α (n = 14), and PBS only (positive control mice; n = 12) was evaluated by clinical scores (fundus score on day 14 or 21; grade 0–4). Fundoscopy was used to determine the individual clinical score. Bars indicate the overall mean EAU. Photomicrographs (right panels) indicate the evaluation of histological findings in EAU donors (day 21). Scale bars, 50 μm. *p < 0.05, **p < 0.005, between two groups.**
also prepared. As shown in Fig. 6A, Behçet’s disease Th22 cells expressed mRNA for both chemokines at much higher levels than did control Th22 cells from a healthy donor. In contrast, Behçet’s disease Th1 cells expressed MCP-1 mRNA, but the expression was lower compared with the results from Th22 cells (Fig. 6A).

We next confirmed whether T cells exposed to supernatants from Behçet’s disease Th22 cells can express mRNA for these inflammatory chemokines. Compared with control results, T cells exposed to supernatants from Behçet’s disease Th22 cells highly expressed mRNA for these chemokines (Fig. 6B), as did T cells exposed to control supernatants from Behçet’s disease Th1 cells. Taken together, these data suggest that Th22-type cytokines or cells may be involved in the pathogenesis of Behçet’s disease.

Discussion

It is widely accepted that effector Th cells have inflammatory capabilities. Th22 cells are CD4⁺ effector Th cells that produce IL-22 and TNF-α. They are a unique subset of Th cells that develop along a pathway distinct from the Th1-, Th2-, and Th17-differentiation pathways (10–12). IL-22 is an inflammatory cytokine that promotes inflammatory responses and correlates with autoimmune disorders (12, 25–27). In addition, Th22 cells play an important role in the pathogenesis of experimental autoimmune disease models (26, 28, 29).

IL-22 secreted by Th22 cells primarily affects epithelial and stromal cells rather than other hematopoietic cells, which lack a functional IL-22R. Expression of the CCR4 and CCR10 skin-homing receptors on Th22 cells suggests that they are likely to be recruited to the skin, where they may contribute to host defense against microbial pathogens and promote tissue repair or remodeling. Multiple studies indicate that Th22 cells may also be involved in the pathogenesis of inflammatory skin disorders, such as psoriasis, atopic eczema, and allergic contact dermatitis (10, 12, 27–29). Although several investigators reported that Th22 cells are involved in the pathogenesis of inflammatory skin disorders, to the best of our knowledge there have been no reports of a relationship between Th22 cells and Behçet’s disease. Therefore, we designed experiments to determine whether Th22 cells are involved in the pathogenesis of uveitis in Behçet’s disease.

In the current study, we showed that Th22-type TCCs established from ocular samples from patients with active uveitis, including those with Behçet’s disease, produced large amounts of the Th22-associated cytokines IL-22 and TNF-α but not Th1/Th17 cytokines. In addition, CD4⁺ T cell lines from PBMCs produced large amounts of IL-22, as well as other inflammatory cytokines, suggesting that Behçet’s disease T cells established from inflamed eyes and peripheral blood cells may include Th22-type effector cells. Additionally, fresh CD4⁺ T cells from patients with active uveitis and Behçet’s disease, without systemic treatment, highly expressed IL-22, TNF-α, and CCR10. We also demonstrated that Th22 cells exposed to infliximab in vitro failed to produce IL-22 and TNF-α, suggesting that TNF-α is required for Th22 differ-

FIGURE 5. Expression of IL-22 in Th1/Th17 cells. Polarized mouse Th1 and Th17 cells induced by Th1- or Th17-differentiation factors were used. (A) For IL-22 ELISA, the supernatants of conventional CD4⁺ T cell lines (open bar; control) and Th1 and Th17 cells (black bars) were collected. The bar graph shows the amount of mouse IL-22, as determined by ELISA (ng/ml). (B) By flow cytometry, polarized Th1 and Th17 cells were stained with anti-mouse IL-22 and anti-mouse CD4 Abs after permeabilization. The percentages of cells that were double positive for IL-22/CD4 are shown. *p < 0.05, **p < 0.005, compared with control data.

