

Plasmacytoid Dendritic Cells Induce CD8⁺ Regulatory T Cells In Human Ovarian Carcinoma

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Abstract

To directly dissect the role of each immune component in human tumor immunopathogenesis, we have studied the interaction between dendritic cells and T cells in the tumor environment of patients with ovarian carcinoma. We previously reported that functional plasmacytoid dendritic cells, but not functionally mature myeloid dendritic cells, accumulated in tumor microenvironments. We now show that tumor ascites macrophage-derived dendritic cells induced tumor-associated antigen-specific CD8⁺ T cells with effector functions. Strikingly, tumor ascites plasmacytoid dendritic cells induced interleukin-10⁺CCR7⁺CD45RO⁺CD8⁺ regulatory T cells. Four characteristics have been identified in tumor plasmacytoid dendritic cell-induced CD8⁺ regulatory T cells: (a) induction of CD8⁺ regulatory T cells is independent of CD4⁺CD25⁺ T cells; (b) CD8⁺ regulatory T cells significantly suppress myeloid dendritic cell-mediated tumor-associated antigen-specific T cell effector functions through interleukin-10; (c) repetitive myeloid dendritic cell stimulation can recover CD8⁺ regulatory T cell-mediated poor T cell proliferation, but not T cell effector function; (d) CD8⁺ regulatory T cells express functional CCR7, and efficiently migrate with lymphoid homing chemokine MIP-3β. Primary suppressive CCR7⁺CD45RO⁺CD8⁺ T cells are found in the tumor environment of patients with ovarian cancers. Thus, tumor-associated plasmacytoid dendritic cells contribute to the tumor environmental immunosuppressive network. Collectively, tumors manipulate tumor microenvironmental dendritic cell subset distribution and function to subvert tumor immunity. The data are relevant to understanding tumor immunopathology as well as reevaluating tumor immunotherapeutic strategies. (Cancer Res 2005; 65(12): 5020-6)

Introduction

Human cancers are able to prime tumor-specific immunity. There exist tumor-specific T lymphocytes in blood, tumor tissues, and lymph nodes in cancer patients. Isolated tumor-associated antigen-specific T cells can kill autologous tumor cells. Functional tumor-associated antigen-specific T cell lines can be established from tumor T cells (1–5). Furthermore, studies have shown increases in tumor-associated antigen-specific cytotoxic CD8⁺ T cells without evidence of clinical regression in some patients (2, 6, 7). This suggests that tumor-associated antigen-

specific T cells might be functionally inhibited *in vivo*. The underlying mechanisms were extensively reviewed (8).

Dendritic cells play a critical role in establishing tumor immunity. However, because of the rarity of dendritic cells *in vivo*, primary dendritic cell studies are confined to *in vitro* differentiated myeloid dendritic cells. Published, detailed studies of functional human dendritic cells in tumor environment are rare (9). Furthermore, another dendritic cell subset, plasmacytoid dendritic cells are technically more difficult to study. T cell activation pattern induced by plasmacytoid dendritic cells are largely determined in the allogeneic system. Little is known of the role of tumor environmental plasmacytoid dendritic cells in tumor immunity (9, 10). These limitations may significantly underestimate the impact of the dendritic cell system in tumor pathology. We have studied malignant ascites of patients with ovarian carcinoma. Malignant ascites contains viable tumor and immune cells, and serves as a model for the tumor microenvironment (9–13). We identified a significant population of plasmacytoid dendritic cells in malignant ascites. In an allogeneic system, normal blood plasmacytoid dendritic cells (14, 15) and tumor plasmacytoid dendritic cells (9) induce interleukin (IL)-10 expressing suppressive T cells. In this report, we further studied the pathologic interaction between tumor ascites T cells and tumor ascites plasmacytoid dendritic cells, and its impact on tumor-associated antigen-specific T cell immunity.

Materials and Methods

Human subjects. We studied previously untreated patients with epithelial ovarian carcinomas, International Federation of Gynecology and Obstetrics stages I, II, III, and IV. Patients gave written, informed consent. Clinical specimens were collected at the Tulane University, New Orleans. The study was approved by local Institutional Review Boards.

Ascites, ascites cells, and tumor-draining lymph nodes. We collected and prepared single cells from ascites, blood, and lymph nodes as previously described (9, 16). CD3, CD14, CD16, CD19, and CD56-expressing cells were depleted using paramagnetic beads (Miltenyi, Auburn, CA), and plasmacytoid dendritic cells were sorted with FACSAria gating on CD4⁺CD123⁺HLA-DR^{bright}CD11c⁻ cells. Cell populations were ≥99% pure by flow cytometry analysis (fluorescence-activated cell sorting, FACS). Tumor ascites T cells were obtained by depleting CD14, CD16, CD19, CD56, and CD123-expressing cells using microbeads (Miltenyi), and sorting CD45⁺ cells by flow cytometry. Cellular purity was ≥98% as determined by FACS staining with anti-human CD3 antibody. Antibodies for flow cytometry and depletion were purchased from BD PharMingen, San Jose, CA. Autologous ascites cells were used throughout this study.

Peripheral blood cells. Peripheral blood was collected aseptically from normal volunteers or patients by venipuncture or by cytophoresis, and mononuclear cells were then obtained by Ficoll-Hypaque density centrifugation. Blood plasmacytoid dendritic cells were obtained as described for tumor ascites plasmacytoid dendritic cells. Cells were ≥98% pure as determined by FACS.

Note: S. Wei and I. Kryczek contributed equally to this work.

