Lack of Th17 Cell Generation in Patients with Severe Burn Injuries

Akihito Inatsu, Mari Kogiso, Marc G. Jeschke, Akira Asai, Makiko Kobayashi, David N. Herndon and Fujio Suzuki

*J Immunol* 2011; 187:2155-2161; Prepublished online 5 August 2011; doi: 10.4049/jimmunol.1003235

http://www.jimmunol.org/content/187/5/2155

---

**References**
This article **cites 44 articles**, 10 of which you can access for free at:
http://www.jimmunol.org/content/187/5/2155.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2011 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Lack of Th17 Cell Generation in Patients with Severe Burn Injuries

Akihito Inatsu,* Mari Kogiso,* Marc G. Jeschke,† Akira Asai,* Makiko Kobayashi,*† David N. Herndon,† and Fujio Suzuki*†

Immunodeficient patients with severe burn injuries are extremely susceptible to infection with *Candida albicans*. In addition to Th1 cells, IL-17–producing CD4+ T cells (Th17 cells) have recently been described as an important effector cell in host anti-*Candida* resistance. In this study, therefore, we tried to induce Th17 cells in cultures of severely burned patient PBMC by stimulation with the *C. albicans* Ag (CAg). In the results, the biomarkers for Th17 cells (IL-17 production and intracellular expression of IL-17 and retinoic acid receptor-related orphan receptor γt) were not displayed by burn patient PBMC stimulated with CAg, whereas these biomarkers of Th17 cells were detected in cultures of healthy donor PBMC stimulated with CAg. Burn patient sera were shown to be inhibitory on CAg-stimulated Th17 cell generation in healthy donor PBMC cultures; however, Th17 cells were induced by CAg in healthy donor PBMC cultures supplemented with burn patient sera that were previously treated with anti–IL-10 mAb. Also, the biomarkers of Th17 cells were not induced by CAg in healthy donor PBMC cultures supplemented with rIL-10. IL-10 was detected in serum specimens derived from severely burned patients. These results indicate that Th17 cells are not generated in burn patient PBMC cultures supplemented with CAg. IL-10, produced in response to burn injuries, is shown to be inhibitory on Th17 cell generation. The high susceptibility of severely burned patients to *C. albicans* infection might be influenced if burn-associated IL-10 production is intervened. *The Journal of Immunology*, 2011, 187: 2155–2161.

Despite major advances in the care of severely burned patients, infectious complications remain a leading cause of morbidity and mortality (1, 2). Fungal infections are histopathologically detected in 44% of autopsied patients with an attributable mortality of 33% (3). Adequately managing fungal infections is very important to burned patients with total body surface area (TBSA) burn of >30% because high mortality caused by fungal infections was demonstrated in patients with high TBSA burns (4, 5). *Candida albicans* infection, especially, causes a high mortality rate in patients with extensive burn injuries (6).

In addition to Th1 cells, IL-17–producing CD4+ T cells (Th17 cells) have been recently described as an anti-*Candida* effector cell in the host’s antifungal resistance (7). In response to stimulation with *C. albicans* Ag (CAg), some of the human peripheral blood CD4+ T cells converted to Th17 cells (8). IL-17, produced by Th17 cells, recruits neutrophils into infected skin tissues and increases the antibacterial functions of recruited neutrophils (9, 10). Also, IL-17 enhances the function of epithelial cells, endothelial cells, and macrophages to produce proinflammatory cyto- kines and chemokines, which are factors involved in systemic inflammatory response syndrome (9). These soluble factors contribute to the local eradication of infected cells because the anti-

---

*Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77550; and †Shriners Hospitals for Children, Galveston, TX 77550

Received for publication September 29, 2010. Accepted for publication June 29, 2011.

This work was supported in part by Shriners of North America Grant 8840 (to F.S.), National Institutes of Health Grant P50 GM060338 (to D.N.H.), and a James W. McLaughlin postdoctoral fellowship (to A.A.).

Address correspondence and reprint requests to Dr. Fujio Suzuki, Department of Internal Medicine, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0435. E-mail address: suzuki@utmb.edu

Abbreviations used in this article: CAg, *Candida albicans* Ag; RORγt, retinoic acid receptor-related orphan receptor γt; TBSA, total body surface area.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/S16/00
acute burn phase (within 1 wk of burn injury), and patients 11–26 were in the chronic burn phase (>2 wk after burn injury). Blood samples were individually withdrawn on 0 (on the admission day, inpatients) to 1488 d (outpatients) after burn injury (Table I). As controls, blood specimens were obtained from five healthy donors (three males and two females; mean age 30.8 ± 4.1 y old), and subjected to the same assays.

