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Cutting Edge: Human CD4⁺CD25⁺ T Cells Restrain the Maturation and Antigen-Presenting Function of Dendritic Cells¹

Namita Misra, Jagadeesh Bayry, Sébastien Lacroix-Desmazes, Michel D. Kazatchkine, and Srini V. Kaveri²

The characteristics and functions of CD4⁺ CD25⁺ regulatory cells have been well defined in murine and human systems. However, the interaction between CD4⁺ CD25⁺ T cells and dendritic cells (DC) remains unclear. In this study, we examined the effect of human CD4⁺ CD25⁺ T cells on maturation and function of monocyte-derived DC. We show that regulatory T cells render the DC inefficient as APCs despite prestimulation with CD40 ligand. This effect was marginally reverted by neutralizing Abs to TGF-β. There was an increased IL-10 secretion and reduced expression of costimulatory molecules in DC. Thus, in addition to direct suppressor effect on CD4⁺ T cells, regulatory T cells may modulate the immune response through DC. The Journal of Immunology, 2004, 172: 4676–4680.

A subset of CD4⁺ T cells expressing the α subunit of the IL-2R (CD25) plays a pivotal role in controlling immune response and in maintenance of T cell homeostasis (1, 2). Although the possible mechanisms of suppression by CD4⁺ CD25⁺ T cells are being explored in mice, they are conflicting and unclear in humans (3–5). The role of IL-10, TGF-β, and CTLA-4 as mediators of suppression has been shown in the murine model (6) but is not clear in humans (4). However, it is clear that human regulatory T cells act through direct cell-cell contact with CD4⁺ cells and may transfer the suppressor activity to the target cells (3, 7). Although the regulation of CD4⁺ cells by CD4⁺CD25⁺ T cells is well characterized, their effect on monocytes/dendritic cells (DC), B cells, and NK cells is not clearly defined. There are conflicting reports on the possible role of APCs as mediators of the suppression in murine (8, 9) and in the human system (5, 10).

In this study, we have examined the regulatory role of human peripheral CD4⁺CD25⁺ T cells on maturation and function of monocyte-derived DC. Our results indicate that regulatory T cells affect maturation of DC, and these DC in turn function as poor APCs.

Materials and Methods

Abs and reagents

Recombinant human (rh)IL-4, CD40 ligand (CD40L), anti-IL-10, and anti-TGF-β Abs were from R&D Systems (Minneapolis, MN), and rhGM-CSF was from Schering-Plough (Kenilworth, NJ). Conjugated Ab to CD16, CD40, CD80, CD83, CD14, and anti-CD3 mAb (UCHT1) were from Immunotech (Marseilles, France). Conjugated Abs to HLA-DR and CD1c and mouse IgG1 were from BD Biosciences (Mountain View, CA). Ab to CD86 and CD95 were from BD PharMingen (San Diego, CA). Ab to CD1a (clone OKT6) was from Ortho Diagnostics (Raritan, NJ). Saponin and rhIL-2 were from Sigma-Aldrich (St. Louis, MO), and purified protein derivative RT48 (PPD) was from Statens Serum Institut (Copenhagen, Denmark).

Generation and culture of DC

DC were generated from peripheral blood monocytes of healthy individuals as previously described (11). Human peripheral blood was obtained from healthy donors in accordance with local ethical committee approval. Adherent monocytes were cultured in complete RPMI 1640 medium with 500 IU of rhIL-4 and 1000 IU of rhGM-CSF per 10⁶ cells and were replenished every 2 days. After 5 days, DC were harvested, washed, and used for subsequent experiments. Flow-cytometric analysis demonstrated that >90% of the cells were immature DC (imDC; CD1a high-positive cells).