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In vitro tumor-associated antigen-specific T cell stimulation. Tumor myeloid dendritic cells were differentiated from tumor macrophages as previously described (17). Tumor plasmacytoid dendritic cells were sorted from ascites as we previously described (9). Dendritic cells were activated with CD40L (200 ng/mL) for 24 hours (17), and loaded with three distinct HLA-A2-binding Her-2/neu peptides (13, 18) at 5 µg/mL each: p369-384 (KIFGSLAFLPESFDGDDPA), p688-703 (RRLLQETELVEPLTPS), and p971-984 (ELVSEFSRMARDPQ). Peptide-loaded myeloid dendritic cells (tumor-associated antigen-myeloid dendritic cell) or peptide-loaded plasmacytoid dendritic cells (tumor-associated antigen-plasmacytoid dendritic cells; 10^2 - 10^4 /mL) were used to stimulate autologous tumor ascites T cells (5×10^5 /mL) in the presence of IL-2 (50 units/mL; R & D Systems, Minneapolis, MN) for 6 days (one round of stimulation). In some experiments, CD25⁺ or CCR7⁺ ascites T cells were initially depleted with magnetic beads, and CD25⁻ T cells or CCR7⁻ T cells were then stimulated with tumor-associated antigen-plasmacytoid dendritic cells. T cells might receive several rounds of the identical stimulation as noted. T cell phenotypes, tumor-associated antigen-specific T cell proliferation, cytokines and cytotoxic activity were detected as we described (9, 16). In some cases, T cells were stained with mouse anti-human CCR7-PE (clone 150503, mouse IgG_{2a}, R & D), mouse anti-human CD8-APC (289-13804, IgG_{2a}, PharMingen) and anti-human IL-10 (JES-19F1, rat anti-human IgG_{2a}, PharMingen). At least 5,000 gated events per condition were analyzed using CellQuest software (Becton Dickinson, Mountain View, CA).

In vitro allogeneic T cell immunosuppression assay. Normal peripheral blood CD3⁺ T cells (4×10^5 /mL) were stimulated with 2.5 µg/mL anti-human CD3 (BD PharMingen) and blood monocytes (1×10^5 /mL) in the presence of different concentrations of freshly sorted allogeneic CCR7⁺CD45RO⁺CD8⁺ T cells from peripheral blood in normal donors or ascites in patients with ovarian cancer. Seventy-two hours after coculture, T cell proliferation was evaluated with thymidine incorporation.

In vitro tumor-associated antigen-specific immunosuppression assay. After two rounds of stimulation with tumor-associated antigen-plasmacytoid dendritic cells, ascites T cells (0.5 - 2×10^5 /mL; on day 13) were added into tumor-associated antigen-myeloid dendritic cells (10^3 /mL) and primary ascites T cell (5×10^5 /mL) coculture for 6 days. Tumor-associated antigen-specific T cell proliferation and cytokines were detected as we described (9, 13, 16). In some cases, anti-human IL-10 receptor (250 ng/mL; 3F8, rat anti-human IgG_{2a}, PharMingen) and anti-human IgG (250 ng/mL; R35-95, rat anti-human IgG_{2a}, PharMingen) were added into the coculture.

Migration assay. After two rounds of stimulation with tumor-associated antigen-plasmacytoid dendritic cells, or tumor-associated antigen-myeloid dendritic cells, day 13 T cells (2×10^5) were initially incubated with 500 ng/mL mouse anti-human CCR7 monoclonal antibody (clone 150503, mouse IgG_{2a}, R & D) or control monoclonal antibody (clone G155-178, mouse IgG_{2a}, BD PharMingen) for 2 hours, and then subjected to *in vitro* migration assay as we have previously described (9). Recombinant, human chemokines (MIP-3α and MIP-3β, 100 ng/mL each; R & D) were added to the lower chamber. The identity of migrating cells was confirmed by FACS for CD8 and CCR7.

T cell repetitive stimulation with tumor plasmacytoid dendritic cells and myeloid dendritic cells. Autologous tumor T cells were labeled with 10 µm 5,6-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) for 10 minutes in the dark at 37°C. 5,6-Carboxyfluorescein diacetate succinimidyl ester-labeled tumor T cells (5×10^5 /mL) were divided into three groups. Group 1 received three rounds of identical stimulation with tumor-associated antigen-plasmacytoid dendritic cells. Group 2 received three rounds of identical stimulation with tumor-associated antigen-myeloid dendritic cells. Group 3 initially received one round of stimulation with tumor-associated antigen-plasmacytoid dendritic cells and followed two rounds of stimulation with tumor-associated antigen-myeloid dendritic cells. Tumor-associated antigen-specific T cell proliferation and cytokines were detected as we previously described (9, 13, 16). Autologous tumor-associated antigen-dendritic cells (10^3 - 10^4 /mL) were used.

Real-time reverse transcriptase PCR. CCR7⁺CD45RO⁺CD8⁺ T cells were sorted from blood, tumor ascites, and tumor-draining lymph nodes. cDNA was prepared from CCR7⁺CD45RO⁺CD8⁺ T cells. Real-time reverse transcriptase-PCR was carried out for *IL-10* (upstream 5'-atgcttcgagatctcc-

gaga-3', downstream 5'-aatcatgatgacagcgccgta-3'), *IFN-γ* (upstream 5'-cgagaccaaattgtctct-3', downstream 5'-atgctcttcgacctcgaac-3') and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Complementary DNA was normalized against, and expressed as fold differences relative to, *GAPDH* (19) as we have previously described (13).

Statistical analysis. Differences in cell surface molecule expression were determined by χ^2 test, and in other variables by unpaired *t* test, with *P* < 0.05 considered significant.

