Impaired and imbalanced cellular immunological status assessed in advanced cancer patients and restoration of the T cell immune status by adoptive T-cell immunotherapy

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ARTICLE INFO

Article history:
Received 25 July 2013
Received in revised form 7 November 2013
Accepted 7 November 2013
Available online 21 November 2013

Keywords:
Immunological status
T cell
Adoptive immunotherapy
Flow cytometry

ABSTRACT

Recent progress has been made in understanding the mechanisms of antitumor immune responses, which may further clarify the immune status of cancer patients. In this study, we performed a detailed evaluation of the immunological status of 47 patients with advanced solid cancer, who had received no immunosuppressive treatment, and compared the results with 32 healthy subjects. Flow cytometry data for peripheral blood were obtained using 19 monoclonal antibodies against various cell surface and intracellular molecules. Absolute numbers of T cells, several T cell subsets, B cells, and NK cells were significantly decreased in patients compared with healthy subjects. The percentage of CD27+CD45RA− T cells was lower and that of CD27−CD45RA− T cells was higher in patients with compared with controls. Regulatory and type 2 helper T cells were elevated in patients relative to healthy subjects. The percentage of perforin + NK cells was significantly lower in patients than in controls. These results suggest a dysfunctional anti-tumor immune response in cancer patients. Furthermore, peripheral blood from 26 of 47 cancer patients was analyzed after adoptive T cell immunotherapy (ATI). ATI increased the number of T cell subsets, but not B and NK cells. The number and percentage of regulatory T cells decreased significantly. These results suggest that ATI can restore impaired and imbalanced T cell immune status.

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1. Introduction

The concept that the immune system recognizes and eliminates tumor cells is well accepted [1,2]. Dysfunction of host systemic immunity, especially cell-mediated immunity (CMI), may be involved in the establishment of advanced cancer. Peripheral blood (PB) is widely used to assess systemic immune function. Previous studies noted that the number of lymphocytes and levels of several cytokines involved in CMI were reduced in PB from cancer patients [3–5]. The cytokine profile of CD4+ helper T cells revealed an imbalance between Th1 (IL-12) and Th2 cells that produce interleukin (IL)-4. The shift from Th1 to Th2 was previously observed in cancer patients [5–7]. Regulatory T (Treg) cells have been reported to be increased in several types of cancer patients [8]. T cells can be divided into various subpopulations that differ in their surface or intracellular markers. Flow cytometry (FCM) to detect those markers might have the potential to clarify the immune status of cancer patients.

The benefits of immunotherapy for human cancer have gradually gained acceptance. In 2010, the United States Food and Drug Administration approved sipuleucel-T, an autologous cellular immunotherapy for the treatment of prostate cancer [9]. Sipuleucel-T is considered to be an active component of antigen-presenting cells that induces a specific immune response against prostatic acid phosphatase [10]. Administration of tumor-infiltrating lymphocytes after lymphodepleting pretreatment was shown to mediate tumor regression in patients with metastatic melanoma [11]. Chimeric antigen receptor-modified T cells targeting CD19 were shown to be beneficial in patients with chronic lymphocytic leukemia [12] and acute lymphoid leukemia [13]. Alternatively, a non-specific immunotherapy approach using autologous T or NK cells was also efficacious [14–18]. We recently reported a survival advantage of adoptive T cell immunotherapy (ATI) in advanced lung cancer patients [19]. In these clinical studies, the accurate assessment of immune effects in patients is of particular importance to understanding the impact of treatment. In the case of antigen-specific approaches, antigen-specific immune responses such as cytotoxic T-cell responses are one of the most important parameters for assessment. In contrast, there are no reliable parameters for non-specific immunotherapy regimes.

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To resolve this, we initially investigated the detailed immunological status of advanced solid cancer patients who had not received prior immunosuppressive treatment, using FCM, and compared it with healthy subjects. In addition, changes in the immunological status of cancer patients receiving ATI were studied. We report that impaired and imbalanced CMI in cancer patients might be restored by ATI.