**Reagents and C. albicans**

FITC-labeled anti-human IL-17A, PE-labeled anti-human retinoic acid receptor-related orphan receptor γt (RORγt), and isotype control mAbs were purchased from eBioscience (San Diego, CA). Human rIL-10 and Cytofix/Cytoperm Plus Fixation/Permeabilization kit with GolgiStop were purchased from BD Biosciences (San Diego, CA). Anti-human IL-4, anti-human CCL2, and anti-human CD3 mAbs were purchased from BioLegend (San Diego, CA). ELISA kits for IL-17A and IL-10 were purchased from Biolegend (San Diego, CA). Anti-human IL-10, anti-human IL-4, anti-human CCL2, and anti-human CD3 mAbs were purchased from eBioscience (San Diego, CA). Human rIL-10 and FITC-labeled anti-human IL-17A, PE-labeled anti-human RORγt mAbs were purchased from Biolegend. For the preparation of CAg, C. albicans serotype A (ATCC 36801) was grown for 2 d at 27˚C in yeast peptone dextrose broth. The heat-killed C. albicans hyphae was designated as CAg. In the experiments, PBMC were stimulated with a constant amount of CAg (corresponded to 10⁵ CFU/ml viable C. albicans) for 1–5 d.

**Isolation of PBMC**

PBMC were isolated from heparinized whole blood using Ficoll-Hypaque density gradient centrifugation. For the cultivation of PBMC, RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) was used.

**Induction of Th17 cells**

IL-17 (a biomarker of Th17 cells) was produced in cultures of PBMC stimulated with CAg. Also, CD4+ T cells in cultures of PBMC stimulated with CAg were detected by FACSCanto as cells expressing intracellular IL-17 and RORγt. When IL-17+ RORγt+ CD4+ T cells with abilities to produce IL-17 were detected in CAg-stimulated PBMC preparations, we considered these cell preparations to include Th17 cells.

**Production and assay of IL-17.** To determine the ability of PBMC to produce IL-17, PBMC (2 × 10⁶ cells/ml) from 26 burn patients were individually stimulated with CAg or anti-CD3 mAb (2.5 μg/ml). As controls, PBMC from five healthy donors were stimulated with CAg in the same conditions. Culture fluids were harvested 1–5 d after cultivation and assayed for IL-17 by ELISA. In our assay system, the detection limit was 8 pg/ml for IL-17.

**Intracellular staining for IL-17 and RORγt.** To determine the percentage of CD4+ T cells that expressed intracellular IL-17 and RORγt in PBMC, PBMC preparations (2 × 10⁶ cells/ml) were stimulated with CAg for 3 d. Then, CD4+ T cells were isolated from these cells using magnetic beads and cultured for an additional 3 h with GolgiStop (0.7 μg/ml). The cells obtained were washed twice with PBS, incubated with Cytofix/Cytoperm solution at 4˚C for 20 min, and then washed with Perm/Wash solution. The cells were incubated with FITC-labeled anti-IL-17A mAb and PE-labeled anti-RORγt mAb, or isotype control mAb at 4˚C for 30 min. After washing, the cells were analyzed for intracellular IL-17A and RORγt expression by FACSCanto.

**Effect of burn patient sera on Th17 cell generation**

To determine the inhibitory effect of burn patient sera on Th17 cell generation, PBMC (2 × 10⁶ cells/ml) from five healthy donors were individually cultured for 3 d with CAg in media supplemented with 15% healthy donor 3 serum, or sera from burn patients 10, 11, 12, 15, and 17. Culture fluids harvested from the above cultures were assayed for IL-17 by ELISA, and cells harvested were analyzed for intracellular IL-17 and RORγt expression by FACSCanto. In the same fashion, the inhibitory effect of IL-10, IL-4, and CCL2 on Th17 cell generation was assayed in cultures of healthy donor PBMC supplemented with sera from burn patients 10, 11, 12, 15, and 17 that were previously treated with 2.5 μg/ml anti-IL-10 mAb, anti-IL-4 mAb, anti-CCL2 mAb, or isotype control Ab (rat IgG2a). To determine the effect of IL-10 on Th17 cell generation, healthy donor PBMC (2 × 10⁶ cells/ml) were stimulated with CAg in the presence of rIL-10 (0.1, 1, or 10 ng/ml). Culture fluids harvested 1–5 d were assayed for IL-17.
5 d after stimulation were assayed for IL-17 by ELISA, and cells harvested 3 d after the stimulation were analyzed for intracellular IL-17 and RORγt expressions by FACSCanto.

