Abstract. Monocyte-derived dendritic cells (Mo-DCs) were generated from peripheral blood monocytes of 12 healthy volunteers (hMo-DCs) and 11 patients (pMo-DCs) with malignancies by culture for 7 days with granulocyte-macrophage colony-stimulating factor and interleukin-4. In this study, we focused on the cytogram pattern by FACS analysis. A gate (R1) was set up by which more than 95% of hMo-DCs were contained. Mo-DCs having lower side scatter than the R1 (R2) comprised 4.5% of hMo-DCs and 24.2% of pMo-DCs. Expressions of antigen presentation-related molecules and phagocytic ability in the R2 of pMo-DCs were lower than those in the R1 population. The R2, but not R1, in pMo-DCs decreased in number between days 7 and 14, and expression levels of antigen presentation-related molecules in the living pMo-DCs on day 14 increased. The 11 patients received dendritic cell vaccine therapy with autologous, tumor-pulsed mature Mo-DCs (day 7). The low R2 group (R2≤10%, 3 patients) had a significantly higher positive delayed-type hypersensitivity reaction against autologous tumor-pulsed Mo-DCs than that of the high R2 group (R2>10%, 8 patients) (p<0.001). These results indicate that the R2 of pMo-DCs may be a dysfunctional and short-lived subset.

Dendritic cells (DCs), which are known as professional antigen-presenting cells (APCs), can induce both the generation and proliferation of specific cytotoxic T lymphocytes (1-6). DCs capture and process antigens, move to the T-dependent areas of secondary lymphoid organs and stimulate naive T cells. Only DCs are capable of inducing primary sensitization against specific antigens in naive T cells (1). The ability to present exogenous antigens to CD8+ T cells through "cross-presentation" is an important feature of DCs (7, 8). Recent advances in biotechnology have made it possible to generate DC-like APCs (Mo-DCs) from peripheral blood mononuclear cells (PBMCs) with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) (9). Thus, Mo-DCs have become popular candidates for DC-based immunotherapy for patients with malignancy of various types (10-19). Unfortunately, the therapeutic efficacy of these DC vaccines has been quite limited. One possible reason is that the antigen-presentation capacity of Mo-DCs generated from cancer patients (pMo-DCs) is impaired (20-26). It has been proposed that DC mediate both T cell immunity and T cell tolerance and that these opposite functions may be linked to the dynamic maturation of DCs (27). If pMo-DCs have impaired maturation ability, pMo-DCs administered to cancer patients may remain immature and sensitize T cells to cancer-related antigens. Although it has been shown that several tumor-secreted factors such as venous endothelial growth factor, IL-6, IL-10 and transforming growth factor β1 (TGF-β1) are able to inhibit the full maturation of functional DCs (28-30), little is known about the reasons why the antigen-presenting ability of pMo-DCs is impaired. To potentiate the efficacy of Mo-DC-based vaccine therapy, a greater understanding of antigen presentation-related functions of pMo-DCs is needed.

Our previous study showed that pMo-DCs of advanced gastrointestinal cancer patients are not only dysfunctional in antigen-presenting ability, but that they also have a relatively short lifespan (26). In this study, therefore, we focused on the identification of this dysfunctional and short-lived pMo-DC subset.

Materials and Methods

Patients. Eleven patients with stage IV carcinoma (3 pancreas, 3 rectum, 2 colon, 2 stomach and 1 gall bladder carcinoma), for whom no other standard therapy option was possible, were enrolled in the
Tumor cells. Autologous tumor cells were collected from malignant effusions, CT-guided biopsy specimens, or probe laparotomy specimens. Tumor specimens were minced with scalpels and passed through metal meshes of decreasing pore size. Cells were cultured in serum-free enriched culture medium (EBM2; Sanko Junyaku, Tokyo, Japan) containing basic fibroblast-growth factor, epidermal growth factors and insulin. To avoid any decrease in tumor-associated antigens, no chemical digestion was done. This procedure yielded a tumor-enriched cell line for Mo-DC-vaccine therapy. A human gastric adenocarcinoma cell line, GCTM-1, was used for in vitro experiments.

Procedure for Mo-DC vaccine. Autologous tumor cells were resuspended in 2 ml of serum-free RPMI medium and lysed by 5 freeze and thaw cycles. Immature Mo-DCs were incubated with the lysed tumor cells overnight (Mo-DCs:tumor cells=5:1). Tumor-pulsed Mo-DCs were further cultured in the presence of 40% monocyte-conditioned medium for Mo-DC maturation, as previously described (32). Tumor-pulsed mature Mo-DCs (10^5 cells/ml) were used for delayed-type hypersensitivity (DTH) skin-test reaction. For testing the tumor-specific response, tumor-pulsed Mo-DCs (10^5 cells/ml) were administered intradermally before and after the treatment. A positive DTH skin-test reaction was defined as an induration greater than 5 mm after 48 h.

