Generation of Immune Inhibitory Dendritic Cells and CD4+T Regulatory Cells Inducing by TGF-β

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ABSTRACT

Variety of positive as well as negative regulatory signals are provided by antigen presenting cell in particular by dendritic cells. In this research, we studied the capacity of dendritic cells to expand antigen-specific T regulatory cells. We also investigated the role of TGF-beta in induction inhibitory functions of dendritic cells in mixed leukocyte reactions.

Dendritic cells were generated from blood CD14+ monocytes with granulocyte-Monocyte colony stimulating factor and interleukin-4 with or without TGF-beta (TGF-β-GM-DC or GM-DC). CD4+ T cell were isolated to assess lymphocyte proliferation by lymphocyte transformation test assay and the ratio of CD4+FOXP3+ CD25+ T cells were determined by fluorescence-activated cell sorter.

T cell proliferation responses in GM–DC showed a significance antigen-specific proliferative response comparing with TGF-β-GM –DC. T Cell proliferation was inhibited in co-culture system containing DC-treated TGF-β.

It can be suggested that the expansion of T regulatory by TGF-β-GM-DC provides a means for antigen specific control of unwanted immune reactions.

Key words: Dendritic cell; TGF-β; FOXP3; T regulatory

INTRODUCTION

Regulatory T cells actively suppress the activation and expansion of self-reactive T cells, thereby preventing autoimmune diseases.¹ CD25+ CD4+ natural T regulatory cells not only inhibit autoimmune responses but also suppress a variety of physiological and pathological immune responses to non-self antigens. The Foxp3+ CD25+ CD4+ T regulatory cells prevent autoimmune responses, as well as over-reaction of the immune system.²⁻⁴

The major cellular population involved in suppression are the FoxP3+ CD25+ CD4+ T regulatory cells. These cellular functions are affected by dendritic cells (Des) and a complex array of cytokines of which interleukin-2 (IL-2), interleukin-10 (IL-10) and Transforming growth factor-β(TGF-β) are especially significant.³⁻⁵ The TGF-β is a cytokine with profound effects in many developmental processes including cell
proliferation, differentiation, cellular adhesion, skeletal development, hematopoiesis and inflammatory responses as well as profound effects in the adult organism in inflammation, wound healing and host immunity.8-10

During antigen capture and processing, mature DC express large amounts of peptide-MHC complexes and accessory molecules on their surface.11

Dendritic cells are professional antigen presenting cell (APC) that have the unique capacity to initiate specific immune responses.11 They have the potential to induce immune tolerance and thus, play a critical role in transplantation.2,3 DC cultured in vitro acquire different phenotypic and functional properties depending on the cocktail of lymphokines provided including the source of serum supplement used.12 Although mature DCs are potent antigen presenting cells that initiate primary immune responses,13 Recently, it has been reported that splenic DCs are capable of differentiating Foxp3 regulatory T cells from Foxp3 precursors, along with TGF-β signaling.14 Thus, in the present study, we evaluated the effects of TGF-β on dendritic cell immune function using induction of T regulatory cells production and by lymphocyte transformation test (L.T.T) assay in MLR.

MATERIALS AND METHODS

The blood samples were provided by blood transfusion organization from the blood donor volunteers. To study the immune phenotype and cell surface expression of monocyte derived dendritic cell, 50ml blood samples were collected from 5 normal subjects. Written permission were obtained from all adult blood donor volunteers who donated blood samples for this study.

Generation of Dendritic Cells from Adherent Blood Mononuclear Cells

Blood mononuclear cells (MNC) were isolated from heparinized blood by centrifugation on a Ficoll histipaque 1.077 (sigma, U.S.A). The cells from the interphase were collected and washed three times with RPMI 1640 medium (sigma). Cell viability was determined by trypan blue exclusion. Monocytes and CD4+ T cells were purified from blood samples using MACS CD14+ magnetic beads and CD4+ T cell isolation kit (Miltenyi Biotec) respectively. CD4+T cells were counted using monoclonal antibody CD4 (>95% pure as analyzed by flow cytometry) and were then frozen in 10% dimethylsulfoxide containing a mixture of 60% RPMI and 30% FCS until use.

