CD8<sup>bright</sup>CD56<sup>+</sup> T Cells Are Cytotoxic Effectors in Patients with Active Behçet's Uveitis

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CD8<sup>bright</sup>CD56<sup>+</sup> T Cells Are Cytotoxic Effectors in Patients with Active Behçet’s Uveitis<sup>1</sup>

Jae Kyoun Ahn,<sup>2,*</sup> Hum Chung,<sup>2,*</sup> Dong-sup Lee,<sup>†</sup> Young Suk Yu,* and Hyeong Gon Yu<sup>3,*</sup>

Behçet’s uveitis, characterized by chronic recurrent uveitis and obliterating retinal vasculitis, frequently causes bilateral blindness. Intraocular infiltration of TCR<sup>αβ</sup>CD8<sup>bright</sup>CD56<sup>+</sup> cells was a distinct feature in Behçet’s uveitis. However, phenotypic and functional characteristics of these cells have remained elusive. This study was conducted to determine phenotypic and functional characteristics and cytotoxic mechanisms of CD8<sup>bright</sup>CD56<sup>+</sup> T cells in Behçet’s uveitis. CD11b<sup>+</sup>CD27<sup>−</sup>CD62L<sup>−</sup> phenotypes of CD8<sup>bright</sup>CD56<sup>+</sup> T cells were increased in patients with active Behçet’s uveitis compared with inactive Behçet’s patients and normal controls. Interestingly, CD45RA<sup>dim</sup>/CD45RO<sup>+</sup> phenotypes were expanded, and CD94 expression was markedly up-regulated in contrast to the down-regulation of NKG2D. Furthermore, these subsets were polarized to produce IFN-γ and contained high amounts of preformed intracellular perforin while exclusively expressing surface FasL upon PI stimulation. Moreover, the cytolytic functions of freshly isolated CD8<sup>bright</sup>CD56<sup>+</sup> T cells were up-regulated against both K562 (NK-sensitive) and Raji (NK-resistant) cells, which were effectively inhibited by perforin inhibitor (concanaamycin A). Their cytolytic activity against HUVECs was also increased and was effectively suppressed by Fas ligand inhibitor (brefeldin A) and partly by perforin inhibitor. Furthermore, cytolytic functions of PMA and ionomycin-stimulated CD8<sup>bright</sup>CD56<sup>+</sup> T cells against HUVECs were greatly enhanced, by pretreatment of recombinant human IFN-γ on HUVECs. Therefore, CD8<sup>bright</sup>CD56<sup>+</sup> T cells in Behçet’s uveitis are characterized by cytotoxic effector phenotypes with functional NK receptors and function as strong cytotoxic effectors through both Fas ligand-dependent and perforin-dependent pathways.

Recently, we reported that the intraocular infiltration and peripheral expansion of TCR<sup>αβ</sup>CD8<sup>bright</sup>CD56<sup>+</sup> cells were distinct developments in patients with Behçet’s uveitis in contrast to endogenous uveitis of other etiologies (11). In addition, these immune cells were down-regulated following combined low-dose cyclosporine and prednisone treatment (12). Because the immunological roles of CD8<sup>bright</sup> T cells are distinctly different in each subset, phenotypic and functional separation of CD8<sup>bright</sup> T cells is crucial to identify the immunopathogenic effectors (13). However, there is little evidence regarding the phenotypic natures and functional characteristics of TCR<sup>αβ</sup>CD8<sup>bright</sup>CD56<sup>+</sup> cells in Behçet’s uveitis.

Contact-dependent, cell-mediated cytotoxicity mechanisms contact from mHC- or mHC-restricted mechanisms. mHC-restricted cytotoxicity is mainly mediated by CD8<sup>+</sup> CTLs through two distinct perforin- and Fas-based pathways resulting in tissue destruction (14). Concanaamycin A (CMA) perforin inhibitor and brefeldin A (BFA) Fas ligand (FasL) inhibitor are useful tools for evaluation of each pathway in CD8<sup>+</sup> T cell-mediated cytotoxicity (15). IFN-γ, a cytokine associated with cell-mediated immunity, represents an additional effector molecule released by activated CD8<sup>+</sup> CTL and promotes the cytolytic effector functions of CD8<sup>+</sup> CTL (16). TCR<sup>αβ</sup>CD8<sup>bright</sup>CD56<sup>+</sup> T cells in Behçet’s uveitis may act as potent cytolytic effectors upon intraocular infiltration. However, there is a paucity of reports concerning the cytotoxic mechanisms of TCR<sup>αβ</sup>CD8<sup>bright</sup>CD56<sup>+</sup> cells in Behçet’s uveitis.

This study was conducted to determine the phenotypic and functional characteristics of CD8<sup>bright</sup>CD56<sup>+</sup> T cells in Behçet’s uveitis and to investigate cytotoxic mechanisms of these expanded subsets.