2. Materials and methods

2.1. Patients and healthy subjects

Forty-seven patients with histologically confirmed cancer of various organs and a performance status of 0, 1, or 2 were enrolled in the study. All patients had unresectable advanced solid cancers and an extended primary tumor with multiple metastases. Patients were excluded if they had an infectious disease, interstitial pneumonia, or autoimmune disease, or if they had received immunosuppressive treatments such as chemotherapy, irradiation, or adrenocorticosteroid hormone in the previous 60 days. Patient characteristics and primary organs are shown in Table 1. Ages ranged from 43 to 84 years, with a median of 69 years. The performance status was 0 in 27, 1 in 17 and 2 in three patients. Thirty-two healthy subjects, aged 40–69 years, were used as controls. The research ethics committee of the Seta Clinic Group, Tokyo, Japan, approved this study. Written informed consent was obtained from all patients and healthy subjects prior to blood collection.

2.2. FCM of peripheral blood mononuclear cells (PBMCs)

Heparinized PB was collected from patients and healthy subjects. We previously determined the influence of temperature and time from blood collection to the start of assay on the results of FCM, and established that storage of PB at 18–22 °C for 1 day followed by phenotypic analyses provided consistent results. Phenotypic analysis of PBMCs was performed by whole-blood staining with OptiLyse C Lysis solution. Absolute cell numbers were determined using Flow-Count™ fluoroscein internal standard beads. The OptiLyse C, Flow-Count beads, and monoclonal antibodies (mAbs) against CD3, CD4, CD8, CD14, CD16, CD19, CD27, CD45, CD45RA, CD56, TCR pan αβ, TCR pan γδ, TCR Vγ9 and NKG2D were purchased from Beckman Coulter (Brea, CA, USA). Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) was used with gradient centrifugation to isolate PBMCs. Isolated PBMCs were used for cytokine production assays, Foxp3, and perforin staining. For Foxp3 staining, PBMCs were fixed and permeabilized using a fixation/permeabilization kit (BioLegend, San Diego, CA, USA) according to the manufacturer’s protocol, and Foxp3 was stained with anti-Foxp3 mAb (clone 259D, BioLegend). For perforin staining, PBMCs were fixed and permeabilized using the IntraPrep™ leukocytic permeabilization reagent (Beckman Coulter), and perforin stained by anti-perforin mAb (eBioscience, San Diego, CA, USA). A Cytomix FC500 and/or Gallios flow cytometer (Beckman Coulter) was used for data acquisition, and the data were analyzed with CXP and/or Kaluza software (Beckman Coulter).

2.3. Cytokine production assay

For analysis of intracellular cytokine production, PBMCs were suspended in RPMI1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) containing 20 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO, USA), 2 μg/mL ionomycin (Sigma-Aldrich), and 20 μg/mL brefeldin A (Sigma-Aldrich), at 1 × 10⁶ cells/mL. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂ for 4 h for the IFN-γ/IL-4 assay or 5 h for the IFN-γ/IL-17 assay. Activated cells were fixed and permeabilized using IntraPrep™. Intracellular cytokines were stained with anti-IFN-γ (Beckman Coulter), IL-4 (Beckman Coulter), or IL-17A mAbs (BioLegend).

2.4. Adoptive T cell immunotherapy

The method for generating activated T cells for treatment was described previously [17,20]. Briefly, PBMCs were cultured with immobilized anti-CD3 antibody and IL-2 for approximately 14 days, and 5 × 10⁹ lymphocytes were harvested. Expanded lymphocytes were infused intravenously and injections were repeated every 2 weeks. The cultured lymphocytes consisted of mainly CD8⁺ or CD4⁺ T cells with small percentages of NK cells and γδ T cells [21].

PB was obtained for FCM analyses just before the first infusion. At the start of treatment, conventional therapies such as chemotherapy or radiotherapy were not indicated in any patients. Tumors were evaluated by radiology after five rounds of ATI. Some patients showed signs of disease progression during ATI, necessitating the use of conventional therapies in combination with ATI. Twenty-six of 47 patients received more than five courses of ATI in the absence of conventional therapy, and were thus evaluated after ATI. In these 26 patients, PB was obtained for FCM analysis 2 weeks after the fifth ATI infusion.

