Flow cytometric assessment of human peripheral blood mononuclear cells in response to a coccidioidal antigen

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The in vitro responses of peripheral blood mononuclear cells (PBMC) from healthy immune and non-immune donors were assessed by flow cytometry after incubation with the coccidioidal antigen toluene spherule lysate (TSL). After 120 h of incubation with 100 μg ml⁻¹ of TSL, expression of the activation markers CD69, CD25 and human leukocyte antigen-DR were all significantly increased in CD3⁺ lymphocytes from immune donors compared to non-immune donors (P < 0.03 for all). No differences in the surface expression of the costimulatory molecules CD28, CD152 or CD154 was seen between immune and non-immune donors after either 24 or 120 h of TSL incubation, nor were differences detected in the expression of the B7 ligands CD80 or CD86 on CD14⁺ monocytes. The percent of CD3⁺ lymphocytes expressing intracellular interferon-γ (IFN-γ) was significantly increased in immune compared to non-immune donors and was further increased by the addition of 10 ng ml⁻¹ of human recombinant interleukin (IL)-12 (P < 0.05 for both). Both CD4⁺ and CD8⁺ lymphocytes contributed to IFN-γ production. These data indicate that coccidioidal antigen stimulation of lymphocytes from healthy immune donors leads to specific expression of activation molecules and production of intracellular IFN-γ. Addition of IL-12 leads to a significant recruitment of cells producing IFN-γ among immune donors.

Keywords coccidioidomycosis, cellular immunology, flow cytometry, intracellular cytokine production

Introduction

Coccidioidomycosis is a disease due to the soil-dwelling dimorphic fungus Coccidioides immitis. Infection is endemic to the desert southwest regions of the United States and northern Mexico and typically occurs after inhalation of dust containing coccidioidal arthroconidia [1]. In humans, successful host immunity appears to reside in an appropriate cellular immune response, with those who control infection developing positive delayed-type hypersensitivity (DTH) to coccidioidal antigens [2].

In previous studies, we have shown that peripheral blood mononuclear cells (PBMC) from healthy, DTH-positive donors produce more interleukin (IL)-2 and interferon-γ (IFN-γ) and undergo enhanced lymphocyte transformation in response to in vitro incubation with the coccidioidal antigen toluene spherule lysate (TSL) than do PBMC from healthy, non-immune donors [3]. Moreover, IFN-γ production by PBMC from donors with disseminated coccidioidomycosis is significantly less than that by PBMC from healthy, asymptomatic donors [4]. While IFN-γ production by PBMC from coccidioidal DTH-positive donors is dramatically increased by IL-12, no increase is seen in DTH-negative donors with disseminated coccidioidomycosis [5].
To further explore the characteristics of the in vitro human immunological response in coccidioidomycosis, we used flow cytometry to examine the expression of surface activation and costimulatory molecules on PBMC from healthy immune and non-immune donors after incubation with TSL. In addition, we examined the intracellular expression of IFN-γ and the effect of IL-12 on IFN-γ expression by flow cytometry.

Materials and methods

Subjects and DTH assessment

The use of human subjects in this study was performed under the guidelines and approval of the Human Subjects Committee of the University of Arizona. All subjects underwent skin testing using usual-strength Spherulin® (ALK Laboratories, Berkeley, CA, USA) for the assessment of DTH as previously described [5]. There were two groups of subjects in the study. The first, comprising the immune group, consisted of healthy individuals who expressed positive coccidioidal DTH and had no evidence of active coccidioidomycosis. The second, making up the non-immune group, consisted of healthy individuals who did not express coccidioidal DTH and were also without evidence of active coccidioidomycosis.

Isolation of PBMC and lymphocyte transformation assay

PBMC were isolated from heparinized blood using Ficoll-Hypaque (Pharmacia, Piscataway, NJ, USA) as previously described [5]. To reduce red cell contamination, 2 ml of cold 0.2% saline was added to the PBMC pellet for 30 s followed by 2 ml of cold 1-6% saline.