Materials and Methods

Patients and classification of disease activity

Forty-five patients with BD according to the international criteria (17) were recruited from the Uveitis Clinic of Seoul National University Hospital.
from November 2003 to December 2004. Other etiologies were excluded following a complete ocular and systemic examination. The patients were clinically divided into two groups according to the degree of uveitis and systemic disease. The active group consisted of 24 patients with panuveitis (anterior chamber cells ≥ 2, vitreous haziness ≥ 2, exudative chorioretinal lesion ≥ 1 according to the criteria of the International Uveitis Study Group (18)) and angiographically proven retinal vasculitis at the time of study. They also showed concurrent systemic manifestations such as oral ulcer and skin lesion. Inactive group consisted of 21 patients who had not experienced attacks of uveitis for ≥ 6 mo with or without treatment. All inactive patients showed normal levels of C-reactive protein, white blood cell counts, and erythrocyte sedimentation rate without any major systemic symptoms such as orogenital ulcers or skin lesions. Twenty-four non-BD uveitis patients were included in this study. These patients were diagnosed with the following: 10 with HLA-B27-related anterior uveitis, 7 with idiopathic anterior uveitis, 3 with Vogt-Koyanagi-Harada syndrome, 2 with idiopathic panuveitis, and 2 with pars planitis. All patients had received no medications within 4 wk at the time of venipuncture. In addition, 10 insulin-dependent diabetes (IDDM) patients were recruited for the analysis of the proportions of CD8brightCD56− or CD8brightCD56+ cells in peripheral blood. Twenty age- and sex-matched healthy subjects were used as controls. All procedures adhered to the tenets of the Declaration of Helsinki, and local approval was received from the Investigational Review Board of the Seoul National University Clinical Research Institute. Informed consents were obtained from all patients and controls subjects. Demographic features in enrolled patients are described in Table I.

### Cells, reagents, and mAbs

Target cells purchased from American Type Culture Collection for ex vivo cytotoxicity assays were lymphoblast K562 (NK sensitive), Burkitt’s lymphoma Raji (NK resistant), human vascular endothelial cell lines from the

![Figure 1](http://www.jimmunol.org/)  
**Figure 1.** Intraocular and peripheral populations of CD8bright T cells with or without CD56 in uveitis patients and IDDM patients. Representative flow cytometric data of aqueous humors and peripheral blood using anti-CD8 mAb and anti-CD56 mAb are illustrated in patients with HLA-B27-related uveitis and with BD panuveitis (A). The percentages of each subset are shown in each upper right quadrant. Each proportion of CD8brightCD56+ T cells (B) and CD8brightCD56− T cells (C) in the eye was plotted vs the corresponding proportion in the peripheral blood from the same uveitis patients (BD uveitis, 21; non-BD uveitis, 24). The populations of CD8brightCD56+ T cells and CD8brightCD56− T cells were compared between the 24 active BD uveitis patients and the 10 IDDM patients (D).
Table II. Phenotypic characteristics of CD8bright T cells with or without CD56 in BD

<table>
<thead>
<tr>
<th>Phenotypic characteristics</th>
<th>Controls (n = 20)</th>
<th>Active BD (n = 24)</th>
<th>Inactive BD (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8brightCD56+ T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of PBMCs</td>
<td>2.1 ± 0.7</td>
<td>7.2 ± 2.4abc</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>CD11b</td>
<td>68 ± 15</td>
<td>110 ± 12abc</td>
<td>72 ± 18</td>
</tr>
<tr>
<td>CD62L</td>
<td>281 ± 73</td>
<td>134 ± 63a</td>
<td>298 ± 103</td>
</tr>
<tr>
<td>CD27</td>
<td>47 ± 11</td>
<td>31 ± 6a</td>
<td>62 ± 14</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>25 ± 10</td>
<td>242 ± 104abc</td>
<td>48 ± 14</td>
</tr>
<tr>
<td>CD25</td>
<td>2 ± 0.2</td>
<td>2 ± 0.4</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>CD45RAbright</td>
<td>49 ± 7</td>
<td>54 ± 9</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>CD45RO</td>
<td>34 ± 14</td>
<td>5 ± 2</td>
<td>12 ± 4d</td>
</tr>
<tr>
<td>NKG2D</td>
<td>82 ± 24</td>
<td>42 ± 10a</td>
<td>20 ± 7d</td>
</tr>
<tr>
<td>CD94</td>
<td>10 ± 4</td>
<td>37 ± 11a</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>CD8brightCD56− T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of PBMCs</td>
<td>23.6 ± 4.3</td>
<td>26.5 ± 5.8</td>
<td>25.1 ± 4.8</td>
</tr>
<tr>
<td>CD11b</td>
<td>27 ± 10</td>
<td>35 ± 11</td>
<td>34 ± 7</td>
</tr>
<tr>
<td>CD62L</td>
<td>861 ± 93</td>
<td>360 ± 184abc</td>
<td>896 ± 163</td>
</tr>
<tr>
<td>CD27</td>
<td>86 ± 5</td>
<td>70 ± 9a</td>
<td>86 ± 8</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>29 ± 14</td>
<td>120 ± 12a</td>
<td>30 ± 12</td>
</tr>
<tr>
<td>CD25</td>
<td>2 ± 0.3</td>
<td>2 ± 0.5</td>
<td>2 ± 0.4</td>
</tr>
<tr>
<td>CD45RAbright</td>
<td>55 ± 14</td>
<td>60 ± 12</td>
<td>52 ± 15</td>
</tr>
<tr>
<td>CD45RO</td>
<td>23 ± 3</td>
<td>11 ± 5</td>
<td>17 ± 8</td>
</tr>
<tr>
<td>NKG2D</td>
<td>65 ± 11</td>
<td>24 ± 4a</td>
<td>15 ± 6d</td>
</tr>
<tr>
<td>CD94</td>
<td>0.7 ± 0.6</td>
<td>7 ± 2a</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