2.5. Statistical analysis

Statistical analysis was conducted using JMP 6.0.0 software (SAS Institute, Cary, NC, USA). Wilcoxon’s rank sum test was used to analyze the data in Table 2 and Wilcoxon’s signed-rank test was used to analyze the data in Table 3. A value of P < 0.05 was considered statistically significant.

3. Results

3.1. Phenotypic characterization of PBMCs

We measured the percentages and absolute numbers of PBMCs collected from 47 cancer patients. The phenotypes were compared with those of 32 healthy control subjects. The statistical values are shown in Table 2 and the important parameters are represented diagrammatically in Figs. 1–3. The numbers of CD4⁵⁺ leukocytes were similar in patients and controls, although the number and percentage
of PBMCs were significantly lower in patients ($P < 0.001$). The percentage and number of CD3$^+$ cells among PBMCs and the numbers of CD3$^+$ CD56$^+$ and CD19$^+$ cells were significantly lower in cancer patients than in controls ($P < 0.001$). In contrast, the percentage of CD14$^+$ cells among PBMCs was significantly higher in patients than in controls ($P < 0.001$).

### 3.2. T cell subsets

We measured the absolute numbers and percentages of T cell subsets (Fig. 2). The numbers of CD3$^+$ TCR$\alpha$+$\beta$+$^+$, CD3$^+$ TCR$\gamma$+$\delta$+$^+$, CD3$^+$ TCR$\alpha$+$\beta$+$^+$, CD3$^+$ TCR$\gamma$+$\delta$+$^+$, CD3$^+$ CD4$^+$ CD8$^+$, and CD3$^+$ CD4$^+$ CD8$^+$ T cells were significantly lower in patients ($P < 0.001$, $P = 0.006$, $P = 0.002$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively) compared with controls. The percentages of CD3$^+$ TCR$\alpha$+$\beta$+$^+$ and CD3$^+$ CD56$^+$ PBMCs were also significantly lower in patients ($P < 0.001$) than in controls. Four T cell subpopulations were distinguished based on CD27 and CD45RA expression. The absolute number of cells in each subpopulation tended to be lower in patients than in controls. The percentage of CD27$^+$ CD45RA$^+$ T cells was significantly lower, and the percentage of CD27$^+$ CD45RA$^+$ T cells was significantly higher in patients ($P < 0.001$, $P = 0.016$) compared with controls.

### 3.3. Subsets of CD4$^+$ T cells

We analyzed the intracellular cytokine profile of CD4$^+$ T cells and the number of regulatory T cells to characterize their anti-tumor function (Fig. 3). The numbers of CD3$^+$ CD4$^+$ IFN-$\gamma$ IL-4$^+$ (Th1), CD3$^+$ CD4$^+$ IFN-$\gamma$ IL-4$^+$ (Th2), and CD3$^+$ CD4$^+$ IL-17$^+$ (Th17) cells were significantly lower in patients than in healthy subjects ($P < 0.001$, $P = 0.016$, $P < 0.001$, respectively). The percentage of Th1 cells among CD3$^+$ CD4$^+$ cells was significantly lower ($P = 0.048$), and that of Th2 cells was significantly higher, in patients ($P = 0.022$) compared with healthy subjects. The Th1/Th2 ratio was lower in patients than in healthy subjects ($P = 0.001$). The overall percentage of Th17 cells was similar in both groups, but an elevated-Th17 subgroup was observed in patients (data not shown). The number of Treg cells was similar, but the percentage of Foxp3$^+$ CD3$^+$ CD4$^+$ Treg cells was significantly higher in patients ($P = 0.001$), compared with controls.

### 3.4. Expression of CD16, NKG2D, and perforin

The number of CD3$^+$ CD56$^+$ CD16$^+$ cells was lower in patients ($P \leq 0.001$), though the percentage of CD16$^+$ cells among CD3$^+$ CD56$^+$ cells was significantly higher ($P = 0.005$) than in healthy subjects. The number of CD3$^+$ CD56$^+$ NKG2D$^+$ cells was significantly lower in patients ($P < 0.001$), though the percentages of NKG2D$^+$ cells among CD3$^+$ CD56$^+$ cells were similar. The number and percentage of CD3$^+$ CD56$^+$ perforin$^+$ cells was lower in patients ($P < 0.001$, $P = 0.005$) than in healthy subjects.