Results

Tumor plasmacytoid dendritic cells and myeloid dendritic cells induce distinct T cell activation. To determine the pathologic significance of tumor environmental plasmacytoid and myeloid dendritic cells on tumor-associated antigen-specific immunity, we used our previously described *in vitro* tumor-associated antigen-specific culture system (9, 13, 16). Myeloid dendritic cells were differentiated *in vitro* from ascites macrophages of HLA-A2⁺ patients as described in ref. (17), and loaded with HLA-A2-specific Her-2/neu peptides (tumor-associated antigen-myeloid dendritic cells; refs. 13, 18). Autologous CD40L-activated tumor-associated antigen-myeloid dendritic cells induced specific T cell activation as expected (Fig. 1).

To study the function of tumor plasmacytoid dendritic cells in the context of tumor-associated antigen-specific T cell immunity, we sorted tumor plasmacytoid dendritic cells from ascites (9, 20). The functional identity of plasmacytoid dendritic cells were confirmed by their capacity to produce copious amounts of type I IFN after adenovirus activation (9). Tumor plasmacytoid dendritic cells were loaded with HLA-A2-specific Her-2/neu peptides (tumor-associated antigen-plasmacytoid dendritic cell). Autologous CD40L-activated tumor-associated antigen-plasmacytoid dendritic cells induced a modest T cell proliferation. Tumor plasmacytoid dendritic cells were significantly less efficient in inducing T cell proliferation than myeloid dendritic cells (*n* = 4; *, *P* < 0.001 for all; Fig. 1A). Strikingly, tumor plasmacytoid dendritic cells induced strong T cell IL-10 (221 ± 53 pg/mL; Fig. 1B), little T cell IFN-γ (Fig. 1C), and poor cytotoxic activity (Fig. 1D; *n* = 4; *, *P* < 0.001 for all, compared with myeloid dendritic cell). No significant T cell IL-4, IL-5, IL-13 and transforming growth factor-β (TGF-β) were detected in the culture. Primary tumor ascites tumor-associated antigen-plasmacytoid dendritic cells (without CD40L activation) did not induce important T cell proliferation (<1,000 c.p.m.) and cytokine production (IL-10, <30 pg/mL; IFN-γ, <0.1 ng/mL). The results indicate that tumor plasmacytoid and myeloid dendritic cells induced distinct T cell responses.

Tumor ascites contain CD4⁺CD25⁺FOXP3⁺ T cells (CD4⁺ regulatory T cells; ref. 13). To determine whether CD4⁺ regulatory T cells affected IL-10 production induced by tumor plasmacytoid dendritic cells, we did similar experiments by initially deleting CD25⁺ ascites T cells with magnetic beads, and stimulated CD25⁻ ascites T cells with tumor-associated antigen-plasmacytoid dendritic cells. We detected similar level of IL-10 (195 ± 73 pg/mL versus 221 ± 53 pg/mL, *n* = 4) in the supernatant from CD25⁻ ascites T cell and tumor-associated antigen-plasmacytoid dendritic cell coculture (*P* = 0.078, compared with no CD25 depletion). The data suggest that CD4⁺ regulatory T cells are not essential for tumor plasmacytoid dendritic cells to induce IL-10-expressing T cells.

Tumor plasmacytoid dendritic cells induced IL-10⁺CCR7⁺CD45RO⁺CD8⁺ T cells. We next examined the phenotype of IL-10-expressing T cells induced by tumor plasmacytoid dendritic

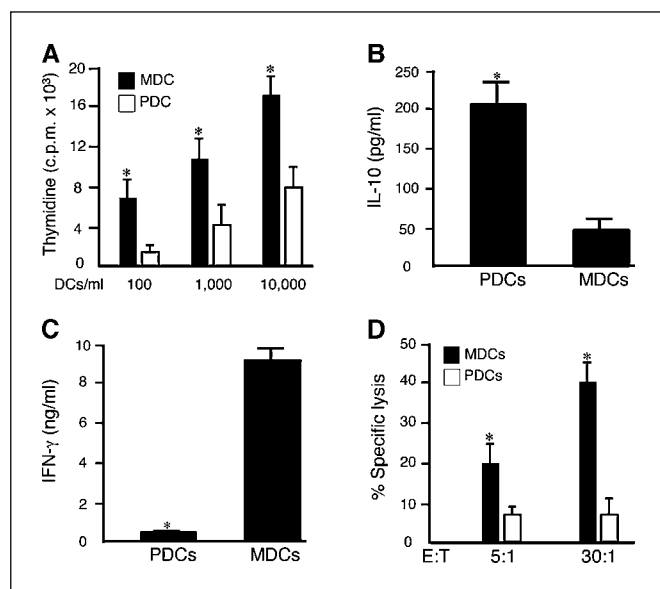


Figure 1. Distinct T cell activation induced by tumor plasmacytoid and myeloid dendritic cells. Primary tumor ascites T cells (5×10^5 /mL) received two rounds of stimulation with different concentrations of autologous tumor-associated antigen-plasmacytoid dendritic cells or tumor-associated antigen-myeloid dendritic cells as we described in Materials and Methods. **A**, T cell proliferation was detected by thymidine incorporation. **B**, IL-10 was detected in the culture supernatant by ELISA. **C**, IFN- γ was detected in the culture supernatant by ELISA. **D**, tumor T cell-mediated specific lysis of T2 cells was detected by FACS as we described in Materials and Methods. T cell cytokines and CD8⁺ T cell activity were shown when tumor ascites T cells (5×10^5 /mL) were stimulated with 10^4 /mL tumor-associated antigen-dendritic cells ($n = 4$; *, $P < 0.001$, compared with plasmacytoid dendritic cells).

cells. Intracellular staining showed that tumor plasmacytoid dendritic cells induced IL-10⁺CD8⁺ T cells ($n = 4$; Fig. 2A). Multiple-color FACS analysis further showed that 100% of IL-10⁺ T cells (gate 1) were CCR7⁺CD45RO⁺CD8⁺ T cells and IL-10⁻CD8⁺ T cells (gate 2) were CCR7⁻CD45RO⁺ T cells, and IL-10⁻CD8⁻ cells (gate 3) were largely CCR7⁻CD45RO⁺ cells (Fig. 2A).