Statistical analyses

Statistical analyses were performed using Prism 4.0 software (GraphPad, San Diego, CA). ANOVA with posthoc Bonferroni correction and unpaired Student t test were used. Data are shown as the mean ± SEM. A p value <0.05 was considered to be statistically significant.

Results

Impaired generation of Th17 cells in cultures of severely burned patient PBMC

PBMC (2 × 10^6 cells/ml), isolated from three burn patients (1, acute-phase burn; 12 and 17, chronic-phase burn) and three healthy donors (1–3), were stimulated with CAg in cultures for 1–5 d. Culture fluids harvested were assayed for IL-17. The production of IL-17 by healthy donor PBMC peaked 3 d after cultivation, but declined to undetectable levels within 5 d of cultivation. However, IL-17 was not produced in cultures of burn patient PBMC stimulated with CAg (Fig. 1A). In the next experiments, all 26 burn patient PBMC and 5 healthy donor PBMC were stimulated with CAg or anti-CD3 mAb (2.5 μg/ml) for 3 d. Culture fluids harvested were assayed for IL-17. In the results, IL-17 was not produced by any in the 16 chronic-phase burn patient PBMC stimulated with CAg (Fig. 1A). In the next experiments, all 26 burn patient PBMC and 5 healthy donor PBMC were stimulated with CAg or anti-CD3 mAb (2.5 μg/ml) for 3 d. Culture fluids harvested were assayed for IL-17. In the results, IL-17 was not produced by any in the 16 chronic-phase burn patient PBMC stimulated with CAg (Fig. 1B). After stimulation with CAg, PBMC from acute-phase burn patients produced 0–0.32 ng/ml IL-17 (Fig. 1B). During the acute-phase burn injuries, production of various cytokines has been demonstrated (16). Healthy donor PBMC stimulated with CAg produced 2.2–3.4 ng/ml IL-17 (Fig. 1B). After stimulation with anti-CD3 mAb, chronic-phase burn patient PBMC produced <0.56 ng/ml IL-17 into their culture fluids (Fig. 1C). IL-17 at amounts of 0.1–0.75 ng/ml was produced by all patient PBMC when they were stimulated with anti-CD3 mAb, whereas 2.7–3.8 ng/ml IL-17 were produced by healthy donor PBMC stimulated with anti-CD3 mAb. These results indicate that, in cultures of PBMC isolated from burn patients, IL-17–producing cells are not generated, significantly, after stimulation with CAg.

In the next experiments, PBMC (2 × 10^6 cells/ml) from burn patient 10 and healthy donor 1 were stimulated with CAg for 3 d and analyzed for IL-17+ RORγt+ CD4+ T cells by flow cytometry. The same experiments were performed using healthy donors 2–5 and burn patients 11 and 15. Fig. 2 shows the

FIGURE 2. Detection of IL-17+ RORγt+ CD4+ T cells in cultures of PBMC stimulated with CAg. PBMC (2 × 10^6 cells/ml) isolated from three burn patients (10, 11, and 15) and five healthy donors (1–5) were stimulated with CAg or without for 3 d. Then, CD4+ T cells isolated from these cells were analyzed for IL-17 and RORγt by flow cytometry.