Purification of T cell subsets from peripheral mononuclear cells and cocultures with DC

T cell subsets were isolated from PBMCs using MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ T cells were purified by using CD4⁺ T cell isolation kit (Miltenyi Biotec), and CD4⁺ CD25⁺ T cells were then selected using anti-CD25 Ab-coupled magnetic beads. The remaining CD4⁺ T cells were used as CD4⁺ CD25⁻ T cells. The T cells were stimulated with plate-bound anti-CD3 Ab (1 μg/ml) and IL-2 (10 U/ml) for 24 h in 24-well plates. imDC were washed and added to T cell cultures at a ratio of 1:5. Cocultures were maintained for 72 h, and supernatants were collected after 48 h for cytokine estimation. For some experiments, DC were stimulated with rhCD40L before coculture with different subsets of T cells, or neutralizing anti-TGF-β Abs were added following cocultures as indicated in figures.

Flow-cytometric analysis of DC cocultured with T cell subsets

DC were washed and stained for 20 min at 4°C with optimal dilution of relevant Ab. Cells were analyzed by flow cytometry (FACSCalibur and CellQuest software, BD Biosciences). For intracellular staining, cells were fixed and incubated with Ab in saponin buffer (PBS, 2% BSA, and 0.5% saponin) for 25 min.

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3 Abbreviations used in this paper: DC, dendritic cell; rh, recombinant human; CD40L, CD40 ligand; PPD, purified protein derivative RT48; imDC, immature DC; MFI, mean fluorescence intensity.
Cells were washed twice in 0.1% saponin buffer and once in PBS before analysis.

In vitro lymphoproliferation assays

Following the cocultures with the CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells, DC were examined for their ability to stimulate allogeneic CD4⁺ T cells in MLR and to present the recall Ag PPD. DC were separated from the T cells using anti-CD1a mAb-coupled magnetic beads. The CD4⁺ T cells from allogeneic donor were isolated as described above. Graded doses (2,000–20,000) of DC were seeded with 10⁴ responder T cells in complete RPMI 1640 medium supplemented with 10% human AB serum in 96-well plates. After 4 days, the cells were pulsed for 16 h with 1 μCi of [³H]thymidine. Results were expressed as cpm (mean ± SD of triplicate values). To test Ag presentation function, DC were preincubated with 5 μg/ml PPD for 24 h followed by addition of autologous CD4⁺ T cells. DC–T cell cultures were set up as explained above. The differences in the capacity of DC, cultured with CD25⁺ or CD25⁻ T cells, to stimulate T cells were expressed as percentages. Percentage for each DC-T cell ratio was calculated as follows: 1 – (mean cpm in MLR with DC cultured with CD4⁺CD25⁺ T cells/mean cpm in MLR with DC cultured with CD4⁺CD25⁻ T cells) × 100.

Transwell experiments

Transwells of 0.4-µm pore size (Costar, Cambridge, MA) were used. The CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were stimulated with plate-bound anti-CD3 Ab and IL-2 for 24 h in lower chambers of 24-well plates. imDC were added to the upper chambers of the Transwell plates at DC:T cell ratio of 1:5. Transwell cultures were maintained for 72 h, and DC from the upper wells were analyzed.

Statistical analysis

Nonparametric Mann-Whitney and two-factor ANOVA were used to determine the statistical significance of the data. A value of p < 0.05 was considered to be statistically significant.

Results and Discussion

We examined the effect of the activated peripheral blood CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ regulatory T cells on the phenotype of DC during maturation in vitro. CD4⁺CD25⁺ T cells were refractory to stimulation through TCR and exerted suppressor effect on CD4⁺CD25⁻ T cells (not shown). Autologous monocytes were cultured in the presence of GM-CSF and IL-4. On day 5 of culture, >90% of the cells displayed DC phenotype (12) with expression of CD1a, CD11c, and HLA-DR, and costimulatory molecules CD86, CD80, and CD40. The expression of CD14 was down-regulated, and that of CD16 was absent. DC, thus obtained, were cocultured with CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells for 72 h. DC cultured in medium with or without cytokines were used as controls. DC cocultured with CD4⁺CD25⁻ T cells showed a marked increase in their costimulatory molecules compared with control DC and DC with CD4⁺CD25⁺ T cells (Table I). There was a significant reduction in percentage of DC expressing CD83 (p < 0.05) and CD86 (p < 0.004) and the mean fluorescence intensity (MFI) of CD40 (p < 0.03) on coculture with CD4⁺CD25⁺ T cells as compared with cocultures with CD4⁺CD25⁻ T cells (Fig. 1A, Table I). Similarly, the expression of CD80 and HLA-DR (MFI) tend to be reduced on DC cocultured with CD4⁺CD25⁺ T cells (Fig. 1A, Table I). Morphology of DC cocultured with CD4⁺CD25⁺ T cells was unaltered compared with DC cultured with cytokines or CD4⁺CD25⁻ T cells. In contrast, DC left in medium alone, reverted back to monocyte/macrophage phenotype and also regained adherience to culture wells (not shown).