a Values of p < 0.05 compared with inactive BD and normal controls.
b Data represents the values of MFI in gated cells.
c Data represents the values of percentages of positive cells in gated cells.
d Values of p < 0.05 compared with normal controls.

**Phenotypic analysis**

Ten microliters of heparinized venous blood was obtained from uveitis patients, IDDM patients, and normal controls. PBMCs were recovered at the buffy coat layer using a Ficoll-Hyphaque (Pharmacia) gradient, and then washed twice to remove RBCs. Directly conjugated mAbs were added at predetermined optimal dilutions. After a 30-min incubation, cells were washed twice in FACS buffer. Phenotypic analysis of fresh PBMCs was performed using anti-CD8 mAb and anti-CD56 mAb in conjunction with a three- or four-color immunofluorescence test for the expression levels of the following molecules: CD11b, CD25, CD45RA, CD45RO, CD62L, CD94, NKG2D, and HLA-DR. In 21 active BD uveitis and 24 non-BD uveitis patients, phenotypic analysis of intraocular infiltrating cells were performed through aqueous humor sampling using a 30-gauge needle. One hundred to 200 μl of aqueous humor were aspirated and immediately placed into a microcentrifuge tube precoated with EDTA and subjected to flow cytometry analysis using anti-CD3, anti-CD4, anti-CD8, and anti-CD56 mAb. Data were acquired using a FACSCalibur flow cytometer and umbilical vein (HUVECs). Each cell line was maintained in the specific medium according to the manufacturer’s recommendations (IMDM for K562; RPMI 1640 for Raji; Ham’s F12 medium for HUVEC). PMA and ionomycin (PI), CMA, BFA, and recombinant human IFN-γ (rhIFN-γ) were purchased from Sigma-Aldrich. All mAbs, including anti-CD8-CyChrome or -allophycocyanin (RTA-T8), anti-CD11b-PE (ICRF44), anti-CD25-CyChrome (M-A251), anti-CD27-PE (M-T271), anti-CD45RA-PE (HI100), anti-CD45RO-PE (UCHL1), anti-CD56-FITC or -PE (NCAM 16.2), anti-CD62L-PE (Dreg 56), anti-NKG2D-allophycocyanin (1D11), anti-perforin-PE (6G9), anti-IFN-γ-PE (25723.11), and anti-IL-4-PE (3010.211) were purchased from BD Pharmingen, except anti-Fasl-PE mAb (MBL).

**FIGURE 2.** Cell surface marker analysis of CD8bright T cells with or without CD56. Fresh PBMCs were stained simultaneously with anti-CD8 mAb, anti-CD56 mAb in conjunction with anti-CD27 mAb, anti-CD62L mAb, anti-CD11b mAb, anti-HLA-DR, and anti-CD25. CD8brightCD56− T cells (A) and CD8brightCD56+ T cells (B) were gated and then analyzed for each cell surface marker expression. Expression levels are represented as the MFI and the frequencies of positive cells in gated cells.
analyzed with the CellQuest software program. Expression levels are represented by the frequencies of positive cells and the mean fluorescence intensity (MFI) of gated cells.