The lymphocyte transformation assay was performed as previously described [3]. Briefly, 5×10⁵ viable PBMC were placed into each flat-bottomed well of a 96-well plate (Corning Glass Works, Corning, NY, USA) in RPMI-1640 (GIBCO, Grand Island, NY, USA) containing 10% heat-inactivated pooled human AB serum (GIBCO). TSL, a gift from Dr John Gallgani (University of Arizona, Tucson, AZ, USA), was added to appropriate replicate wells at a concentration of 100 μg ml⁻¹ and the plate incubated for 120 h at 37 °C in 95% air/5% CO₂. After that time, [³H]-thymidine (0.5 μCi ml⁻¹; NEN, Boston, MA, USA) was added to each well and, after an additional 18–24 h of incubation, each well was harvested onto glass filter paper and counted by scintillation spectrometry. Results are expressed as a stimulation index (SI), determined from the counts per minute (cpm) of the wells containing the antigen divided by the control wells without antigen.

Analysis of cell surface markers

PBMC were placed in 2 ml of RPMI-1640 (GIBCO) with 10% heat-inactivated pooled AB human serum (GIBCO) in 5 ml snap-cap polypropylene tubes at a concentration of 3×10⁶ cells ml⁻¹ and incubated at 37 °C in 5% CO₂/95% air from 6–120 h with either RPMI-1640 alone (control) or with 100 μg ml⁻¹ of TSL. At the end of this time, the cells were centrifuged (30 min, room temperature, 300 g) in staining buffer (phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.1% NaN₃) at 4 °C and the pellet resuspended in fixing buffer (PBS with 1% paraformaldehyde) and incubated for 15 min in the dark on ice. After an additional centrifugation (30 min, room temperature, 300 g), the pellet was placed into freeze buffer (PBS with 10% dimethylsulfoxide (DMSO) and 1% BSA) and frozen at −70 °C. Cells were later thawed in a 37 °C water bath, washed in staining buffer, and resuspended in staining buffer containing the appropriately labeled monoclonal antibody (mAb) for 30 min in the dark on ice. The following mAbs were used to label the cells: fluorescein isothiocyanate (FITC)-labeled anti-CD3 and anti-CD14; phycoerythrin (PE)-labeled anti-CD69, anti-CD25, anti-CD152, anti-CD154, anti-human leukocyte antigen (HLA)-DR, anti-CD80, and anti-CD86 (BDIS, San Jose, CA, USA). In preliminary experiments, results of staining of cells thawed from a frozen state were compared to cells that had not been frozen. No difference in results was appreciated.

For determination of intracellular IFN-γ production, a modification of a previously described technique was used [6]. PBMC were incubated for 24 h, a time found to be optimal for IFN-γ expression in our system (data not shown). Tubes received either RPMI-1640 alone; 10 ng ml⁻¹ of human recombinant IL-12 (Pharmingen, La Jolla, CA, USA); 100 μg ml⁻¹ of TSL; or 100 μg ml⁻¹ of TSL plus 10 ng ml⁻¹ of IL-12. All tubes contained 10 μg ml⁻¹ of anti-CD28 mAb (Pharmingen). During the final 6 h of incubation, 10 μg ml⁻¹ of brefeldin A (Sigma Chemical, St. Louis, MO, USA) was added to all tubes.

After 24 h, cells were washed in staining buffer, placed into fixing buffer for 15 min in the dark on ice, and then frozen at −70 °C in freeze buffer. Frozen cells were thawed in a 37 °C water bath, aliquoted to 5–10×10⁵ cells per polystyrene tube and resuspended in 0.5 ml of 1× Permiabilizing Solution (BDIS). Tubes
were then incubated for 10 min in the dark at room temperature, washed in Permiabilization Wash Solution (Pharmingen), then placed into staining buffer containing the appropriate labeled mAb and incubated for 30 min in the dark on ice. Cells were triple-labeled with the following mAbs: FITC-labeled anti-IFN-γ, PE-labeled anti-CD69, and PerCP-labeled anti-CD3, anti-CD4 or anti-CD8 (all from BDIS).