It was well established that CD4⁺CD25⁺ T cells exert their regulatory function on target T cells in a contact-dependent yet cytokine-independent manner (3). To examine whether suppressor effect of regulatory T cells on DC is also contact dependent, we performed Transwell experiments separating DC and T cells by 0.4-µm membrane. The down-regulation of the co-stimulatory molecules was observed only when the CD4⁺CD25⁺ T cells were in contact with the DC (Table I) with an exception of MFI for CD40 (p < 0.05). Together, the results suggest that cell contact was necessary for DC to maintain an activated state in cocultures with CD4⁺CD25⁻ T cells and a suppressive state in cocultures with CD4⁺CD25⁺ T cells. Interestingly, percent expression of CD86 remained up-regulated (p < 0.05) in the absence of cell contact with CD4⁺CD25⁺ T cells.

We then examined the functional relevance of the suppression of DC by CD4⁺CD25⁺ T cells. Activated CD4⁺CD25⁻ T cells were potent stimulators of imDC that in turn stimulated allogeneic CD4⁺ T cells in MLR (Fig. 1B). In contrast, there was up to 3-fold reduction in the capacity of DC cocultured with CD4⁺CD25⁺ T cells to stimulate allogeneic T cells compared with DC cocultured with CD4⁺CD25⁻ T cells (p < 0.0001) and to DC cultured with IL-4, GM-CSF (p < 0.0001) (Fig. 1B). Furthermore, we tested whether DC cocultured with CD4⁺CD25⁻ T cells were able to process and present an Ag (PPD) to autologous CD4⁺ T cells. DC cocultured with CD4⁺CD25⁻ T cells were ineffective APCs and showed significant reduction in proliferation compared with DC preincubated with CD4⁺CD25⁺ cells (p < 0.0001), or with cytokines (p < 0.0001) or in medium alone (p < 0.02) (Fig. 1B).

<table>
<thead>
<tr>
<th>Table 1. Phenotype analysis of DCs matured in the presence of T cell subsets</th>
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<td>DC in Contact Cultures</td>
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</tr>
<tr>
<td>DC (medium)</td>
</tr>
<tr>
<td>CD1a</td>
</tr>
<tr>
<td>CD83</td>
</tr>
<tr>
<td>CD86</td>
</tr>
<tr>
<td>CD80</td>
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<tr>
<td>CD40</td>
</tr>
<tr>
<td>HLA-DR</td>
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<tr>
<td>CD95</td>
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</table>

*imDCs of 5 days were cultured in contact and in Tranwell with CD4⁺CD25⁻ and CD4⁺CD25⁺ T cell subsets. DCs in control cultures were either left in medium alone or supplemented with IL-4 and GM-CSF. Results are represented as mean ± SE of percentage/MFI of cells positive for the indicated markers. MFI are indicated in parentheses. Results are from four of the cell-cell contact and two of the Tranwell experiments. Statistical significance, as determined by nonparametric Mann-Whitney, is indicated.