**Intracellular staining of IFN-γ and IL-4**

To measure the IFN-γ and IL-4 production by circulating CD8<sup>bright</sup> cells with or without CD56, flow cytometric measurement was performed as described previously (19). Briefly, fresh PBMCs (10<sup>6</sup>/well) from BD patients were stimulated in a 96-well round-bottom plate for 6 h at 37°C containing PMA (30 ng/ml) and ionomycin (1 μM) in the presence of protein-secreting inhibitor BFA (10 μg/ml). Unstimulated control wells were incubated with BFA alone. This short-term stimulation did not alter the membrane phenotype with respect to the CD56 expression. Cells were fixed with 4% paraformaldehyde at the end of a 6-h incubation period. They were also permeabilized with buffer to allow intracellular access of anti-IFN-γ-PE and anti-IL-4-PE mAb. For the phenotype staining, cells were incubated for 30 min at 4°C with anti-CD8-CyChrome mAb and anti-CD56-FITC mAb. Cells were immediately analyzed on FACSCalibur and CellQuest software. Intracellular IFN-γ and IL-4 expression is represented by the frequencies of positive cells in gated cells. In both unstimulated and stimulated wells, analysis gates were set for lymphocytes using forward and side scatter properties, and the frequencies of cytokine-producing cells were acquired by phenotype gating using anti-CD8 and anti-CD56. We preliminarily confirmed that the frequencies of IFN-γ and IL-4-producing CD8<sup>bright</sup> T cells reached a peak at 6 h stimulation in normal controls and that the specificity of IFN-γ and IL-4 was tested in patients with BD and atopic dermatitis.

**Intracellular perforin and surface FasL expression**

To determine intracellular amounts of perforin in unstimulated CD8<sup>bright</sup> T cells with or without CD56, PBMCs were first stained with anti-CD8 mAb and anti-CD56 mAb, washed, and then fixed and permeabilized in 1 ml of perm/wash solution containing saponin (permeabilizing buffer) at room temperature for 40 min. Cells were washed twice with buffer and incubated with intracellular anti-perforin mAb for 40 min at 4°C. Cells were then washed and immediately analyzed on a FACSCalibur. PE-labeled mouse IgG1 was used as negative control. In some experiments, we measured the amounts of intracellular perforin after 5 h of PI stimulation. The amounts of intracellular perforin were represented by the frequencies of positive cells and the MFI of gated cells.
To investigate the expression levels of surface FasL, fresh PBMCs (10⁶/well) were stained with anti-CD8, anti-CD56, and anti-FasL after 5 h of PI stimulation without BFA. Unstimulated wells and PE-labeled-mouse IgG1 were used as controls. Surface FasL expression is represented as the frequencies of positive cells in gated cells. In some experiments, the inhibitory effect of 0–500 nM CMA and 0–100 μM BFA on surface FasL expression was determined. The frequencies of membrane FasL-expressing CD8brightCD56+ T cells reached a peak at 5 h in stimulation in healthy controls.

Cell sorting

Fresh PBMCs from 60 ml of heparinized peripheral blood were stained with anti-CD8-CyChrome mAb and anti-CD56-FITC mAb. CD8brightCD56+ cells and CD8brightCD56- cells were purified by FACStar (purity of each population was >97%). Cells of each population (>2 × 10⁶ cells in RPMI 1640 supplemented with 10% FCS and antibiotics) were directly subjected to ex vivo cytotoxicity assays. We confirmed that the proportions of apoptotic cells in sorted cells were <10% using Annexin V et al. and propidium iodide.

Ex vivo cytotoxicity assays

To investigate the killing ability of sorted cells, K562 cells and Raji cells were labeled with 100 μCi Na251CrO4 (Amersham Biosciences) for 90 min at 37°C in each growth medium, washed twice with medium, and then subjected to cytotoxicity assays. The labeled targets (3 × 10⁴ cells per 100 μl/well) were incubated with sorted effector cells (3 × 10⁵ cells per 100 μl/well) (E:T ratio = 10:1) in 96-well round-bottom microtiter plates. The plates were centrifuged after incubation for 4 h, after which the 100-μl supernatant was harvested and counted with a gamma counter. Specific 51Cr release (percentage) was calculated using the following formula: (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100. Maximum release was determined by the incubation of target cells with 100 μl of 1 N hydrochloric acid. The spontaneous release was <10% of the maximum release. Specific 51Cr release (percentage) was calculated as described previously.

Modulation of cytotoxicity

Target cell treatment. To determine the promoting effect of IFN-γ on the cytotoxicity of HUVECs, HUVECs were preincubated with 100 U/ml rhIFN-γ for 24 h at 37°C in Ham’s F12K medium, extensively washed with medium, and then subjected to 6-h 51Cr release assays with the E:T ratio of 10:1. Furthermore, we investigated the effect of IFN-γ on the Fas and HLA-DR expression of HUVECs.

Effector cell treatment. To explore the cytotoxic mechanisms, the sorted effector cells were preincubated with 100 nM CMA or 20 μM BFA for 2 h in a final volume of 3 × 10⁴ cells per 100 μl/well, mixed with 100 μl of target cells (E:T ratio = 10) in the presence of CMA or BFA, and then subjected to standard 4-h (K562, Raji) or 6-h (HUVECs) 51Cr release assay. Furthermore, PI-stimulated effector cells for 5 h were used against HUVECs in some experiments.