Then, 5×10^5 of CD14+ cells were cultured in 1ml RPMI 1640 medium supplemented with 10% fetal calf serum, 50units/ml penicillin and 50μg/ml streptomycin with different combinations of the following amounts of purified recombinant human cytokines: 1000 units/ml rhGM-CSF (Serotec, UK), 500unit/ml rh IL-4 (Serotec,) to generate DC and 10 ng/ml Transforming growth factor-beta 1 (TGF-β1) (Peprotech). To induce maturation of DC (Produced with GM-CSF, IL-4±TGF-β), 1μg/ml bacterial lipopolysaccharide(LPS) from Salmonella Minnesota (Sigma-Aldrich) was added to cultures on day 5 and cells were incubated for an additional 24hrs.

Flow cytometry

Surface expressions of dendritic cells were determined by flow cytometry on a fluorescent activated cell sorter (B.D). Harvested cells were washed twice with PBS supplemented with 1% BSA. Fc receptors on cells were pre-blocked with excess human IgG (Sigma-Aldrich) on ice for 15 min. Cells were stained for 30 min at 4°C with the following FITC-conjugated antibodies: anti-HLR-DR, anti CD86 and anti-CD83 and PE-conjugated antibody: isotype control (eBioscience). For intracellular staining of Foxp3 expression in CD4+ T cells, T cells were first stained with anti-CD4-FITC and anti-CD25-PE, then T cells were fixed and permeablized before staining with anti-Foxp3-APC (BD Bioscience).

Finally in order to identity and enumerate the cells, the cell suspensions were analyzed by flow cytometry.

Co Culture Experiments with T Cells

Allogenic isolated T cells and dendritic cells were co cultured in 96 U-shaped–bottomed plates, 10×10^3 Tcell were cultured in each well with 1×10^3 - 1×10^4 DC with final volume of 200 μl per well.

All samples were run in triplicates. Cultures were incubated at 37°C in a humidified 5% CO2 atmosphere and then T cell proliferation was measured by addition of 1 μCi/well [methyl-3H]thymidine (MP Biomedicals, UK) for the last 18h before the end of culture on day 5. The incorporation of [methyl-3H]thymidine was measured in a liquid scintillation counter. Results are expressed as stimulation index(SI) which is cpm of stimulated cultures divided by cpm of the control.

Statistical Analysis

For statistical analysis, we used paired t-tests. The P values were determined in all cases and they were considered statistically significant at P<0.05.
RESULTS

Dendritic Cell Production

Dendritic cells generated from blood CD14+ cells, which showed phenotypes of mature dendritic cells. Thus, we generated two populations of DC. One population consisting of DC treated with TGF-β, GM-CSF, IL-4 and a population of DC treated with GM-CSF, IL-4. The later DC served as a control (Figure 1, Table 2).

Inhibition Of Allogenic T Cell Activation By DC-Treated With TGF-β

The functional potential of mature monocyte-derived DCs to induce proliferation of T lymphocytes was investigated by MLR. We compared the T cell proliferation responses in TGF-β-GM-DC population and in GM-DC population using [methyl-3H]thymidine (1 µCi/well). GM–DC showed a significant antigen-specific proliferative response(SI=3.1) when compared with TGF-β-GM –DC (SI=1.7). Therefore T Cell proliferation was inhibited in co-culture system containing TGFβ-GM dendritic cell (Figure 2, Table 1).

Table 1. Effect of TGF-β-GM-DC and GM-Dc on CD4+ T cell proliferation in MLR, evaluated by L.T.T assay. The data are mean ± SD of stimulation index(SI)

<table>
<thead>
<tr>
<th>MLR</th>
<th>Total samples</th>
<th>SI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β-GM-DC+T (1/10)</td>
<td>5</td>
<td>1.7± 0.2</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>GM-DC+T [(1/10) control]</td>
<td>5</td>
<td>3.1± 0.42</td>
<td></td>
</tr>
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</table>

Stimulation index (SI)