### 3.5. Changes in the number and percentage of immune cells in PB after ATI

To evaluate the anti-tumoral immune responses induced by ATI, we analyzed PB from 26 patients collected 2 weeks after the fifth transfusion. The statistical values are shown in Table 3 and the important parameters are represented diagrammatically in Fig. 4. The total number of cells infused from the first to fifth ATI ranged from $1.8 \times 10^{10}$ to $4.6 \times 10^{10}$ cells. The numbers of CD45$^+$ leukocytes, PBMCs, and CD3$^+$ cells in PB increased significantly after five infusions ($P = 0.010$, $P = 0.001$, $P < 0.001$, respectively), whereas the numbers of CD3$^+$ CD56$^+$ cells, CD19$^+$ cells and CD14$^+$ cells remained unchanged. The percentages and numbers of TCR$\alpha$+$\beta$+$^+$, TCR$\gamma$+$\delta$+$^+$, TCR$\gamma$+$\delta$+$^+$, and CD4$^+$ CD8$^+$ T cells among PBMCs increased significantly after ATI.
(P < 0.001). The percentage of CD4⁺CD8⁺ T cells and the CD4/CD8 ratio decreased significantly after ATI (P < 0.001). The number of CD27⁻/CD45RA⁺ T cells increased significantly following ATI (P < 0.001), whereas the number of CD27⁺CD45RA⁻ cells was unchanged. The percentage of CD27⁺CD45RA⁻ T cells increased significantly (P < 0.001) after ATI, whereas the percentage of CD27⁻CD45RA⁺ T cells decreased (P = 0.033). There were no differences in the percentages of Th1, Th2, and Th17 cells among the CD3³ CD4⁺ subset, though the number of Th2 cells was elevated after ATI (P = 0.005). The percentage and number of Treg cells decreased following ATI. The percentages of CD16¹ cells among CD3⁺ and CD3⁺CD56⁺ cells decreased significantly (P = 0.001, P = 0.039), though their absolute numbers were unaltered. The percentages of NKG2D⁺ cells among the CD3⁺ and CD3⁺CD56⁺ subsets were significantly increased (P < 0.001, P = 0.018), while the number of NKG2D⁺CD3⁺ cells was also significantly increased (P < 0.001). Neither the percentage nor the number of perforin⁺ cells was altered by ATI.

<table>
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<th>Leukocytes</th>
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<td>[CD45⁺⁺ gated] PBMC</td>
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<td>9.4 9.6</td>
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<td>[PBMC gated] CD3⁺⁺</td>
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4. Discussion

The anti-cancer immune response is composed of an orchestrated combination of various immune cells and cytokines. We performed a detailed examination of the immune status in 47 patients with advanced solid cancers with primary sites in various organs. All had unresectable advanced cancer, a performance status of 0, 1 or 2 and adequate physiological functions. Patients with advanced cancer often need conventional therapies; however, such therapies might have toxic effects such as myelosuppression, which could affect the treatment results. In this study, we therefore only analyzed data from patients who did not receive such conventional therapies before or during ATI. To investigate the potential effects of ATI on immune status, we examined PBMCs 2 weeks after the fifth course of ATI. Among the 47 patients, 21 patients required conventional therapy before the fifth ATI infusion, and the results of the remaining 26 patients who continued ATI without conventional therapies were thus used to analyze the effects of ATI.