**Flow cytometry analysis**

Prior to flow cytometry, all cells were washed and resuspended in PBS. Data were acquired using a FACScan single argon laser flow cytometer (BDIS) and analyzed using CellQuest software (BDIS). For each analysis, 25,000 events were acquired. For T-lymphocyte analysis, a gate was set on cells with forward- and side-scatter characteristics of live lymphocytes. Further gating was performed on cells expressing either the CD3, CD4 or CD8 antigen. For monocyte analysis, a gate was set on CD14+ cells with forward- and side-scatter characteristics of live monocytes. Unstained samples and samples stained with isotype-matched negative control reagents were used to verify staining specificity and to determine thresholds for positivity.

**Statistical analysis**

Data were analyzed based on the percentage of cells expressing a fluorescent intensity above threshold. Data are expressed as mean ± SEM. Continuous, unpaired variables in two groups were analyzed using the Student t-test and the paired t-test was employed for continuous, paired data. A two-tailed P-value < 0.05 was considered to be significant.

**Results**

**Surface marker expression**

Expression of surface activation markers on CD3+ lymphocytes was examined in PBMC that had been incubated for 120 h with or without 100 μg ml⁻¹ of TSL. As shown in Figure 1, the mean ± SEM percent expression of CD69, CD25 and human leukocyte antigen (HLA)-DR was significantly higher among immune donors compared to non-immune donors (P < 0.03 for all). These differences paralleled the difference seen in lymphocyte transformation between the two groups.

On the other hand, no significant differences were observed between CD3+ lymphocytes from immune and non-immune donors for the expression of the costimulatory molecules CD28, CD152 (CTLA-4) and CD154 (CD40-ligand) after incubation with TSL for either 24 or 120 h (Table 1). Overall, CD28 was expressed on > 80% of CD3+ lymphocytes from both immune and non-immune donors, while CD152 and CD154 were expressed on < 2% of cells from both donor groups at both periods of incubation with TSL antigen.

The expression of B7 molecules on CD14+ monocytes from immune and non-immune donors after TSL incubation was also assessed. Based on previously published data [7] and preliminary experiments, an incubation time with 100 μg ml⁻¹ of TSL for 6 h was employed. The mean ± SEM percent of CD14+ monocytes that expressed CD80 on their surface from four immune donors was 0.3 ± 0.2 when incubated in cell culture medium alone compared to 3.32 ± 2.23 when incubated with TSL (P = 0.06). The mean ± SEM percent for three non-immune donors when incubated with cell culture medium alone was 0.2 ± 0.1 compared with 2.68 ± 2.10 when in...
Table 1  Percent of CD3+ lymphocytes expressing CD28, CD152 and CD154 on their surface using PBMC from immune and non-immune donors and incubated with cell-culture medium alone (Control) or with 100 μg ml⁻¹ of TSL in vitro for 24 and 120 h