Flowcytometry Results

Phenotype analysis of monocyte-derived DCs was carried out using flowcytometry.
Table 2. Effect of TGF-β on expression of costimulatory molecules on DCs in vitro evaluated by flowcytometry. The results are mean ±SD. These results indicate maturation two populations of DC, regarding CD83, CD86 and HLA-DR.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Total sample</th>
<th>CD83</th>
<th>CD86</th>
<th>HLA-DR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature TGFβ-GM-DC</td>
<td>5</td>
<td>44±2.73</td>
<td>70.8±3.70</td>
<td>94.2±3.19</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Immature TGFβ-GM-DC</td>
<td>5</td>
<td>1.2±1.0</td>
<td>46±3.93</td>
<td>77.8±2.58</td>
<td></td>
</tr>
<tr>
<td>Mature GM-DC</td>
<td>5</td>
<td>45.6±4.7</td>
<td>74.6±3.36</td>
<td>96.2±2.79</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Immature GM-DC</td>
<td>5</td>
<td>2±1.2</td>
<td>49.6±3.64</td>
<td>78.2±5.31</td>
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</table>

Analysis of phenotype showed the expression of HLA-DR and minimal level of CD83 on their surface before stimulation with antigen. After L.P.S activation, TGFβ-GM-DC acquired significantly increased expression of CD86, HLA-DR and CD83, although their levels were lower than GM-DC.

Expression of the CD83 marker and HLA-DR indicating the maturation of DCs(Figure3, Table 2). TGFβ-GM dendritic cell were much more effective than GM-DC for expanding CD25+CD4+Foxp3+ Tregulatory cells (Figure 4, Table 3).

Table 3. Effects of TGF-β-GM-DC and GM-DC on expression of FOxp3 and CD25 on CD4+ T cell in MLR as evaluated by flowcytometry. Results are percent of CD4+ T cell bearing FOxp3 and CD25 (results are expressed as the average mean fluorescence intensity of 5 independent experiments).

<table>
<thead>
<tr>
<th>MLR</th>
<th>Total sample</th>
<th>CD25</th>
<th>FOxp3</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>T + TGFβ-GM-DC</td>
<td>5</td>
<td>81.4±12.07</td>
<td>71.4±12.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>T + GM-DC (control)</td>
<td>5</td>
<td>41.8±12.7</td>
<td>47.6±11.52</td>
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DISCUSSION

The principal finding of this study is production of a dendritic cell population with TGF-β. Because of the importance of TGF-β in the immunomodulation, Geissmann et al. (1998) demonstrated that langerhan cells may originate from monocyte in the presence of TGF-β1 in vitro. We showed that TGF-β, in combination with GM-CSF and IL-4 induced monocytes to differentiate into dendritic cells with capability of inhibiting T cell proliferation in vitro.

It has been shown by Fogel-petroic et al. (2007) that physiological concentrations of TGF-β1 altered adherent-cells function when co-cultured with Peripheral blood mononuclear cells (PBMC) in MLR. In this study, we found that TGF-β-GM-DC induced the expression of Foxp3 in the CD4+ T cell in the MLR. (Figure 4, Table 3). Foxp3 is known to play a critical role for regulatory T cell function. Thus, our results showed that activated TGF-β-GM-DC induced allogeneic CD4+ T cells to express the highest level of CD25 and Foxp3 (Figure 4, Table 1). Our observation is consistent with other reports suggesting that T cell differentiation is determined by the duration and quality of the activating signals. In this study after LPS activation, TGF-β-GM-DC acquired the ability to increase significantly expression of CD86, HLA-DR and CD83 (Table 2, Figure 3). Comparing with control, the ability of TGF-GM-DC to induce normal allogeneic T cell activation leading to proliferation was markedly impaired, this inhibition can reflect levels of CD83 and CD86 costimulatory molecules. Indeed, our results showed the increased density of CD83/CD86 relative to Foxp3 expression on the T CD4+ cells.

Soto et al. found that IL-10-producing CD4+ T cells and CD4+CD25+CD152+ T regulatory were increased in alternatively activated-dendritic cells recipients. Consistent with this result; we observed that TGF-β-GM-DC could expand suppressive CD4+CD25+Foxp3+ T cells (Table 3, Figure 4).
Generation of Immune Inhibitory Dendritic Cells

In conclusion, the present study extends insight into the functional biology of TGF-β-GM-DC and their interactions with allogeneic T cells and indicate that TGF-β-GM-DC has regulatory properties and therapeutic potential. We suggest that the expansion of T regulatory via TGF-β-GM-DC will provide a means for antigen specific control of unwanted immune reactions.

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REFERENCES