Statistical analysis

The MFI and frequencies of positive cells were compared using the Mann-Whitney U test. Values of p < 0.05 were considered significant.

Results

Intraocular infiltration and peripheral expansion of CD8brightCD56+ T cells are distinct features in BD uveitis patients

In comparison to non-BD uveitis patients, intraocular infiltration and peripheral expansion of CD8brightCD56+ T cells were remarkable in BD uveitis patients (Fig. 1, A and B). These findings were more outstanding in aqueous humor than peripheral blood. The proportion of the CD8brightCD56+ T cell was also increased in BD uveitis patients and was higher in aqueous humor than peripheral blood (Fig. 1C). As shown in Fig. 1D, the peripheral expansion of the CD8brightCD56+ T cell was not noted in IDDM patients.

The activated effector phenotypes with functional NK receptors of CD8brightCD56+ T cells expand in active BD uveitis patients

In active BD uveitis, CD8brightCD56+ T cells had a number of features compatible with primed effector phenotypes (Table II). Compared with inactive BD uveitis and normal controls, CD27 and CD62L of CD8brightCD56+ T cells were found to be down-regulated, whereas HLA-DR and CD11b were highly expressed (Fig. 2). CD8brightCD56− T cells showed the similar findings in active BD uveitis, but the changes were more remarkable on the CD8brightCD56+ T cells (Fig. 2). No significant difference was observed in CD25 expression levels. Furthermore, in active BD uveitis, the proportions of CD45RAlowCD45RO− phenotypes were markedly increased in the CD8brightCD56+ T (Fig. 3). CD94 was expressed exclusively in active BD on the CD8brightCD56+ T.
cells. In contrast, NKG2D expression was significantly down-regulated on both the CD8<sup>bright</sup>CD56<sup>+</sup> T cells and CD8<sup>bright</sup>CD56<sup>−</sup> T cells in BD compared with normal controls. However, NKG2D expression on CD8<sup>bright</sup> T cells with or without CD56 was up-regulated in active BD compared with inactive BD.

**CD8<sub>bright</sub>CD56<sup>+</sup> T cells in active BD uveitis are polarized to produce IFN-γ**

Because IFN-γ-positive CD8<sub>bright</sub> T cells were <1% of unstimulated cells from patients and controls, we measured the capacities of IFN-γ production after in vitro stimulation. After 6 h of PI stimulation, the frequencies of IFN-γ-producing CD8<sub>bright</sub>CD56<sup>+</sup> T cells were significantly higher in active BD than those in inactive BD and normal controls (Fig. 4). In contrast, the frequencies of IL-4-producing CD8<sub>bright</sub>CD56<sup>+</sup> T cells were lower in active BD uveitis. Similarly, the IFN-γ-productions of CD8<sub>bright</sub>CD56<sup>−</sup> T cells were also elevated in active BD uveitis but smaller than those of CD8<sub>bright</sub>CD56<sup>+</sup> T cells (Tables III and IV; Fig. 4).

**CD8<sub>bright</sub>CD56<sup>+</sup> T cells in active BD contain high amounts of perforin and exclusively exhibit higher expression of surface FasL upon PI stimulation**

Because CD8<sub>bright</sub>CD56<sup>+</sup> T cells shed the preformed intracellular perforin after in vitro PI stimulation, we measured the amounts of intracellular perforin of CD8<sub>bright</sub> T cells without in vitro stimulation. CD8<sub>bright</sub>CD56<sup>−</sup> T cells in active BD uveitis contained higher levels of intracellular perforin than those in inactive BD uveitis and normal controls (Table III; Fig. 5). The perforin-producing proportion was also increased in CD8<sub>bright</sub>CD56<sup>−</sup> T cells, although the level was not as high as that of CD56<sup>+</sup> subsets. Because CD8<sub>bright</sub>CD56<sup>−</sup> T did not express the surface FasL without stimulation, we measured the expression levels of surface FasL after 5 h of PI stimulation. Surface FasL was strongly and exclusively expressed in CD8<sub>bright</sub>CD56<sup>−</sup> T cells of both patients and normal controls and was markedly up-regulated in active BD (Table III; Fig. 5).

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**Table III. Functional characteristics of peripheral CD8<sub>bright</sub>CD56<sup>+</sup> T cells with or without CD56 in BD**

<table>
<thead>
<tr>
<th>Cytokine production</th>
<th>Controls (n = 10)</th>
<th>Active BD (n = 10)</th>
<th>Inactive BD (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8&lt;sub&gt;bright&lt;/sub&gt;CD56&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 ± 6</td>
<td>65 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34 ± 18</td>
</tr>
<tr>
<td>IL-4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5 ± 1.4</td>
<td>0.9 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1 ± 1.1</td>
</tr>
<tr>
<td>CD8&lt;sub&gt;bright&lt;/sub&gt;CD56&lt;sup&gt;−&lt;/sup&gt; T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16 ± 7</td>
<td>35 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25 ± 14</td>
</tr>
<tr>
<td>IL-4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.7</td>
<td>0.7 ± 0.3</td>
<td>0.9 ± 0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data represents the frequencies of positive cells in gated cells after 6 h of PI stimulation.