FACS analysis revealed that primary tumor ascites contained CCR7⁺CD45RO⁺CD8⁺ T cells (Table 1). The level of CD45RO⁺CCR7⁺CD8⁺CD3⁺ cells was significantly higher in ascites, blood, and draining lymph nodes in patients with ovarian cancer than that in blood from normal donors ($P < 0.01$ for all; Table 1). Furthermore, the level of CCR7⁺CD45RO⁺CD8⁺CD3⁺ cells was also significantly higher in malignant ascites and tumor-draining lymph nodes than in peripheral blood ($P < 0.01$) in the same individual patients (Table 1).

To determine whether original ascites CCR7⁺CD8⁺ T cells affected the induction of IL-10⁺CCR7⁺CD45RO⁺CD8⁺ T cells by tumor plasmacytoid dendritic cells, we did the similar experiments by initially deleting CCR7⁺ ascites T cells with magnetic beads, and stimulated CCR7⁻ ascites T cells with tumor-associated antigen-plasmacytoid dendritic cells. We detected similar levels of IL-10 (243 ± 83 pg/mL versus 221 ± 53 pg/mL) in the supernatant from CCR7⁻ ascites T cell and tumor-associated antigen-plasmacytoid dendritic cell coculture ($P = 0.12$, compared with no CCR7 depletion). The data suggest that original CCR7⁺CD8⁺ T cells are not essential for tumor plasmacytoid dendritic cells to induce IL-10-expressing T cells.

Tumor plasmacytoid dendritic cell-induced IL-10⁺CD8⁺ T cells migrate with MIP-3 β . To further determine whether CCR7 is functional in tumor plasmacytoid dendritic cell-induced

IL-10⁺CD8⁺ T cells, we conducted a chemotaxis assay with MIP-3 β (CCL19), the ligand for CCR7. We observed that a fraction of tumor plasmacytoid dendritic cell-activated T cells efficiently migrated with MIP-3 β , but not with MIP-3 α and medium (data not shown), and neutralizing mouse anti-human CCR7 monoclonal antibodies efficiently blocked this migration ($n = 4$; *, $P < 0.001$; Fig. 2B). FACS analysis revealed that the migrated cells were uniquely CCR7⁺CD8⁺ T cells (Fig. 2B). Tumor myeloid dendritic cell-activated T cells poorly migrated in these conditions. CCR7 was solely expressed on IL-10⁺CD8⁺ T cells (Fig. 2A). Thus, tumor plasmacytoid dendritic cell-induced IL-10⁺CD8⁺ T cells expressed functional CCR7, which would allow them to return to the tumor-draining lymph nodes.

Tumor plasmacytoid dendritic cell-induced IL-10⁺CCR7⁺CD8⁺ T cells are suppressive. We next studied whether tumor plasmacytoid dendritic cell-induced IL-10⁺CCR7⁺CD8⁺ T cells are suppressive. To this end, autologous tumor ascites T cells were initially activated with tumor-associated antigen-plasmacytoid dendritic cells as described (Fig. 1A). Then, different concentrations of tumor plasmacytoid dendritic cell-activated T cells were added into autologous tumor-associated antigen-myeloid dendritic cell and T cell coculture system. As expected, myeloid dendritic cells induced strong tumor-associated antigen-specific T cell proliferation in the absence of plasmacytoid dendritic cell-activated T cells (Fig. 3A and B). Interestingly, tumor plasmacytoid dendritic cell-activated T cells significantly inhibited myeloid dendritic cell-mediated, Her2/neu-specific T cell proliferation in a dose-dependent manner ($n = 4$; * $P < 0.05$, ** $P < 0.01$, compared with controls; Fig. 3A). Tumor-associated antigen-specific T cell IFN- γ production was also suppressed ($n = 4$; * $P < 0.01$; Fig. 3C). Interestingly, mouse neutralizing anti-human IL-10 receptor antibody significantly recovered T cell functions (Fig. 3B and C). The data indicate that tumor plasmacytoid dendritic cell-induced CD8⁺ T cells are suppressive and that IL-10 is critical for this suppression.

Myeloid dendritic cell stimulation fails to recover tumor plasmacytoid dendritic cell-elicited poor T cell function. Myeloid dendritic cells have been used as immune-boosting vaccines to treat patients with cancers. It is unknown whether repetitive myeloid dendritic cell vaccination could override the T cell immunosuppressive function induced by tumor plasmacytoid dendritic cells. To test this possibility, autologous tumor ascites T cells were initially stimulated with tumor plasmacytoid dendritic cells followed by repetitive myeloid dendritic cell stimulation. As expected, repetitive myeloid dendritic cell stimulation (without plasmacytoid dendritic cells) induced strong tumor-associated antigen-specific T cell proliferation ($n = 4$; Fig. 3D). Repetitive plasmacytoid dendritic cell stimulation (without myeloid dendritic cells) induced a modest tumor-associated antigen-specific T cell proliferation ($n = 4$; Fig. 3D). Interestingly, after two rounds of myeloid dendritic cell stimulation, T cells prestimulated with tumor plasmacytoid dendritic cells exhibited strong T cell proliferation and cellular divisions, comparable to T cells that received three rounds of myeloid dendritic cell stimulation (Fig. 3D and E). However, after myeloid dendritic cell stimulation, the T cells previously stimulated with tumor plasmacytoid dendritic cells produced IL-10 (170 ± 68 pg/mL) and expressed little IFN- γ (0.12 ± 0.05 ng/mL), which was largely observed in the first two cell divisions ($5 \pm 2\%$ IFN- γ ⁺ T cells; Fig. 3E). The IL-10-expressing cells were CD8⁺ T cells, similar to tumor plasmacytoid dendritic cell stimulation alone