Statistical analysis. Fisher’s exact probability test was used for statistical analyses. Calculations were carried out with StatView software (Abacus Concepts, Berkeley, CA, USA). All results with a p value of less than 0.05 were considered statistically significant.

Results

Cytogram pattern of Mo-DCs on day 7. R1 and R2 populations of immature Mo-DCs on day 7 were identified

Table I. Characteristics of study patients.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age^a</th>
<th>Gender</th>
<th>Primary site</th>
<th>% of R2</th>
<th>DTH</th>
<th>Survival time^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43 M</td>
<td>stomach</td>
<td>2.8</td>
<td>+</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>38 M</td>
<td>rectum</td>
<td>5.7</td>
<td>+</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>49 F</td>
<td>rectum</td>
<td>6.2</td>
<td>+</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>64 F</td>
<td>colon</td>
<td>14.6</td>
<td>+</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>65 F</td>
<td>pancreas</td>
<td>18.0</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72 M</td>
<td>colon</td>
<td>23.4</td>
<td>-</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>45 F</td>
<td>rectum</td>
<td>23.4</td>
<td>-</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>65 M</td>
<td>stomach</td>
<td>31.7</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>49 M</td>
<td>gall bladder</td>
<td>41.6</td>
<td>-</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>55 M</td>
<td>pancreas</td>
<td>45.1</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>72 F</td>
<td>pancreas</td>
<td>54.0</td>
<td>-</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Eleven patients with advanced cancer (stage IV), who were enrolled in the present study and were not involved in any previous chemotherapy, radiotherapy, or immunotherapy. Patients were divided into a low R2 group (R2<10%, 3 patients) and a high R2 group (R2 > 10%, 5 patients).

^a years

^b months

Generation of Mo-DCs. PBMCs were isolated from heparinized peripheral blood from the patients and healthy volunteers by Ficoll Paque (Life Technologies, Gaithersburg, MD, USA) density gradient centrifugation. PBMCs were resuspended in GMP-grade RPMI 1640 (Hy-Media, Nipro, Tokyo, Japan) with 1% human albumin (RPMI medium), plated at a density of 2x10^6 cells/ml and allowed to adhere in 24-well culture plates (Nalge Nunk International, Chiba, Japan). After 4-h incubation at 37°C, the non-adherent cells were removed, and the adherent cells were allowed to adhere in 24-well culture plates (Nalge Nunk) containing basic fibroblast-growth factor, epidermal growth factors and insulin. To avoid any decrease in tumor-associated antigens, no chemical digestion was done. This informed consent was also obtained from the healthy volunteers.

Flow cytometry (FACS) analysis. To analyze the cytogram and the expression of antigen presentation-related molecules in Mo-DCs, the cells were incubated for 1 h with anti-CD80 or anti-HLA-ABC (BD Pharmingen, San Diego, CA, USA) conjugated to FITC or anti-CD1c or anti-HLA-DR conjugated to PE (BD Pharmingen). The isotype controls were IgG1 and IgG2a (BD Pharmingen). For staining, cells were washed two times with phosphate-buffered saline (PBS) and then incubated for 1 h at 4°C in (FACS buffer) containing 3% BSA (Sigma, St. Louis, MO, USA) and 0.1% NaN3 (Sigma) in PBS as well as the appropriate concentration of labelled mAb. After a washing with FACS buffer, the fluorescence intensity of gated Mo-DC populations was measured with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the data were analyzed by CELLQuest software (Becton Dickinson).

Cytogram pattern analysis of Mo-DCs. The cytogram pattern of hMo-DCs was more homogeneous than that of pMo-DCs. Mo-DCs were divided into two populations according to the cytogram pattern. A gate was set up in which more than 95% of hMo-DCs were contained; these hMo-DCs were designated as the R1 population in this study; hMo-DCs in the lower side scatter (SSC) gate were designated as the R2 population.

Capture of lysed GCTM-1 tumor cells by Mo-DCs. The membrane components of lysed GCTM-1 cells were labelled with the PKH 67 green fluorescent cell linker kit (Sigma), and Mo-DCs were labelled with PE-conjugated HLA-DR mAb (BD Pharmingen), according to the manufacturers’ protocol. Fluorescence-labelled Mo-DCs and lysed tumor cells were co-cultured at an original cell ratio of 1:1 for 4 h at 37°C or 4°C, washed, and then applied to a FACS Calibur flow cytometer. The fluorescence intensity data were analyzed with CELLQuest software. Both PKH 67-positive and HLA-DR-positive cells (double-positive cells) in gated Mo-DCs populations were defined as lysed tumor cell-captured Mo-DCs.