<table>
<thead>
<tr>
<th>Marker</th>
<th>Group</th>
<th>Incubation time (h)</th>
<th></th>
<th></th>
<th></th>
<th>Non-immune</th>
<th>Immune</th>
<th>P*</th>
<th>Non-immune</th>
<th>Immune</th>
<th>P*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Donors (n)</td>
<td></td>
<td>24</td>
<td>120</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>3</td>
<td>4</td>
<td>0.67</td>
<td>89.9 ± 3.1</td>
<td>88.8 ± 3.6</td>
<td>0.84</td>
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<tr>
<td>CD28</td>
<td>TSL</td>
<td>90.3 ± 2.9</td>
<td>0.07</td>
<td>0.59</td>
<td>0.57</td>
<td>86.9 ± 5.8</td>
<td>84.3 ± 5.2</td>
<td>0.74</td>
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<tr>
<td>CD152</td>
<td>Control</td>
<td>0.86 ± 0.64</td>
<td>0.71 ± 0.48</td>
<td>0.84</td>
<td>0.10 ± 0.04</td>
<td>0.15 ± 0.06</td>
<td>0.55</td>
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<tr>
<td>TSL</td>
<td>P†</td>
<td>0.25 ± 0.13</td>
<td>0.68 ± 0.25</td>
<td>0.24</td>
<td>0.41 ± 0.20</td>
<td>0.96 ± 0.37</td>
<td>0.25</td>
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<tr>
<td>CD154</td>
<td>Control</td>
<td>0.07 ± 0.05</td>
<td>0.11 ± 0.06</td>
<td>0.70</td>
<td>0.04 ± 0.01</td>
<td>0.12 ± 0.09</td>
<td>0.44</td>
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<tr>
<td>TSL</td>
<td>P†</td>
<td>0.58 ± 0.37</td>
<td>1.52 ± 1.34</td>
<td>0.60</td>
<td>0.04 ± 0.02</td>
<td>0.20 ± 0.09</td>
<td>0.14</td>
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<tr>
<td></td>
<td></td>
<td>0.25</td>
<td>0.37</td>
<td>0.99</td>
<td>0.57</td>
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</table>

Results are expressed as mean ± SEM.
* P-values comparing immune and non-immune donors;
† P-values comparing medium alone to TSL among immune and non-immune donors.

cubated with TSL (P = 0.15). There was no significant difference between the percent expression of CD80 for immune and non-immune donors after incubation with TSL (P = 0.72). For these same donors and under the same conditions, the mean ± SEM of cells from immune donors expressing CD86 was 89.3 ± 4.9 for culture medium alone and 64.6 ± 20.5 for TSL (P = 0.03), compared with 97.4 ± 1.2 for culture medium alone and 72.2 ± 27.5 for TSL from non-immune donors (P = 0.22). There was no significant difference in the expression of CD86 between immune and non-immune donors after incubation with TSL (P = 0.69).

Intracellular IFN-κ expression

The percent of CD3+ lymphocytes expressing intracellular IFN-γ was examined after 24 h of incubation with cell culture medium alone (control), with 10 ng ml⁻¹ of IL-12, with 100 μg ml⁻¹ of TSL, or with the combination of TSL and IL-12. Figure 2 displays the flow cytometric results from a representative immune and non-immune donor. The percentage of CD3+ lymphocytes producing intracellular IFN-γ was significantly higher in cells incubated with TSL alone from immune compared to non-immune donors (Table 2). Moreover, the combination of TSL and IL-12 resulted in a significant increase in the percentage of CD3+ cells producing IFN-γ compared to those incubated with TSL alone in immune donors (P = 0.01) but not in non-immune donors (P = 0.22). Although the addition of IL-12 alone appeared to increase the amount of intracellular IFN-γ produced compared to control among both immune and non-immune donors, these increases were not significant (P = 0.09 and 0.07, respectively).

The production of CD4 and CD8 lymphocytes in the production of IFN-γ was also assessed. Using PBMC incubated with 100 μg ml⁻¹ of TSL alone from four immune donors, 1.15 ± 0.57% of CD3+ lymphocytes produced IFN-γ, compared to 0.56 ± 0.26% of CD4+ lymphocytes and 1.96 ± 1.33% of CD8+ lymphocytes. Using cells incubated with the combination of 100 μg ml⁻¹ of TSL and 10 ng ml⁻¹ of IL-12 from these same donors, 4.51 ± 1.67% of CD3+ cells produced IFN-γ, compared to 3.55 ± 2.17% of CD4+ cells and 3.90 ± 1.74% of CD8+ cells. Hence, both CD4+ and CD8+ cells contributed to the production of intracellular IFN-γ by T-lymphocytes from immune donors.