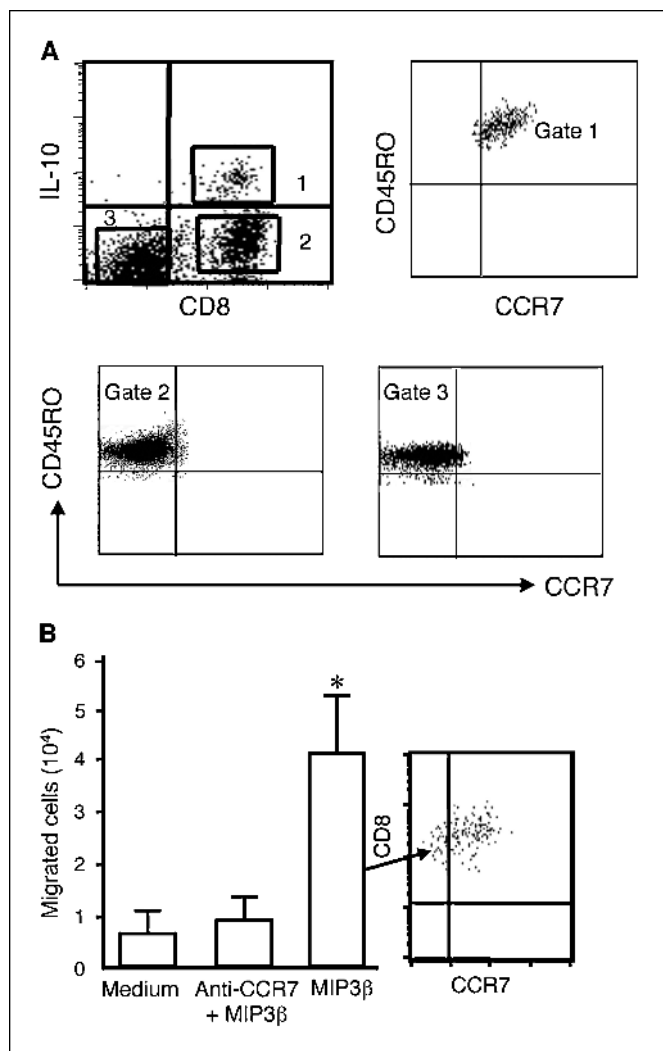


Figure 2. Tumor plasmacytoid dendritic cells induce IL-10⁺CCR7⁺CD45RO⁺ CD8⁺ T cells. Primary tumor ascites T cells (5×10^5 /mL) received two rounds of stimulation with autologous tumor-associated antigen–plasmacytoid dendritic cells (10^6 /mL). A, intracellular staining showed that tumor-associated antigen–plasmacytoid dendritic cells induced IL-10⁺CD8⁺ T cells. FACS showed that 100% of IL-10⁺ cells were CCR7⁺CD45RO⁺CD8⁺ T cells (gate 1). IL-10⁺CD8⁺ T cells were CD62L⁺CD45RO⁺ (gate 2). IL-10⁺CD8⁺ cells were largely CD62L⁺CD45RO⁺CD4⁺ T cells (gate 3). B, *in vitro* migration of tumor plasmacytoid dendritic cell–induced IL-10 expressing T cells. After two rounds of stimulation with tumor-associated antigen–plasmacytoid dendritic cells or tumor-associated antigen–myeloid dendritic cells, tumor T cells (5×10^5 /mL) were subject to a chemotaxis assay. A significant migration mediated by MIP3 β was observed with tumor-associated antigen–plasmacytoid dendritic cells–stimulated T cells. The migrated cells were largely CCR7⁺CD8⁺ T cells as shown by FACS ($n = 4$; *, $P < 0.001$, compared with myeloid dendritic cells or medium or anti-CCR7).

(Fig. 2A). Whereas T cells stimulated with myeloid dendritic cells (without plasmacytoid dendritic cells), expressed strong T cell IFN- γ (total $55 \pm 7\%$), 40% of IFN- γ expressing T cells were observed in the fourth cell division, indicating potent effector functions (Fig. 3E). Therefore, repetitive myeloid dendritic cell stimulation is unable to override the T cell–defective function initially induced by tumor plasmacytoid dendritic cells.

Primary tumor IL-10⁺CCR7⁺CD45RO⁺CD8⁺ T cells are suppressive. We next examined the functional characteristics of tumor primary CCR7⁺CD45RO⁺CD8⁺ T cells (Table 1). We sorted CCR7⁺CD45RO⁺CD8⁺ T cells from blood, ascites, and

lymph nodes in the same individual patients ($n = 8$) and from peripheral blood in normal donors ($n = 5$). Direct intracellular staining revealed a negligible level of IL-10 protein in the sorted T cells. However, real-time reverse transcriptase-PCR showed high levels of *IL-10* mRNA in CCR7⁺CD45RO⁺CD8⁺ T cells from peripheral blood, primary tumor ascites, and primary tumor-draining lymph node in patients with ovarian cancer, whereas normal blood CCR7⁺CD45RO⁺CD8⁺ T cells expressed a negligible level of *IL-10* mRNA (*, $P < 0.001$, compared with normal blood; Fig. 4A). Furthermore, the level of *IL-10* mRNA was at least 2-fold higher in CCR7⁺CD45RO⁺CD8⁺ T cells from tumor ascites than that from blood and lymph nodes (Fig. 4A). All the CCR7⁺CD45RO⁺CD8⁺ T cells expressed comparable, but low levels of IFN- γ , whereas activated normal T cells expressed high IFN- γ (Fig. 4B). Altogether, IL-10 is highly expressed in primary CCR7⁺CD45RO⁺CD8⁺ T cells *in vivo* in patients with ovarian carcinoma.