Immunological status in cancer was determined by comparing FCM results between patients and healthy subjects. The numbers of CD45⁺ leucocytes were similar in patients and controls, although the number of PBMCs was significantly lower in patients, indicating that patients had significantly increased numbers of granulocytes. The numbers of T cells, including several subsets, B cells, and NK cells, were significantly lower, and the percentage of monocytes among PBMCs was significantly higher in patients compared with healthy subjects. These findings are similar to those in previous reports [2,3,22] and indicate a total decrease of lymphocytes. T cells, including αβ, γδ, CD4⁺ and CD8⁺ T cells, are a major component of the cells used in ATI, prompting us to perform a detailed evaluation of T cell subsets. The numbers of cells in these T cell subsets were lower in cancer patients, suggesting total T cell impairment. Furthermore, T cells can be divided into subpopulations based on cell surface markers, such as CD27 and CD45RA [23]. The percentage of CD27⁺CD45RA⁻ naïve cells, which demonstrate a high proliferation rate, was lower in patients than in healthy controls. In contrast, the percentage of CD27⁻CD45RA⁺ effector memory cells, which proliferate poorly but produce IFN-γ and exert antitumor immunity, was higher in patients. This suggests a shift from naïve to effector cells in patients. A similar result was previously reported in patients with squamous cell carcinoma of the head and neck [24]. However, the result is controversial, because another report showed that numbers of naïve, memory, and effector CD8⁺ T subsets did not differ between healthy donors and cancer patients [25]. The percentage of IFN-γ⁺ IL-4⁺ Th1 cells among CD4⁺ T cells was lower, but that of IFN-γ⁺ IL-17⁺ Th2 cells was higher in patients compared with controls. These lead to lower Th1/Th2 ratios in patients. Although the number of Th2 which is a part of CD4⁺ T cells was significantly lower in patients, the difference was smaller than that of Th1. These results indicate that the whole CD4⁺ T response might be
Fig. 1. Box plots of percentages and absolute numbers of representative leukocytes from cancer patients and healthy subjects. Percentages of leukocytes in PBMC (upper row) and absolute numbers of leukocytes in peripheral blood (lower row) in 47 cancer patients and 32 healthy subjects were analyzed. The lengths of the boxes represent the interquartile ranges; horizontal lines represent the medians; vertical lines extend to the minimum and maximum values. *P < 0.05.

Fig. 2. Box plots of percentages and absolute numbers of representative T cell subsets from cancer patients and healthy subjects. Percentages (upper row) and absolute numbers (lower row) of T cell subsets in PB from 47 cancer patients and 32 healthy subjects were analyzed. The lengths of the boxes represent the interquartile ranges; horizontal lines represent the medians; vertical lines extend to the minimum and maximum values. *P < 0.05.
reduced and the Th1/Th2 balance was shifted from Th1 to Th2 in patients. This imbalance has been reported previously, and was confirmed in the current study [6,7]. In terms of the immune regulation, the percentages of Th1 and Th2 cells among CD4+ T cells might help to explain the balance between CMI and humoral immunity. Humoral immunity is predominant in cancer patients. IL-17 is known to be involved in autoimmune disease [18]. IL-17-producing CD3+CD4+ cells are known as Th17 cells, and the role of Th17 cells in malignancy is currently under debate [26]. We attempted to examine Th17 cell numbers in both patients and healthy subjects, but their numbers were unfortunately too low to analyze. Several reports have demonstrated increases in Treg cells in PB or in tumor-infiltrating lymphocytes from cancer patients [27–29]; our study also revealed that the percentage of Foxp3+ CD4+ T cells was higher in patients than in healthy controls. These observations suggest an inhibitory effect on CMI in cancer patients. Expression of surface molecules involved in anti-tumor cytotoxic activity was also examined. The percentage of CD16+ T cells was elevated in patients, though the percentages of NKG2D+ T cells were similar in patients and healthy subjects. These observations differ from previous reports that showed CD16 or NKG2D expression was lower in cancer patients [30,31]. Perforin is a cytolytic protein involved in NK cell cytotoxic activity. The percentage of perforin+ NK cells was lower in patients, indicating dysfunctional NK cell cytotoxic activity.