Discussion

Successful host immunity in human coccidioidomycosis depends on an appropriate cellular immune response [2]. We have previously demonstrated that immune individuals can be readily distinguished from non-immune donors through in vitro analysis of PBMC response to the coccidioidal antigen TSL [3–5]. In the present work, we have expanded on this analysis by examining the flow cytometric characteristics of that response among healthy immune and non-immune donors.
Flow cytometry in human coccidioidomycosis

In addition to activation antigens, we also examined the expression of the costimulatory molecules CD28, CD152 (CTLA-4), and CD154 (CD40-ligand) on CD3+ lymphocytes, and the B7 ligands CD80 and CD86 on CD14+ monocytes. Although changes in surface expression of costimulatory molecules in response to antigen stimulation have been reported by others [11,12], we were unable to detect any significant alterations in the surface expression of these molecules under the conditions tested. The reasons for this are not clear. One possibility is that the antigen used in this study, TSL, does not strongly induce these activation antigens, a concept supported by work with Cryptococcus neoformans [13]. It is also possible that changes did occur, but at a frequency too low to be detected.

Using the technique of intracellular cytokine detection, we found a significant increase in the number of CD3+ lymphocytes expressing IFN-γ among immune donors compared to cells from non-immune donors. This validates earlier work where PBMC supernatants were assayed for IFN-γ using enzyme-linked immunosorbent assay (ELISA) [5]. Most striking is the marked increase in the frequency of cells from immune donors expressing intracellular IFN-γ in response to TSL combined with IL-12. These data suggest that the increase in IFN-γ production by IL-12 noted in earlier work [5] is due, in part, to an increase in the frequency of cells producing IFN-γ and not simply due to an increase in IFN-γ production by individual cells. Moreover, both CD4+ and CD8+ were found to contribute to the production of IFN-γ, a finding similar to observations of healthy donors after vaccination with influenza and tetanus [14].

Intracellular cytokine detection by flow cytometry has been used by others as a way to measure the immune response by quantitating the frequency of cells producing cytokines. For example, it has been used to measure CD4+ lymphocyte production of tumor necrosis factor (TNF-α) in response to cytomegalovirus antigen among human immunodeficiency virus (HIV)-infected patients [15]. Our data support the concept that these same methods can be applied to human coccidioidomycosis.

In this work, CD3+ lymphocyte expression of CD69, CD25, and HLA-DR was found to increase in response to TSL among immune but not among non-immune donors. This increased expression paralleled the lymphocyte proliferation assay. CD69 is a type II integral membrane protein which is expressed early on activated lymphocytes and natural killer cells but not on resting cells [7,8]. In other experimental models, expression of CD69 has allowed for rapid assessment of immune response [9,10]. Our results indicate that measurement of expression of CD69, as well as CD25 and HLA-DR, may serve a similar purpose in human coccidioidomycosis.

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Table 2 Percentage of CD3+ lymphocytes producing intracellular IFN-γ after incubation with cell-culture medium alone (Control); with 10 ng ml⁻¹ of IL-12; with 100 μg ml⁻¹ of the coccidioidal antigen TSL; or with the combination of TSL and IL-12 (TSL + IL-12)

<table>
<thead>
<tr>
<th>Subject (n)</th>
<th>Control</th>
<th>IL-12</th>
<th>TSL</th>
<th>TSL + IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immune (5)</td>
<td>0.05 ± 0.02</td>
<td>0.27 ± 0.08</td>
<td>0.11 ± 0.04</td>
<td>0.46 ± 0.25</td>
</tr>
<tr>
<td>Immune (7)</td>
<td>0.09 ± 0.03</td>
<td>2.08 ± 1.01</td>
<td>1.31 ± 0.42</td>
<td>5.72 ± 1.38</td>
</tr>
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</table>

Data shown as mean ± SEM. P-values based on comparison of results from immune and non-immune donors for each column. Number of subjects in each group are shown in parentheses.
Acknowledgements

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