We further tested whether tumor primary CCR7⁺CD45RO⁺CD8⁺ T cells were functionally distinct from normal blood CCR7⁺CD45RO⁺CD8⁺ T cells. To this end, CCR7⁺CD45RO⁺CD8⁺ T cells were sorted from normal blood and ovarian tumor ascites, and cultured with normal allogeneic blood T cells in the presence of anti-CD3 stimulation. Strikingly, CCR7⁺CD45RO⁺CD8⁺ T cells from tumor ascites, but not from normal blood, suppressed T cell proliferation in a dose-dependent manner ($P < 0.01$ for all; Fig. 4C). Thus, tumor environmental CCR7⁺CD45RO⁺CD8⁺ T cells are functionally suppressive T cells (CD8⁺ regulatory T cells). These CD8⁺ regulatory T cells might be induced by tumor environmental plasmacytoid dendritic cells (Fig. 2).

Discussion

Published, detailed functional studies of dendritic cells and T cells in human tumor environment are scarce. Here, we studied the interaction between plasmacytoid dendritic cells and T cells in the tumor microenvironment in ovarian carcinomas. We show that tumor plasmacytoid dendritic cells induce regulatory CD8⁺ T cells.

There are several proposals to explain why the immune system fails to control tumor progression, including deletions or mutations

Table 1. CD45RO⁺CCR7⁺CD8⁺ T cells in ovarian cancer patients

	Tumor ascites (n = 33)	Tumor blood (n = 15)	Tumor lymph node (n = 8)	Normal blood (n = 8)
CD45RO ⁺	59 ± 11*	38 ± 16*	45 ± 7*	19 ± 6
CCR7 ⁺	41 ± 15	48 ± 6	92 ± 6	62 ± 12
CD45RO ⁺ CCR7 ⁺	22 ± 8*	12 ± 5*	20 ± 5*	5 ± 3

NOTE: Fresh T cells were isolated from normal blood, or from ascites, blood, and draining lymph nodes in patients with ovarian cancer, and stained with mouse anti-human CD45RO, CCR7, CD3, and CD8, and then analyzed by flow cytometry analysis. Results were expressed as the percentage of marked cells in CD8⁺CD3⁺ population (mean ± SE). * $P < 0.01$, as compared with normal blood.

affecting the genes encoding proteins in the MHC class I pathway, or the antigen itself (21), altered TCR signal transduction or expression (22), triggering of Fas receptor on CD8⁺ T cells by Fas ligand expressed by tumor cells (23), and producing inhibitory cytokines such as TGF- β and IL-10. We recently showed that tumor environmental CD4⁺CD25⁺FOXP3⁺ T cells contribute to tumor-associated antigen-specific immunosuppression (13). Apart from these possibilities, the dendritic cell system is thought to contribute to tumor immunopathogenesis. Tumor-derived cytokines, such as IL-6, IL-10, M-CSF, and vascular endothelial growth factor inhibit dendritic cell-differentiation,

maturation, and function (8, 16, 24). Immature dendritic cells are found in tumor tissues (25), which might induce IL-10-expressing CD4⁺ regulatory T cells (26, 27). This report, in conjunction with our previous work (9), show for the first time that human tumors mediate plasmacytoid dendritic cell tumor trafficking and enable plasmacytoid dendritic cells to induce CD8⁺ regulatory T cells.

Normal blood plasmacytoid dendritic cells are shown to induce immunosuppressive T cells in an allogeneic T cell reaction (14, 15). We previously showed that tumor environmental plasmacytoid dendritic cells promoted tumor vascularization (10), and induced IL-10-expressing T cells (9). We now show that tumor plasmacytoid dendritic cells induce CD8 regulatory T cells in the context of tumor-specific immunity. Although the mechanisms whereby tumor plasmacytoid dendritic cells induce CD8 regulatory T cells remain to be defined, we have identified four characteristics for these plasmacytoid dendritic cell-induced IL-10⁺CD8⁺ T cells: (a) induction of CD8⁺ regulatory T cells is independent on CD4⁺ regulatory T cells; (b) CD8⁺ regulatory T cells significantly suppress myeloid dendritic cell-mediated tumor-associated antigen-specific T cell effector functions through IL-10; (c) tumor myeloid dendritic cells cannot recover the suppressive functions; (d) CD8⁺ regulatory T cells express functional CCR7 and efficiently migrate with lymphoid homing chemokine MIP-3 β . Furthermore, primary functional IL-10⁺CCR7⁺CD8⁺ T cells are found in blood, malignant ascites, and tumor-draining lymph nodes in patients with ovarian cancers. The data suggests that tumor plasmacytoid dendritic cells may induce IL-10⁺CCR7⁺CD8⁺ regulatory T cells *in vivo*.