FIGURE 3. Effect of burn patient sera on CAg-stimulated Th17 cell generation in healthy donor PBMC cultures. PBMC (2 × 10^6 cells/ml) isolated from healthy donors (1–5) were individually cultured with media alone, media plus CAg, media plus CAg and healthy donor 3 serum (15%), and media plus CAg and sera (15%) from burn patients 10, 11, 12, 15, and 17. Culture fluids harvested 3 d after cultivation were assayed for IL-17 (A). Cells harvested 3 d after cultivation were analyzed for their intracellular expression of IL-17 and RORγt by flow cytometry (B). The results obtained were combined and displayed in the figure by the mean ± SEM of the five experiments. **p < 0.01 versus PBMC cultured with CAg and healthy donor serum.
percentage of IL-17+ RORγt+ cells in these two groups of CD4+ T cells. After stimulation with CAg, the increased generation (9.4-fold increase) of IL-17+ RORγt+ cells (%) was demonstrated in cultures of healthy donor CD4+ T cells. However, the generation of IL-17+ RORγt+ CD4+ T cells did not increase in cultures of burn patient PBMC stimulated with CAg. These results indicate that Th17 cells are not easily generated in burn patient PBMC cultures supplemented with CAg.

Burn patient sera are inhibitory on Th17 cell generation

The effect of burn patient sera on Th17 cell generation in CAg-stimulated healthy donor PBMC cultures was examined. PBMC (2 × 10^6 cells/ml) from healthy donor 1 were cultured with media supplemented together with CAg and sera (15%) from burn patients 10, 11, 12, 15, and 17 for 3 d. The amount of patient serum added to the culture media was decided based on a dose-response curve shown in our preliminary studies. Various concentrations of patient 9 serum were added to the cultures of healthy donor PBMC stimulated with CAg. After cultivation, the ability of these PBMC to produce IL-17 was determined. In the results, 1.8–2.5 ng/ml IL-17 was produced by 2 × 10^6 cells/ml PBMC stimulated with CAg, and the similar amounts of IL-17 were produced by the PBMC cultured with 2.5–5% (v/v) of patient serum. However, IL-17 production was clearly reduced in PBMC cultures supplemented with 10–20% patient serum. Due to the limited amount of patient serum, we have used the patient serum at a concentration of 15% throughout the experiments. As a control, the same PBMC were cultured with media supplemented with CAg and burn patient serum treated with isotype control Ab.

**FIGURE 4.** Generation of Th17 cells in healthy donor PBMC cultures supplemented with burn patient sera previously treated with various mAbs. Five PBMC preparations (2 × 10^6 cells/ml) isolated from healthy donors (1–5) were individually cultured with CAg and sera from burn patients 10, 11, 12, and 15. Before being used to cultures, burn patient sera were treated with 2.5 μg/ml anti–IL-10, anti–IL-4, anti-CCL2 mAb, or isotype control Ab (rat IgG2a) for 30 min at 4°C. Culture fluids harvested 3 d after stimulation were assayed for IL-17, and cells harvested from these cultures were analyzed for intracellular expression of IL-17 and RORγt by flow cytometry. The results obtained were combined and displayed in the figure by the mean ± SEM of the five experiments. **p < 0.01 versus PBMC cultured with CAg and burn patient serum treated with isotype control Ab.

Table I. Burn patients and healthy donors enrolled in the study

<table>
<thead>
<tr>
<th>Healthy Donors (Donor No.)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Age (y)</td>
<td>35</td>
<td>23</td>
<td>45</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>10</td>
<td>40</td>
<td>22</td>
<td>8</td>
<td>15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acute-Phase Burn Patients (Patient No.)a</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Age (y)</td>
<td>54</td>
<td>28</td>
<td>28</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>TBSA burn (%)</td>
<td>38</td>
<td>80</td>
<td>80</td>
<td>70</td>
<td>35</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>3rd degree (%)</td>
<td>38</td>
<td>80</td>
<td>80</td>
<td>70</td>
<td>35</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>2nd degree (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Days after burn injuryb</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>IL-10 (pg/ml)c</td>
<td>120</td>
<td>76</td>
<td>80</td>
<td>145</td>
<td>120</td>
<td>220</td>
<td>180</td>
<td>250</td>
<td>560</td>
<td>868</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chronic-Phase Burn Patients (Patient No.)a</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Age (y)</td>
<td>4</td>
<td>50</td>
<td>3</td>
<td>10</td>
<td>15</td>
<td>4</td>
<td>12</td>
<td>14</td>
<td>13</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>15</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>TBSA burn (%)</td>
<td>42</td>
<td>80</td>
<td>33</td>
<td>61</td>
<td>55</td>
<td>58</td>
<td>49</td>
<td>85</td>
<td>46</td>
<td>52</td>
<td>84</td>
<td>44</td>
<td>53</td>
<td>83</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>3rd degree (%)</td>
<td>42</td>
<td>80</td>
<td>33</td>
<td>37</td>
<td>45</td>
<td>58</td>
<td>34</td>
<td>85</td>
<td>46</td>
<td>52</td>
<td>74</td>
<td>37</td>
<td>53</td>
<td>73</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td>2nd degree (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>10</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Days after burn injuryb</td>
<td>14</td>
<td>17</td>
<td>22</td>
<td>24</td>
<td>29</td>
<td>30</td>
<td>212</td>
<td>305</td>
<td>363</td>
<td>434</td>
<td>449</td>
<td>586</td>
<td>694</td>
<td>704</td>
<td>829</td>
<td>1488</td>
</tr>
<tr>
<td>IL-10 (pg/ml)c</td>
<td>620</td>
<td>880</td>
<td>640</td>
<td>440</td>
<td>380</td>
<td>675</td>
<td>646</td>
<td>310</td>
<td>225</td>
<td>145</td>
<td>220</td>
<td>176</td>
<td>120</td>
<td>140</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>