<sup>b</sup> Values of $p < 0.05$ compared with inactive BD and normal controls.

---

**Table IV. Functional characteristics of peripheral CD8<sub>bright</sub> T cells with or without CD56 in BD**

<table>
<thead>
<tr>
<th>Cytotoxic molecules</th>
<th>Controls (n = 12)</th>
<th>Active BD (n = 15)</th>
<th>Inactive BD (n = 15)</th>
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<tr>
<td>CD8&lt;sub&gt;bright&lt;/sub&gt;CD56&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perforin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30 ± 10 (15 ± 6)</td>
<td>66 ± 7 (71 ± 25)&lt;sup&gt;§&lt;/sup&gt;</td>
<td>33 ± 11 (22 ± 11)</td>
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<tr>
<td>FasL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45 ± 13</td>
<td>86 ± 14&lt;sup&gt;§&lt;/sup&gt;</td>
<td>51 ± 16</td>
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<td>CD8&lt;sub&gt;bright&lt;/sub&gt;CD56&lt;sup&gt;−&lt;/sup&gt; T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perforin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6 ± 2 (6 ± 2)</td>
<td>26 ± 9 (20 ± 7)&lt;sup&gt;§&lt;/sup&gt;</td>
<td>9 ± 5 (9 ± 3)</td>
</tr>
<tr>
<td>FasL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.4</td>
<td>2.5 ± 0.7</td>
<td>1.8 ± 0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data represents the frequencies of positive cells in gated cells without stimulation (MFI).

<sup>b</sup> Data represents the frequencies of positive cells in gated cells after 5 h of PI stimulation.
Both NK-like and MHC-mediated cytolytic functions of CD8<sup>bright</sup>CD56<sup>+</sup> T cells in active BD are up-regulated.

CD8<sup>bright</sup>CD56<sup>+</sup> T cells in active BD uveitis exhibited potent cytolytic activities against NK-sensitive K562 compared with the CD8<sup>bright</sup>CD56<sup>+</sup> T cell (Fig. 6). NK-like cytolytic functions of CD8<sup>bright</sup>CD56<sup>+</sup> T cells in active BD were comparable to CD56<sup>+</sup> (NK) cells (data not shown). Both CD8<sup>bright</sup>CD56<sup>+</sup> T cells and CD8<sup>bright</sup>CD56<sup>-</sup> T cells in patients with active BD demonstrated

**FIGURE 6.** Cytotoxic capacities of CD8<sup>bright</sup> T cells with or without CD56. Unstimulated fresh PBMCs from patients with BD and normal controls were sorted into CD8<sup>bright</sup>CD56<sup>+</sup> T cells and CD8<sup>bright</sup>CD56<sup>-</sup> T cells, and then cytotoxicity was directly analyzed against K562 (A) and Raji (B) in a 4-h 51Cr release assay at the E:T ratio (10:1). Horizontal bar indicates the mean of three independent experiments. To determine the cytotoxic pathways of CD8<sup>bright</sup>CD56<sup>+</sup> T cells, the cytotoxicity assay against K562 and Raji was performed in the presence of 100 nM CMA (C). Spontaneous release of all experiments was <10%.

**FIGURE 7.** Cytotoxic capacities of CD8<sup>bright</sup>CD56<sup>+</sup> T cells against HUVECs. A, In active BD, CD8<sup>bright</sup>CD56<sup>+</sup> T cells exhibited the potent cytolytic functions against HUVECs. Cytolytic activities of freshly sorted CD8<sup>bright</sup>CD56<sup>+</sup> T cells and CD8<sup>bright</sup>CD56<sup>-</sup> T cells against HUVECs were measured by standard 6-h 51Cr release assay with E:T ratio of 10:1. B, Amounts of intracellular perforin in CD8<sup>bright</sup>CD56<sup>+</sup> T cells were reduced after 5-h PI stimulation. Dotted line indicates isotype negative control Ab, thin line indicates unstimulated status, and thick line indicates 5-h PI-stimulated status. BFA down-regulated surface FasL expression of 5-h PI-stimulated CD8<sup>bright</sup>CD56<sup>+</sup> T cells and CMA did not.