FIGURE 6. Expression of inflammatory chemokines in T cells exposed to supernatants from Behçet’s disease Th22 cells. (A) Th22 cell lines from Behçet’s disease patients (BD-1 and BD-2) or a healthy donor (HD) were harvested on day 5 and examined for the expression of CCL2 and CCL5 mRNA by qRT-PCR. As a control, Th1 cell lines from Behçet’s disease patients (BD-3) were also harvested. (B) T cells exposed to supernatants from Behçet’s disease Th22 cells (BD-1 and BD-2) were collected and examined for the expression of chemokine mRNA by qRT-PCR. T cells exposed to supernatants from Behçet’s disease Th1 cells (BD-3) or healthy donor Th22 cells (HD) were also collected. Data show the relative expression of these chemokines (ΔΔCt).
entiation in Behçet’s disease. To confirm this result, we used both human and mouse rTNF-α proteins. CD4⁺ Behçet’s disease T cells exposed to TNF-α in vitro produced IL-22, and murine Th1 and Th17 cells produced large amounts of IL-22 when cultured with mouse rTNF-α.

In addition, fresh T cells from a uveitis patient with Behçet’s disease expressed high levels of Th22-related molecules, such as IL-22, TNF-α, and CCR10, whereas fresh T cells from a Behçet’s disease patient being treated with infliximab expressed low levels of these molecules. Similarly, Th22 clones from Behçet’s disease uveitis patients expressed high levels of Th22-related molecules, but the expression was suppressed in Th22 cells cultured with infliximab. Importantly, the expression of Th22-related molecules was greatly suppressed in Th22 cells cultured with both anti–TNF-α and anti–IL-6 blockers. These results suggest that these blocks may prevent the differentiation of Th22 cells. In fact, infliximab successfully controls inflammation in the eyes of patients with Behçet’s disease (17–24). Thus, suppression of effector T cell differentiation by anti–TNF-α therapy may protect uveitis patients from severe ocular complications.

Fresh intraocular T cells from mice with EAU contained a large population of IL-22⁺ cells, suggesting that CD4⁺ IL-22⁺ Th22-type T cells may be associated with the pathogenic mechanisms of intraocular inflammation. We also showed that retinal Ag-specific CD4⁺ T cells from EAU mice produced large amounts of IL-22 in the presence of retinal Ags (IRBP peptides). Additionally, we showed that anti-mouse IL-22-blocking Abs, together with anti-mouse TNF-α Ab, greatly suppressed intraocular inflammation in EAU models (see Fig. 4C).

IL-22 is an inflammatory cytokine that promotes inflammatory responses and correlates with autoimmune disorders, such as rheumatoid arthritis (25), Crohn’s disease (26), and skin inflammatory diseases (11, 12, 29). In a mouse model of psoriasis, dermal inflammation is suppressed in IL-22-deficient mice (27). IL-22⁺ T cells and IL-22 contribute to the inflammatory response and promote epithelial healing, including the corneal epithelium (30). However, the role of IL-22/Th22 cells in inflammation remains controversial. We found that the production of inflammatory cytokines by T cells is enhanced by exposure to supernatants from Behçet’s disease Th22 cells. T cells exposed to Th22 supernatants expressed high levels of mRNA for chemokines, suggesting that Th22-type cytokines and cells may be involved in the acute immune response in Behçet’s disease. Although the proinflammatory responses are synergistically dependent on IL-22 and TNF-α (12), Behçet’s disease T cells, such as intraocular-infiltrating cells, include Th22 cells, and the T cells produce large amounts of both IL-22 and TNF-α.

In conclusion, the inflammatory cytokine IL-22 seems to enhance the inflammatory response in Behçet’s disease patients who have uveitis. The proinflammatory cytokine TNF-α may promote Th22 differentiation in Behçet’s disease. Thus, Th22 cells that produce IL-22 and TNF-α constitute a novel subset of effector Th cells in Behçet’s disease. We are now conducting experiments to determine whether IL-22/Th22 cells are involved in the pathogenesis of inflammation in other types of uveitis (noninfectious and infectious), and we are also investigating whether ocular-resident tissues, such as retinal pigment epithelium, can suppress these effector T cells in vitro.

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References