, p < 0.05. The indicated p values were obtained on comparison of DC (CD4⁺CD25⁻) and DC (CD4⁺CD25⁺) and on comparison of DC (CD4⁺CD25⁺) in cell-cell contact and Tranwell cultures.
Furthermore, we stimulated 5-day-old DC with rhCD40L before coculture with the regulatory T cells. Triggering of CD40 on DC induced their maturation and increased expression of CD83, CD80, CD86, CD40, and HLA-DR molecules (13). However, following the rhCD40L treatment and coculture with CD4⁺/CD25⁺ T cells, percentage of DC expressing CD80, CD83, and CD86, and MFI for CD40 and HLA-DR tend to be reduced (Table II), although the difference was found to be statistically significant only in the case of CD80 (p < 0.04). The DC pretreated with rhCD40L were potent stimulators of allogeneic T cells (Fig. 1A); DC that were further incubated with CD4⁺/CD25⁺ T cells mounted significantly lower (p < 0.001) response in the same proliferation assay. These results further support the suppressor effect of CD4⁺/CD25⁺ T cells on the DC.

We then tested whether the suppressed DC function was due to altered cytokine secretions. CD4⁺/CD25⁺ T cells render CD4⁺/CD25⁺ target T cells anergic and induce IL-10 secretion, which, in turn, can suppress other T cells in a contact-independent manner (7). We assessed the secreted and intracellular IL-10 levels in DC cocultured with different T cell subsets. Because both activated CD4⁺/CD25⁺ and DC produce substantial IL-10 (14), it was not surprising to find IL-10 in DC cocultured with CD4⁺/CD25⁺ T cells. However, there was 2-fold enhancement of IL-10 levels in the supernatants from DC-CD4⁺/CD25⁺ T cell cultures (Fig. 2A). Furthermore, FACS analysis showed that there was >2-fold increase in IL-10-positive DC cocultured with CD4⁺/CD25⁺ T cells (Fig. 2A). No difference was found in intracellular IL-12 levels of DC in different cocultures (not shown).

**FIGURE 1.** CD4⁺/CD25⁺ T cells down-regulate the maturation and function of the DC. CD4⁺/CD25⁻, CD4⁺/CD25⁺ T cells and autologous DC were generated as described. Five-day-old imDC were cultured with the T cell subsets at a ratio of 1:5 for 72 h. T cells were depleted, and DC were used for phenotypic analysis and functional assays. A, Phenotypic analysis of DC cocultured with different T cell subsets. Percentage of positive cells and MFI (mf) are indicated. Results shown are representative of five independent experiments from different donors. B, Allogeneic and Ag-mediated stimulation of the CD4⁺ T cells by DC as assessed by [³H]thymidine incorporation. MLR and PPD-induced stimulation assays were set up as described. As controls, DC were also cultured in medium alone or with IL-4 and GM-CSF. Data are from five experiments, and statistical analysis was performed using two-factor ANOVA. *, p < 0.0001; †, p < 0.05. C, Functional analysis of rhCD40L-treated DC matured in the presence or absence of CD4⁺/CD25⁺ T cells. Five-day-old DC were incubated with rhCD40L at 1.5 μg/ml for 24 h. DC were washed and subsequently cocultured with activated CD4⁺/CD25⁺ T cells at a DC-T cell ratio of 1:5 for 48 h. Controls were DC incubated with rhCD40L, washed, and cultured in medium alone. T cells were depleted from the DC-T cell cocultures, and DC were used for MLR as explained above.
In view of the emerging evidence that links the membrane-bound form of TGF-β with CD4⁺CD25⁺ T cell-mediated suppression of activated T cells (15), we tested the effect of anti-TGF-β mAb in DC-T cell coculture experiments. On incubation of the DC-CD4⁺CD25⁺ T cell cocultures with anti-TGF-β mAb, the reduction in allostimulatory capacity of DC was found to be 60% (at DC-T cell ratio of 1:5) against 69% in the absence of the Abs. An average reversal of suppression for all DC-T cell ratios was 10% at 10-fold higher concentration of anti-TGF-β mAb. However, there was a marginal increase in proliferation induced by DC treated with CD4⁺CD25⁻ T cells in the presence of anti-TGF-β mAb (Fig. 2B) (4). Therefore, we conclude that, although IL-10 and TGF-β may contribute to the overall suppressor effect of the DC cocultured with CD4⁺CD25⁺ T cells, there may be other factors that are responsible for this phenomenon. Suppressor function of CD4⁺CD25⁺ T cells is a complex process, tightly regulated by multiple factors, including IL-2, CTLA-4, glucocorticoid-induced TNFR, Toll-like receptors (16), and transcription factor FoxP3 (17).