aAll patients were subjected to our standard therapeutic protocol, including early excision of burn wound, systemic antibiotic therapy, and continuous enteral feeding.
bDay of blood sample collection relative to day of injury; day 0 = same day as injury, day 1 = 1 d after injury. Blood samples were taken once from each patient.
cThe amounts of IL-10 in sera of these patients were measured by ELISA.

F, female; M, male.
supplemented with healthy donor 3 serum in the same fashion. Culture fluids harvested were tested for IL-17 by ELISA, and cells harvested from the cultures were analyzed for intracellular IL-17 and RORγt by flow cytometry. The same experiments were performed using PBMC from healthy donors 2–5. Obtained results were combined and displayed in Fig. 3A (IL-17 production) and Fig. 3B (intracellular expression of IL-17 and RORγt) as a mean ± SEM. In the presence of burn patient sera, IL-17 was not produced by healthy donor PBMC stimulated with CAg (Fig. 3A). Also, the generation of IL-17 RORγt CD4+ T cells was not increased in CAg-stimulated healthy donor PBMC cultures supplemented with burn patient sera (Fig. 3B). These results indicate that soluble factors present in the burn patient serum are inhibitory on CAg-stimulated generation of Th17 cells in PBMC cultures.

In previous studies, IL-10, IL-4, and CCL2 were detected in the sera of patients with acute- and chronic-phase burn injuries (17–19). Therefore, the role of IL-10, IL-4, and CCL2 on CAg-stimulated Th17 cell generation in healthy donor PBMC cultures was examined. Thus, in the presence of CAg, healthy donor 1 PBMC (2 × 10^6 cells/ml) were cultured with media supplemented with sera from burn patients 10, 11, 12, 15, and 17 that were previously treated with 2.5 μg/ml anti–IL-10 mAb, anti–IL-4 mAb, or anti-CCL2 mAb. Culture fluids harvested 3 d after cultivation were assayed for IL-17 by ELISA, and cells harvested from these cultures were analyzed for intracellular IL-17 and RORγt by flow cytometry. The same experiments were performed using PBMC from healthy donors 2–5. Obtained results were combined and displayed in Fig. 4 as the mean. By CAg stimulation, IL-17 CD4+ RORγt+ T cells were generated in healthy donor PBMC cultures supplemented with burn patient sera previously treated with anti–IL-10 mAb, whereas these cells were not demonstrated in the same cultures supplemented with burn patient sera that were treated with mAb directed against IL-4 or CCL2. These results suggest that IL-10 present in burn patient sera is inhibitory on CAg-stimulated generation of Th17 cells in PBMC cultures.

As shown in Table I, IL-10 was detected in sera of all burn patients. Therefore, in the next experiments, the inhibitory effect of rIL-10 on CAg-stimulated generation of Th17 cells in healthy donor PBMC cultures was confirmed. Thus, PBMC (2 × 10^6 cells/ml) from healthy donors 1–5 were stimulated with CAg in the presence of various doses of rIL-10 for 1–5 d. Culture fluids harvested were assayed for IL-17 by ELISA. In the results, IL-17 was not produced in CAg-stimulated healthy donor PBMC cultures supplemented with rIL-10 (Fig. 5). The inhibitory effect of rIL-10 on IL-17 production by CAg-stimulated healthy donor PBMC was shown as dose dependent. Also, the generation of IL-17 RORγt CD4+ T cells was greatly decreased by 80% when healthy donor PBMC were cultured with 10 ng/ml rIL-10 (Fig. 6). These results indicate that rIL-10 inhibits CAg-stimulated generation of Th17 cells in healthy donor PBMC cultures.