We previously reported the phenotypes of cells used for ATI infusion in 678 patients [21]. Approximately 90% of cultured cells were αβ T cells, consisting of 30 ± 15% CD3+CD4+ T cells, 61 ± 15% CD3+CD8+ cells, 7 ± 8% γδ T cells, and 7 ± 10% CD3−CD56+ NK cells. The effect of ATI on the immune status of patients was determined in this study by comparing FCM results before and after treatment. The numbers of T cells and their subsets in PB increased significantly, while those of B cells, NK cells, and monocytes did not. Between the before and after treatment, a mean of 3.1 × 10^10 expanded T cells were infused into 26 patients. The total volume of blood in a human is approximately 5000 mL and the median number of T cells in patient PB prior to treatment was 786 cells/μL. Accordingly, the number of T cells in patient whole PB was estimated to be 3.9 × 10^9 cells. Thus, the total number of T cells infused was estimated to be eight times greater than the total number of T cells in whole PB. A significant increase in T cell numbers to 1102 cells/μL in PB might be explained by the infused cells circulating around the body. The changes in percentages of CD4+, CD8+, CD27+/−CD45RA+/−, and NKG2D+ T cells might have resulted from the different percentages of these phenotypes in the infused cells from PBMCs. The numbers of T cell subsets in PB were elevated following ATI, whereas the number of Foxp3+ CD4+ T cells in PB significantly decreased to almost healthy-subject levels (Wilcoxon’s rank sum test). Another recent study investigated the number and percentage of Foxp3+ CD4+ T cells in PB from patients with advanced pancreatic cancer before and after ATI. They reported no significant change in either the number or percentage of Treg cells following ATI [32]. However, most patients in that study had received prior chemotherapy, as well as combined treatments, and chemotherapy is known to have a marked effect on immune status [33]. The number of PBMCs and their phenotypes differed depending on the time from chemotherapy, making evaluation of the effect of ATI on immune status difficult. In the current study, we demonstrated a reduction in the number of Treg cells following ATI in patients who had not received immunosuppressive treatment. This marked reduction of Foxp3+ CD4+ T cells in PB might represent a novel mechanism of ATI, and is worthy of further investigation. Analyses of tumor biopsy specimens might improve our understanding of the anti-tumor immune response. However, this approach is impractical, given that repeated biopsies impose a heavy burden on

Fig. 3. Box plots of percentages and absolute numbers of representative CD4+ T cells subsets and perforin+ NK cells from cancer patients and healthy subjects. Percentages (upper row) and absolute numbers (lower row) of Th1, Th2, Treg and perforin+ NK cells in PB from 47 cancer patients and 32 healthy subjects were analyzed. The lengths of the boxes represent the interquartile ranges; horizontal lines represent the medians; vertical lines extend to the minimum and maximum values. *P < 0.05.
most patients. The prevalence of Treg cells was shown to be increased in the PB, as well as in the tumor site in patients with invasive breast or pancreatic cancer [34]. The significant decrease in Treg cells in the PB following ATI observed in this study thus suggests that Treg cells may also be decreased in the tumor.

It is important to determine whether the change in immunological status by ATI was associated with clinical outcome. However, this issue could not be clarified because the patients in this study had cancers of various organs, and the prognosis thus differed for each patient despite the immunological responses observed. It was previously reported that insufficient lymphocyte numbers might have a negative effect on vaccine therapy for cancer [35,36]. The combination of ATI and antigen-specific immunotherapy might prove beneficial by restoring the number of lymphocytes and imbalanced T cell immune status in patients. Furthermore, the findings of this study indicate a potential application for immunological monitoring of other immunotherapies that enhance T cell immune responses in a non-antigen-specific fashion, including anti-CTLA4 mAb, ipilimumab [37], anti-programmed death 1 (PD-1) antibody [38] and anti-PD-1 ligand antibodies [39].

In summary, the cellular immunological status was impaired in patients with advanced solid cancer, and the impaired T cell immune status was restored by ATI infusions. FCM analysis allows the assessment of cancer patient immune status following immunotherapy.

Sources of funding

The authors received no specific funding for this study.
Acknowledgments

This study was carried out under the joint research of Seta Clinic Group and Medinet. The authors thank Naoko Ariyoshi and Yuichi Takeno (Medinet Co., Ltd.) for technical assistance.

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