CCR7 was used to categorize central memory T cells (CD45RO⁺CCR7⁺) versus effector memory T cells (CD45RO⁺CCR7⁻; ref. 28). Simultaneous staining of peripheral blood cells with CD45RO and CCR7 reveals a high heterogeneity in distribution of these markers (29). Functional variables are suggested to describe different T cell subsets (30). We show that tumor plasmacytoid dendritic cell-induced IL-10⁺ T cells exhibit a central memory T cell phenotype (CCR7⁺CD45RO⁺; ref. 28). These cells are functionally suppressive and migrate in response to MIP-3 β . Furthermore, the *in vivo* primary counterpart (IL-10⁺CD8⁺CD45RO⁺CCR7⁺ T cells) is found in patients with ovarian cancers. Analogously,

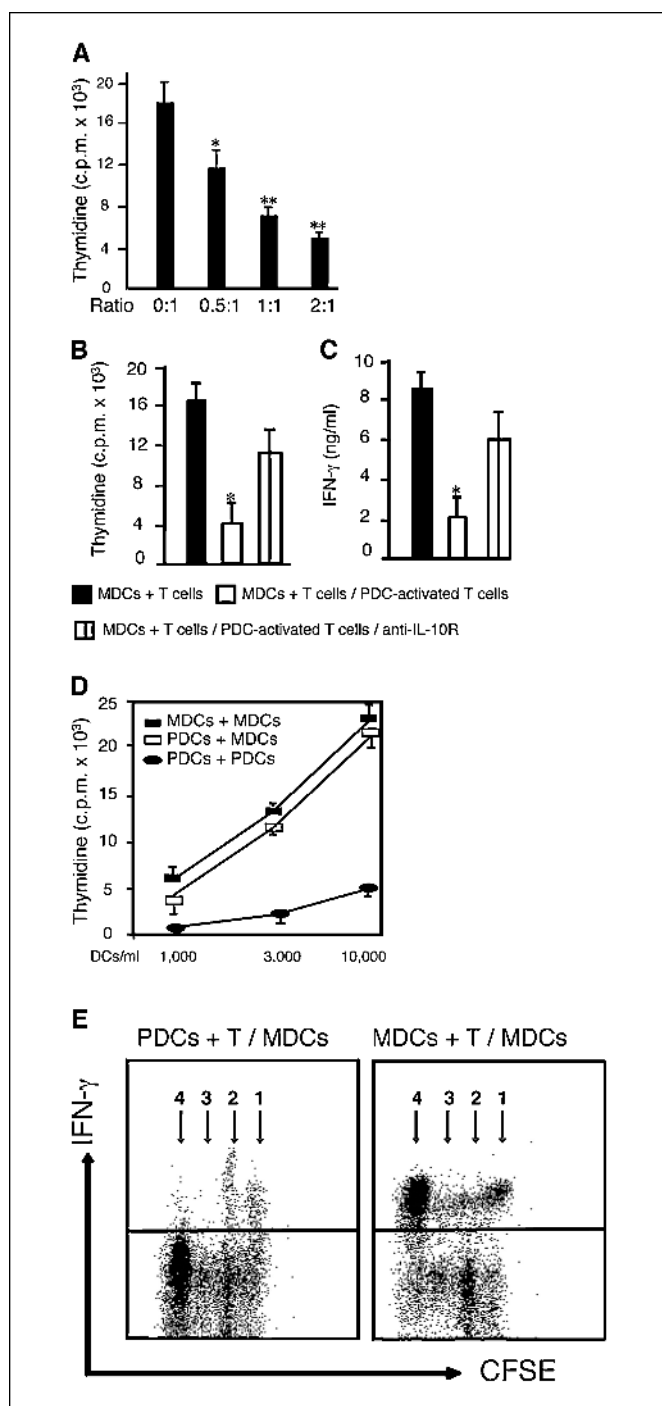


Figure 3. Tumor plasmacytoid dendritic cells induced functionally suppressive IL-10⁺CCR7⁺CD8⁺ T cells. **A**, primary tumor ascites T cells (5×10^5 /mL) received two rounds of stimulation with autologous tumor-associated antigen-plasmacytoid dendritic cells (10^4 /mL). Different concentrations of these tumor-associated antigen-plasmacytoid dendritic cell-stimulated T cells were added into autologous tumor-associated antigen-myeloid dendritic cells (10^4 /mL) and primary T cell (5×10^5 /mL) coculture, and were further cultured for 6 days. T cell proliferation was detected by thymidine incorporation. Ratio, plasmacytoid dendritic cell-activated T cells/primary tumor ascites T cells ($n = 4$, *, $P < 0.05$; **, $P < 0.01$, compared with the group without plasmacytoid dendritic cell-activated T cells). **B** and **C**, anti-human IL-10 receptor was added into the coculture as described above. On day 6, T cell proliferation (**B**) and IFN- γ (**C**) production were detected as described. The ratio between primary tumor ascites T cells and tumor-associated antigen-plasmacytoid dendritic cell-stimulated T cells was 1:1 ($n = 4$; *, $P < 0.01$, compared with the group with anti-human IL-10R). **D** and **E**, 5,6-carboxyfluorescein diacetate succinimidyl ester-labeled autologous tumor T cells (5×10^5 /mL) were divided into three groups. Group 1 received only tumor-associated antigen-myeloid dendritic cell (10^3 - 10^4 /mL) stimulation. Group 2 initially received tumor-associated antigen-plasmacytoid dendritic cell (10^4 /mL) stimulation, and followed with tumor-associated antigen-myeloid dendritic cell (10^3 - 10^4 /mL) stimulation. Group 3 received only tumor-associated antigen-plasmacytoid dendritic cell (10^3 - 10^4 /mL) stimulation. **D**, T cell proliferation was determined by thymidine incorporation. **E**, T cell IFN- γ expression and cell divisions were shown when autologous tumor T cells (5×10^5 /mL) were stimulated with 10^4 /mL tumor-associated antigen-dendritic cells. One of four independent experiments is shown.