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by the cytogram pattern of FACS analysis, and hMo-DCs had a higher SSC pattern, whereas pMo-DCs had a lower SSC pattern (Figure 1A). The percentage of R2 in pMo-DCs (n=11, 24.2±5.2%) was significantly higher than that in hMo-DCs (n=12, 4.5±0.6%) (p=0.001, Figure 1B).

The expressions of antigen presentation-related molecules in R1 and R2 populations of Mo-DCs on day 7. The expressions of antigen presentation-related molecules in R1 and R2 populations were compared between hMo-DCs (n=6) and pMo-DCs (n=6) (Figure 2). The expressions of CD80, CD11c and HLA-ABC in the R2 population of hMo-DCs were significantly lower than those in the R1 population (p=0.031, 0.033 and 0.020, respectively). The expressions of CD80, CD11c, HLA-DR and HLA-ABC in the R2 population of pMo-DCs were also significantly lower than those in the R1 population (p=0.013, 0.033, 0.028 and 0.048, respectively). When the expressions of these molecules in the R1 populations of hMo-DCs and pMo-DCs were compared, only CD80 expression in the R1 population of pMo-DCs was lower than that in the hMo-DCs (p=0.050). Similarly, only CD80 expression in the R2 population of pMo-DCs was significantly lower than that in the R2 population of hMo-DCs (p=0.006).

Phagocytic ability of pMo-DCs in R1 and R2 populations on day 7. A dot plot pattern of a representative case is shown in Figure 3A. In this case, even though 47% of the R1 population of pMo-DCs captured the lysed GCTM-1 cells, only 7.7% of the R2 population of pMo-DCs captured the lysed GCTM-1 cells. Data for pMo-DCs generated from 5 patients are shown in Figure 3B. The percentage (10.0±2.2%) of Mo-DCs capturing the lysed GCTM-1 in the R2 population was significantly lower than that (39.0±7.7%) in the R1 population (p=0.023), suggesting that the phagocytic ability of the R2 population was lower than that of the R1 population. Capture of lysed GCTM-1 cells by pMo-DCs was not due to non-specific binding because the percentage of double-positive pMo-DCs at 4°C was less than 5%.

Cytogram pattern and expressions of antigen presentation-related molecules of Mo-DCs on day 14. The percentage of the R2 population in hMo-DCs and pMo-DCs on day 14 was measured. In hMo-DCs, no significant change in cell number was found between day 7 and day 14. In pMo-DCs, however, a significant decrease in cell number was found on day 14 compared to day 7, as found in our previous study (26). A representative cytogram is shown in Figure 4A. The cytogram pattern of pMo-DCs was very similar to that of hMo-DCs on day 14. No significant difference in the percentage of the R2 population was observed between pMo-DCs (n=8, 5.1±1.0%) and hMo-DCs (n=8, 4.9±1.1%) on day 14 (Figure 4B).

The expressions of antigen presentation-related molecules in the R1 population of hMo-DCs (n=6) and pMo-DCs (n=6) on day 14 are compared in Figure 5. No significant differences in the expressions of molecules examined were observed between hMo-DCs and pMo-DCs that survived until day 14.

Relationship between the DTH reaction after Mo-DC-vaccine therapy and the R2 population of pMo-DCs. Eleven patients received Mo-DC-vaccine therapy with autologous tumor-pulsed mature Mo-DCs. Patient profiles are provided in Table I. Autologous tumor-pulsed mature pMo-DCs on day 7 were injected subcutaneously every 2 weeks. Two months after therapy, the reaction of DTH was estimated using

![Figure 1](image_url)

Figure 1. (A) FACS analysis of representative cytogram patterns of hMo-DCs and pMo-DCs on day 7. The R1 gate, which contains more than 95% of hMo-DCs, is determined by forward and side scatter on day 7. R2 comprises Mo-DCs not in R1. (B) Percentages of the R2 population of hMo-DCs (filled column, n=12) and pMo-DCs (dotted column, n=11) on day 7. The results are presented as mean±SE (bars) values.

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autologous tumor-pulsed Mo-DCs to assess the effectiveness of the immunotherapy. The patients were divided into a low R2 group (R2≤10%, 3 patients) and a high R2 group (R2 > 10%, 8 patients). The low R2 group (3/3) had a significantly stronger positive DTH reaction than that of the high R2 group (2/8) (\(p<0.001\), Table I). Patients in the low R2 group had a significantly longer survival time than that of patients in the high R2 group (Figure 6).