C, rhIFN-γ pretreatment for 24 h up-regulated the surface expression of Fas and HLA-DR on HUVECs. Dotted line indicates unrelated surface mAb (CD3), thin line indicates Fas expression of HUVECs without IFN-γ pretreatment, and thick line indicates Fas expression of HUVECs with IFN-γ pretreatment. D, PI stimulation of CD8<sup>bright</sup>CD56<sup>+</sup> T cells and/or IFN-γ pretreatment of HUVECs up-regulated the cytotoxicity against HUVECs, which were effectively inhibited by BFA. In contrast, CMA partly inhibited the cytolytic activities of unstimulated CD8<sup>bright</sup>CD56<sup>+</sup> T cells.
strong cytotoxicity against NK-resistant Raji (Fig. 6). These findings indicated that, in active BD uveitis, NK-like cytolytic activities were additionally upregulated in CD8brightCD56⁺ T cells. Furthermore, these cytotoxic activities were effectively inhibited by CMA pretreatment, which indicated that NK-like and MHC-dependent cytotoxic mechanisms of CD8brightCD56⁺ T cells in BD might be mediated through the perforin-dependent pathway (Fig. 6).

CD8brightCD56⁺ T cells in active BD uveitis exhibit the potent cytotoxicity against HUVECs through Fas-FasL pathway that is augmented by IFN-γ and PI stimulation

The cytotoxic capacities of unstimulated CD8brightCD56⁺ T cells against HUVECs were upregulated in active BD uveitis (Fig. 7). PI stimulation of CD8brightCD56⁺ T cells caused the discharge of prestored perforin from the cells and elevated surface Fas expression, which was suppressed by BFA (Fig. 7). IFN-γ pretreatment of HUVECs elevated the surface expression of both Fas and HLA-DR (Fig. 7). Moreover, the cytolytic capacities against HUVECs were enhanced by PI stimulation of the effector cells and/or by IFN-γ pretreatment of HUVECs, which were successfully inhibited by BFA (Fig. 7). In contrast, CMA only partially reduced the cytotoxicity of unstimulated CD8brightCD56⁺ T cells against HUVECs irrespective of IFN-γ pretreatment of HUVECs and did not affect the cytolytic activities of PI-stimulated CD8brightCD56⁺ T cells.

Discussion

Previous studies examining the role of T cell-mediated autoimmunity in BD focused on CD4⁺ T cells and found that T cells were autoreactive to heat shock proteins and retinal S Ag in BD patients, especially with ocular involvement (20, 21). However, extrinsic infectious pathogens and HLA-B51 are strongly associated with disease susceptibility (22, 23). A recent study suggested that HLA-B51-restricted cytotoxic CD8⁺ T cells might be involved in the pathogenesis of BD (24). Because the immune reactions evoked by certain infectious agents and by the possible autoantigen presentation with HLA-B51 may occur primarily in the CD8⁺ T cell compartment, we investigated the immunological alterations of CD8⁺ T cell population of Behçet’s uveitis patients.

In this study, the intraocular infiltration and peripheral expansion of CD8brightCD56⁺ T cells were unique features of BD uveitis in contrast to other etiologies of uveitis. This is consistent with previous reports that CD8⁺ T cells are the predominant intraocular infiltrating cells in BD uveitis and that CD8brightCD56⁺ T cells are expanded in the uveitis patients (11, 12). However, the skin biopsy of BD patients demonstrated that CD4⁺ T cells were predominant and that CD8⁺ T cells and CD56⁺ cells were much less predominant (25, 26). This discrepancy may reflect the unique intraocular immunomodulatory environment and more destructive inflammatory nature of BD uveitis compared with skin lesions (27, 28). Furthermore, IDDM patients, a representative immune-mediated inflammatory disease, did not show those characteristics. Therefore, CD8brightCD56⁺ T cells may function as particular immune effectors pertinent to chronic uveitis like BD.

This study demonstrates that the activated effector phenotypes (CD27⁺CD62L⁺CD11b⁺HLA-DR⁺) of CD8bright T cells are expanded in patients with active Behçet’s uveitis. The phenotypic natures of CD8bright T cells in BD were remarkable in the CD56⁺ subsets. CD56 is induced on CTLs from human peripheral blood lymphocytes in a Th1 dominant environment or via chronic antigenic stimulation (7, 8). Strong Th1 polarization and chronic autoantigenic stimulation have been observed in BD (2, 24). Previous studies also showed that CD27 and CD62L expression of human CD8⁺ T cells is inversely related to the effector functions (29, 30) and that CD8⁺CD28⁺CD11b⁺ T cells are expanded in BD (31). Therefore, CD8brightCD56⁺ T cells in active BD uveitis may be induced from CD8brightCD56⁻ T cells.