Discussion

C. albicans is the most significant human fungal pathogen. Although C. albicans is normally commensal, candidiasis may develop in immunosuppressed hosts (20). A major factor in host resistance against systemic C. albicans infection is Th1 cell-mediated immunity (21). In response to the specific Ag, IL-2 and IFN-γ produced by Th1 cells stimulate cytocidal activities of various effector cells against cells infected with C. albicans (21). However, Th1 responses are suppressed by IL-10 (22), which is a regulatory cytokine produced by regulatory T cells, monocytes/macrophages, B cells, and some other T cell popula-

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Effect of rIL-10 on Th17 cell generation in cultures of healthy donor PBMC stimulated with CAg. Five PBMC preparations (2 × 10^6 cells/ml) isolated from healthy donors 1–5 were individually cultured with CAg and various doses of rIL-10 (none, ◊; 0.1 ng/ml, ●; 1 ng/ml, △; 10 ng/ml, ▲). Culture fluids harvested 1–5 d after stimulation were assayed for IL-17. The results obtained were combined and displayed to the figure by the mean ± SEM of the five experiments. *p < 0.05, **p < 0.01 versus cultured with media alone.

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** CAg-stimulated generation of IL-17 RORγt CD4+ T cells in healthy donor PBMC cultures supplemented with rIL-10. Five PBMC preparations (2 × 10^6 cells/ml) isolated from healthy donors 1–5 were individually stimulated with CAg in the presence of rIL-10 (10 ng/ml) for 3 d. Harvested cells were analyzed for their intracellular expression of IL-17 and RORγt by flow cytometry. The obtained results were combined and displayed to the figure as mean ± SEM for IL-17 RORγt CD4+ T cells. *p < 0.05 versus PBMC cultured with media added without rIL-10.

We have demonstrated that severely burned mice were 50 times or more susceptible to the systemic infection with *C. albicans* than that of normal mice (23). A predominance of Th2 cells in humans and rodents following severe burn injury has been demonstrated (23–25).

Th17 cells (IL-17+ RORγt CD4+ T cells), a new subset of CD4+ T cells, are additionally described as host anti-*Candida* effector cells (7, 12). In healthy conditions, Th17 cells are generated by the CAg stimulation from CD4+ T cells (8, 12). IL-17 produced by Th17 cells directly and indirectly induces neutrophil recruitment and activation (10, 26). Indeed, IL-17 deficiencies are associated with the defects of neutrophil responses (27). Due to the neutrophil dysfunction, IL-17R knockout mice are highly susceptible to *C. albicans* infection (12).

In this study, we tried to generate Th17 cells by CAg stimulation in cultures of burn patient PBMC. To detect Th17 cells, three different biomarkers (IL-17 production, intracellular expression of IL-17, and RORγt) were used. RORγt is a key transcription factor for the generation of Th17 cells, and transcription of the genes encoding IL-17 is induced by RORγt (8). In the results, CD4+ T cells with abilities to produce IL-17 and express intracellular IL-
17 and RORγt were generated in cultures of healthy donor PBMC stimulated with CAg. However, cells with abilities to produce IL-17 and express RORγt were not induced by the CAg in cultures of chronic-phase burn patient PBMC. It has been described in previous papers (3, 28–30) that severely burned patients who survive longer after burn injury have a higher incidence of candidiasis. In contrast, PBMC from acute-phase burn patients produced small amounts of IL-17 under CAg stimulation. These results suggest that the generation of Th17 cells is impaired in peripheral blood of severely burned patients exposed to C. albicans infection.

Neutrophils treated with 100 ng/ml or more IL-17 increased their ability to kill Streptococcus pneumoniae (10). Also, the candidacidal activities of neutrophils were enhanced when they were treated with 1 ng/ml or more IL-17 (31). These results indicate that 1 ng/ml or more doses of IL-17 are required for the direct activation of neutrophils. Other reports described that 100 ng/ml IL-17 is needed to induce IL-8 production by epithelial cells. Because IL-8 is known to be a major activator of neutrophils, this suggests that 100 ng/ml IL-17 is required for indirect activation of neutrophils. A recent paper (16) described that IL-17 is detected in the plasma of patients 0–55 days postburn injury. However, the amounts of IL-17 detected in plasma of burn patients were 3–9 pg/ml. Therefore, the influence of Th17 cells that appeared in response to burn injury on the antifungal activities of neutrophils is minimal.