Maturation of DC has a major impact on T cell responses. The high DC density and the high levels of Ag and costimulatory molecules deliver a strong and sustained stimulation to specific T cells, leading to a rapid proliferation. DC-T cell interaction also results in a reciprocal stimulation (18–20). Given the important role played by costimulatory molecules in T cell activation and suppression, attempts have been made to assess whether the regulatory cell function is mediated through the APCs. Effect of CD4⁺CD25⁺ T cells on APCs including DC remains controversial. Although some studies reported down-regulation of costimulatory molecules and T cell stimulatory capacity of APCs (10, 21), others have reported an APC-independent suppressor effect of CD4⁺CD25⁺ T cells on target T cells (5, 9). However, these observations do not rule out the fact that regulatory T cells could exhibit a bystander suppressor effect on other cells such as monocytes/DC, B cells, and NK cells. This effect may not be directly implicated in contact-mediated suppressor effect on target CD4⁺ T cells but may contribute to regulation of the immune response in general. We observe that, whereas CD4⁺CD25⁺ cells up-regulate, CD4⁺CD25⁺ regulatory cells down-regulate the maturation process and Ag-presentation capacity of DC.

A separation of immunogenic and tolerogenic function of the DC during cross-presentation of exogenous Ag to CD8⁺ T cell response is shown to be dependent on the stimulus provided by the CD4⁺Th cell (22). In view of our results, the stimulus to the DC can be a positive signal delivered by CD4⁺CD25⁻ T cells or an inhibitory signal by CD4⁺CD25⁺ regulatory T cells. It is tempting to speculate that either or both of the two important costimulatory signals, CTLA-4-B7 or CD40-CD40L, could be involved in mediating the modulatory effect on the DC. An involvement of another unidentified receptor-ligand-mediated effect cannot be ruled out.

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### Table II. Phenotype analysis of DC pretreated with rhCD40L⁺

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<th>DC (rhCD40L)</th>
<th>DC (rhCD40L, CD4⁺CD25⁺)</th>
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<tbody>
<tr>
<td>CD1a</td>
<td>78 ± 8 (50 ± 10)</td>
<td>73 ± 6.3 (47 ± 4)</td>
</tr>
<tr>
<td>CD83</td>
<td>73 ± 11 (66 ± 28)</td>
<td>48 ± 17 (41 ± 13)</td>
</tr>
<tr>
<td>CD86</td>
<td>86 ± 10 (44 ± 7)</td>
<td>69 ± 7 (56 ± 11)</td>
</tr>
<tr>
<td>CD80</td>
<td>95 ± 2 (81 ± 33)</td>
<td>80 ± 5 (53 ± 16)</td>
</tr>
<tr>
<td>CD40</td>
<td>99 ± 0.2 (715 ± 207)</td>
<td>52 ± 16 (511 ± 135)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>97 ± 1 (380 ± 155)</td>
<td>94 ± 3 (248 ± 95)</td>
</tr>
<tr>
<td>CD95</td>
<td>80 ± 7.4 (68 ± 18)</td>
<td>93 ± 2.4 (64 ± 29)</td>
</tr>
</tbody>
</table>

*imDCs of 5 days were stimulated with rhCD40L at a concentration of 1.5 μg/ml for 24 h prior to coculture with the CD4⁺CD25⁺ T cells for 48 h. DCs in control cultures were left in medium alone following stimulation with rhCD40L. Results are represented as mean ± SE of percentage/MFI of cells positive for the indicated markers. MFI is indicated in parentheses. Results are from four experiments. Statistical significance, as determined by nonparametric Mann-Whitney, is indicated.

α, p < 0.05.
References