In this study, CD45RA⁺CD100⁺CD45RO⁺ phenotypes of CD8bright CD56⁺ T cells were increased in active BD uveitis. CD45RA can be upregulated on CD45RA⁺CD8⁺ T cell effectors during the late immune response following cytokine-driven proliferation (32, 33). Thus, in active BD uveitis, the CD45RA⁺CD100⁺CD45RO⁺ effector phenotypes of CD8brightCD56⁺ T cells may acquire the CD45RA or CD45RO following the repetitive stimulation. Interestingly, CD94 was markedly upregulated on CD8brightCD56⁺ T cells in active BD uveitis and supports the hypothesis that CD8brightCD56⁺ T cells may play an immunopathogenic role in the CD8⁺ T cell-mediated immune response of BD. CD94, a functional NK receptor for HLA-E, is inducible on activated effector CTL phenotypes in vivo and may act as an inhibitory NK receptor for CD8⁺ T cells (34, 35). Inhibitory function of CD94 on CD8⁺ T cells only occurs during chronic inflammation that needs to be controlled (36, 37). Therefore, it is plausible that CD94 on CD8brightCD56⁺ T cells in active BD uveitis may be induced for the regulation of effector functions (4, 38). Moreover, we found that NKG2D was considerably downregulated on CD8bright T cells with or without CD56 in BD compared with controls, suggesting that the activation of CD8bright T cells may be actively regulated in BD. NKG2D, a functional NK receptor for MHC class I-related chain A (MICA), is constitutively expressed on CD8⁺ T cells and may function as a costimulatory receptor of CD8⁺ T cells (39, 40). Because MICA gene polymorphism is associated with the risk of BD (41), it is possible that aberrant expression of NKG2D on CD8bright T cells in BD may be related to the abnormal expression of MICA on target tissues.

In line with previous reports (12, 42), this study confirms that CD8brightCD56⁺ T cells in active BD uveitis are polarized to produce large amounts of IFN-γ upon stimulation compared with the inactive BD and normal controls. Furthermore, these IFN-γ-producing capacities of CD8brightCD56⁺ T cells are 2-fold greater than those of CD56⁻ counterparts in active BD uveitis. Thus, CD8brightCD56⁺ T cells may contribute to Th1 polarization of BD.

Cytotoxic CD8⁺ T cells destroy their target cells using two distinct cytotoxic molecules such as secreted lytic protein perforin and surface FasL. This study demonstrates that CD8brightCD56⁺ T cells in active BD uveitis are heavily armed with both cytotoxic molecules. Recent studies in normal human also showed that in vitro-stimulated CD8brightCD56⁺ T cells contained high amounts of intracellular perforin (9, 10). In this study, CD8brightCD56⁺ T cells in active BD contained comparable levels of intracellular perforin without in vitro stimulation and exclusively expressed surface FasL upon stimulation. Parallelled with the results of surface markers and cytokine production, both cytotoxic molecules were predominant in CD8brightCD56⁺ T cells compared with CD56⁻ counterparts.

The perforin-dependent cytotoxic pathway dominates in CD8⁺ CTLs and NK cells (43). NK cells, part of the innate immune system, can kill target cells within minutes of the first stimulation of activating receptors and respond rapidly to challenge with limited capacity for Ag loads (44). In contrast, CD8⁺ CTLs, part of the adaptive immune system, can kill multiple target cells after TCR activation and exhibit the slower response with higher capacity for Ag loads (45). We found that MHC-mediated cytotoxicities of freshly isolated CD8bright T cells were increased in active BD uveitis. Interestingly, unstimulated CD8brightCD56⁺ T cells in active BD uveitis showed remarkable NK-like cytolytic activities. Moreover, the cytolytic functions of unstimulated CD8brightCD56⁺ T
cells were mediated through the perforin-dependent pathway. Previous studies also reported that CD8\(^{\text{bright}}\)/CD56\(^{+}\) T cells in normal human showed strong NK-like cytotoxicity after in vitro anti-CD3 or cytokine stimulation (9, 10, 45). Therefore, this suggests that CD8\(^{\text{bright}}\)/CD56\(^{+}\) T cells in active BD uveitis may acquire the NK-like cytotoxic activity in addition to the cytolytic functions of conventional CD8\(^{+}\) CTLs.

The essential retinal finding of Behçet’s uveitis is an obliteration, necrotizing retinal vasculitis resulting in ischemic retinal damages. Previous studies suggested that γδ T cells, neutrophils, CD4\(^{+}\) T cells, or anti-endothelial cell Ab might cause vascular endothelial cell damage of BD uveitis (1–3, 46). However, there has been insufficient evidence regarding the immunopathogenic roles of CD8\(^{\text{bright}}\)/CD56\(^{+}\) T cells in vascular endothelial cell damage. In this study, the cytolytic activities of CD8\(^{\text{bright}}\)/CD56\(^{+}\) T cells against HUVECs were increased in active BD uveitis, particularly in the subsets of CD8\(^{\text{bright}}\)/CD56\(^{+}\) T cells. Furthermore, the cytotoxic mechanisms of CD8\(^{\text{bright}}\)/CD56\(^{+}\) T cells against HUVECs were mediated through FasL-dependent and perforin-dependent pathways. Human CD8\(^{+}\) CTLs can use both FasL and perforin pathways (47).

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References