It is well known that pro- and anti-inflammatory cytokine production is elevated following burn injuries (16, 32, 33). Among these cytokines, IL-10 is associated with frequent occurrence of sepsis and high mortality rate in severely burned patients (34). During infection, IL-10 inhibits the activity of Th1 cells, NK cells, and macrophages, all of which play a role in the optimal clearance of the invading pathogen (35). IL-10 allows long-term escape of C. albicans from antifungal host defense and allows persistent infection of the pathogen (36). Recently, the role of IL-10 on Th17 cell generation has been described (37–39). After stimulation with IL-23, IL-6, and TGF-β, IL-17 production and intracellular RORγt expression were greatly increased in cultures of splenic CD4+ T cells isolated from IL-10 knockout mice. When these cells were cultured with IL-23 in the presence of rL-10, the generation of Th17 cells was clearly reduced (37, 38). Also, IL-17 production was not induced by IL-6, TGF-β, and IL-23 in human CD4+ T cell cultures supplemented with rL-10 (39). These findings strongly suggest that IL-10 is inhibitory on Th17 cell generation.

In this study, IL-17+ RORγt+ CD4+ T cells were not generated in healthy donor PBMC cultures supplemented with the burn patient serum. However, these effector cells were generated in healthy donor PBMC cultures supplemented with the same patient serum previously treated with anti–IL-10 mAb. In addition, Th17 cell generation was dose dependently suppressed by rL-10 in cultures of healthy donor PBMC stimulated with CAg. These results indicate that IL-10 present in burn patient serum plays a role in the decreased generation of Th17 cells in PBMC cultures. IL-10 has been widely demonstrated in the sera of severely burned patients (40–42). Th2 cells, M2M, and PMN-II have been described as sources of IL-10 in these patients (40–42). The impaired generation and function of Th1 cells in severely burned patients have been well documented. In severely burned patients lacking in Th1 responses, Th2 responses were shown to develop aggressively (24, 25). Some of Th2 cells were known to remain for a long time in chronic-phase burn patients as IL-4+ cells (19, 43). It has been described in many papers that the generation of Th2 cells occurs under stimulation with small amounts of IL-4 released from IL-4+ cells (44–46). In chronic-phase burn patients who were invaded with pathogens, Th2 responses were easily developed in association with minimal amounts of IL-4 released from IL-4+ cells. In our recent experiments, IL-4+ T cells were detected in the CD3+ cell population derived from patients 434–586 days after burn injury. In cultures of PBMC derived from these chronic burn patients, Th2 cells were predominantly induced by CAg stimuli. Because Th2 cells are major IL-10 producer cells, IL-4+ T cells with the ability to produce small amounts of IL-4 may be involved in inhibiting Th17 cell responsiveness in chronic-phase burn patients.

In this study, the inhibition of Th17 cell responsiveness was demonstrated in severely burned patients. This phenomenon may not be particularly attributable to the pathology of burn injury. Physiological threat such as surgery and trauma, and general burn care treatment may be involved in the impaired Th17 cell responsiveness. Because all patients enrolled in this study were diagnosed with systemic inflammatory response syndrome, but not sepsis, the influence of sepsis on Th17 cell responsiveness is unknown. To clarify the correlation between clinical outcome and suppression of Th17 cell responsiveness in severely burned patients, further studies are required.

In conclusion, IL-17+ RORγt+ CD4+ T cells were not induced by the CAg stimulation in cultures of burn patient PBMC. IL-10, which was widely present in sera of severely burned patients, was shown to be inhibitory on Th17 cell generation in PBMC cultures. IL-10 was shown to be responsible for the lack of Th17 cells in patients with severe burn injury; this cytokine contributed to the homeostasis of excessive inflammation in such patients. Immunological intervention of IL-10 production should be performed under the careful monitoring of the balance of inflammatory and anti-inflammatory parameters.

Disclosures
The authors have no financial conflicts of interest.

References