**Discussion**

The results of our previous study indicated that Mo-DCs from patients with advanced cancer contain a dysfunctional and short-lived Mo-DC subset (26). In this work, we focused on the specification of the dysfunctional and short-lived Mo-DC subset to estimate the nature of pMo-DCs.

It is known that the cytogram pattern of FACS analysis differs between monocytes and Mo-DCs. When monocytes differentiate to Mo-DCs, the Mo-DC cytogram moves to the upper right. Based on these findings, to identify this short-lived Mo-DC subset, the cytogram pattern of Mo-DCs was analyzed by FACS. A gate (R1) was set up in which more than 95% of Mo-DCs generated from 12 healthy volunteers (hMo-DCs) were contained. The area having lower side scatter than R1 was named R2 and is similar to a gate for monocytes. On day 7, the percentage of the R2 population in pMo-DCs was significantly higher than that in hMo-DCs (Figure 1). In addition, pMo-DCs in R2 had significantly
lower expressions of MHC and costimulatory molecules and a lower phagocytic ability than those in R1 (Figures 2 and 3). On day 14, however, the number of pMo-DCs decreased to three-quarters of that of day 7. The cytogram pattern of pMo-DCs also changed between day 7 and day 14 (Figure 4); the percentage of R1 in pMo-DCs increased. In hMo-DCs, however, neither cell number nor cytogram pattern changed between day 7 and day 14 (Figure 4). The cell number in R1 of pMo-DCs did not change significantly between day 7 and day 14, suggesting that mainly pMo-DCs in R2 were dying between day 7 and day 14. If so, R2 is a short-lived subset. Nevertheless, we cannot rule out completely the possibility that pMo-DCs in R2 changed to those in R1. This latter possibility is unlikely since it requires that Mo-DCs in R1 are short-lived.

Consistent with our previously reported findings (26), expressions of antigen presentation-related molecules of pMo-DCs were weak compared with those of hMo-DCs (Figure 2). Interestingly, most pMo-DCs in R2 disappeared between day 7 and day 14, and the difference in expressions of antigen presentation-related molecules between pMo-DCs and hMo-DCs also disappeared on day 14 (Figure 5). We conclude that the R2 population of pMo-DCs is dysfunctional and short-lived. Some investigators have shown that tumors impair dendritic cell differentiation from monocytes (33). In the present study, the R2 population had almost the same cytogram pattern as monocytes. We now speculate that those pMo-DCs which belong to R2 are insufficiently differentiated Mo-DCs. In fact, the mean fluorescence intensity of CD14 in R2 was higher than that in R1 (data not shown).

DC vaccine therapy with autologous tumor-pulsed Mo-DCs for patients with advanced malignancies is being evaluated in our laboratory. Based on our hypothesis that pMo-DCs that belong to R2 are insufficiently differentiated Mo-DCs, we analyzed the relationship between the percentage of the R2 population in Mo-DCs and the induction of tumor-specific immunological response. We used the DTH skin-test reaction against tumor-pulsed Mo-DCs as a tumor-specific response. As expected, a higher DTH-positive reaction after the therapy was induced in patients whose Mo-DCs contained a smaller R2 population (Table I). This suggests that Mo-DCs containing a smaller R2 population have a higher antigen presentation ability in vivo. This possibility is partly supported by the finding of a longer survival time in patients who received Mo-DCs containing a smaller R2 population (Figure 6). Our results indicate that we may be able to improve the efficacy of DC vaccine therapy by treating the R2 population in pMo-DCs. When anti-TGF-β1 antibody was added to the initial culture of monocytes, Mo-DCs in R2 significantly decreased, and both the expressions of MHC and costimulatory molecules as well as the phagocytic ability of pMo-DCs were improved (data not shown). It has been reported that the overexpression of TGF-β1 enhances cell invasion of fibrosarcoma, prostatic carcinoma and mammary adenocarcinoma cells, with a consequent increase in the
metastatic potential of the tumor (34-36). In addition, it has been shown that tumor-derived TGF-β1 reduces the efficacy of DC vaccine (37, 38). Although these findings suggest that TGF-β1 may partly contribute to generation of the R2 population in Mo-DCs obtained from patients with malignancies, we have no definite recommendation for overcoming this problem.

In conclusion, Mo-DCs in the R2 population are a dysfunctional and short-lived subset. The percentage of R2 population in Mo-DCs may be a useful index for evaluating the quality of Mo-DCs to be used in DC-based vaccine therapy.

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