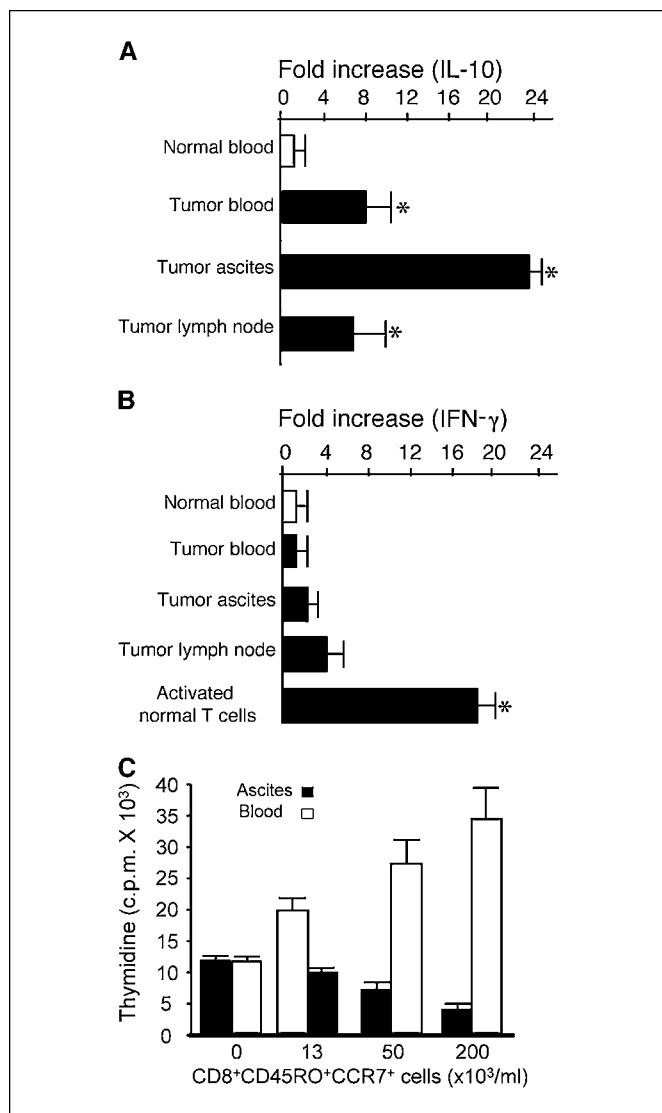


Figure 4. *In vivo* primary functional suppressive IL-10⁺CCR7⁺CD8⁺ T cells. A and B, primary CCR7⁺CD45RO⁺CD8⁺ T cells express IL-10. IL-10 and IFN- γ mRNA were quantified by real-time reverse transcriptase-PCR in sorted primary CCR7⁺CD45RO⁺CD8⁺ T cells from blood, ascites, and tumor-draining lymph nodes. Activated T cells, normal blood T cells were stimulated with anti-CD3 (2.5 μ g/mL) and anti-CD28 (5 μ g/mL) for 48 hours (positive control). C, primary tumor CCR7⁺CD45RO⁺CD8⁺ T cells are suppressive. Primary CCR7⁺CD45RO⁺CD8⁺ T cells were sorted from normal blood and tumor ascites, and cocultured with allogeneic T cells in different ratios in the presence of anti-CD3. T cell proliferation was detected by thymidine incorporation. One of five experiments with five individual donors is shown ($P < 0.05$ for all, compared with blood).

IL-10⁺CD8⁺CD45RO⁺CCR7⁺ T cells may be termed as central regulatory memory T cells.

Notably, the tumor plasmacytoid dendritic cell *in vitro* induced CD8⁺CD45RO⁺CCR7⁺ T cells and primary *in vivo* tumor

CD8⁺CD45RO⁺CCR7⁺ T cells in ovarian tumor-bearing patients are able to inhibit T cell activation. Based on the functional definition of regulatory T cells (31), ovarian tumor-associated CD8⁺CD45RO⁺CCR7⁺ T cells are regulatory CD8⁺ T cells. It remains to be defined whether this population is suppressive in patients with other tumors. Furthermore, normal blood CD8⁺CD45RO⁺CCR7⁺ T cells are not suppressive. Therefore, CD8⁺CD45RO⁺CCR7⁺ T cells are not a common phenotype and marker for defining CD8⁺ regulatory T cells. In fact, CD8⁺ regulatory T cells with distinct phenotypes have been reported. For instance, CD8⁺CD28⁻ alloantigen-specific T suppressor cells were observed in human heart transplant recipients (32). Human CD8⁺CD25⁺ thymocytes are reported to mediate suppressive effects through CTLA4 and TGF- β in normal donors (33). Murine CD8⁺ regulatory T cells with central memory phenotype were also reported to be a novel CD8⁺ regulatory T cell population (34). Our data suggest that tumor plasmacytoid dendritic cells would contribute to CD8⁺ regulatory T cell induction. CD8⁺ regulatory T cells may inhibit tumor-associated antigen-specific effector T cell immunity at the functional sites (tumor sites), and further home to lymph node and suppress tumor-associated antigen-specific naïve T cell central priming at the priming sites.

No immunogenic (functionally mature) myeloid dendritic cells are detected in malignant ascites, suggesting that their absence is immunopathologically relevant, and means to introduce them into the tumor might be therapeutic. However, if myeloid dendritic cells cannot recover T cell effector functions after plasmacytoid dendritic cell induction, as shown in this study, blocking plasmacytoid dendritic cell tumor trafficking or functions may be essential prior to boosting tumor immunity by myeloid dendritic cells. Our data, in conjunction with the work from other groups (35–42), may at least partially explain why tumor-bearing hosts often have established weak tumor immunity, not sufficient to control disease progress, and why only moderate clinical benefits are observed in myeloid dendritic cell-based immunotherapy.

In summary, our study indicates that tumors manipulate tumor environmental dendritic cell subset distribution and functions to subvert tumor immunity (8). Multiple cellular and molecular layers of the suppressive network have been imposed in the tumor microenvironment in patients with cancers (8, 35–42). We suggest that a combination therapy with subverting immunosuppressive mechanisms, including blocking plasmacytoid dendritic cell tumor trafficking or function, is a promising, yet little-studied strategy for treating human cancers.

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