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# Immunohistological analysis of peptide-induced delayed-type hypersensitivity in advanced melanoma patients treated with melanoma antigen-pulsed mature monocyte-derived dendritic cell vaccination

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SUMMARY

*Background:* In melanoma patients vaccinated with monocyte-derived melanoma peptide-pulsed dendritic cells (DC), the delayed-type hypersensitivity (DTH) reactions have been examined as a surrogate marker to determine if acquired immunity is induced by DC vaccination. To date, however, only limited information has been reported as for histopathological analyses of DTH.
*Objective:* To evaluate tumor-specific immunomonitoring histopathologically after DC vaccination in melanoma patients.
*Methods:* Seven patients previously vaccinated with monocyte-derived melanoma peptide-pulsed DCs were challenged with recall antigenic peptide injection in the skin of the forearm. Using immunohistochemical techniques, the presence of immune cells and the expression of CD4, CD8, interleukin (IL)-2, IL-4, IL-10, Foxp3, CD1a, CD1d, and interferon (IFN)-γ was investigated at the site of injection where a DTH reaction developed.
*Results:* Strong DTH reactions from infiltrated erythema to bullae formation were detected in all 7 cases. Biopsies taken from the DTH site revealed heavy infiltration of mononuclear cells and eosinophils in the dermis and subcutaneous tissue. Cells staining positively for CD4, CD8, IL-2, IL-4, Foxp3, CD1d, and IFN-γ were increased at the site 48 h after antigen injection in all cases. Cells positive for IL-10 were never found in any patient. Regulatory T cells appeared 6 h after injection and reached their maximum at day 7.
*Conclusions:* The significant induction of $CD8^+$T cells as well as both Th1 and Th2-type cells at the site of DTH suggests that effective antigen presentation leading to anti-tumor immune responses has taken place. Inhibitory mechanisms may also develop as the disappearance of the DTH response could be related to an increase in Foxp3+ cells.


## 1. Introduction

The incidence and mortality of malignant melanoma is steadily increasing among not only Caucasians, but also other populations including the Asian [1]. Surgical treatment is effective in early stage melanoma, but new strategies are required for patients with advanced melanoma that are highly resistant to conventional chemo- and radiation therapies. One of those new strategies is the active immunotherapy that has been developed in recent years but still needs many improvements. A very attractive option is the development of various vaccination strategies using autologous dendritic cells (DC). In these methods, dendritic cells (DC) are differentiated from peripheral blood monocytes, and after pulsing with tumor antigens, they are administered into patients with the aim to induce an active immune response [2–5]. In melanoma patients this procedure can lead to the induction of an anti-tumor effect [2,3,5] due to the proliferation and activation of melanoma-specific cytotoxic T cells. Potential disadvantages that have been reported may be the induction of tolerance caused by cytokines produced by regulatory T cells [4,6], loss of MHC class I expression [4,5,7] and mutations in or downregulation of tumor antigens [5].

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The in vivo response to treatment can be examined using imaging to determine the shrinkage and disappearance of metastasis of the primary melanoma, while the in vitro evaluation of peripheral blood mononuclear cells (PBMCs) activity can be performed in ELISPOT [5,8] and ELISA [8] assays. These assays measure cytokine production of cells after proper tumor antigen presentation by antigen presenting cells to T cells, like IFN-γ, or the release of granzymes by NK cells. Alternatively, a delayed-type hypersensitivity (DTH) test provides a simple approach to study the in vivo immune response induced by DC vaccination [2,5,9,10]. In melanoma patients, the DTH reaction has been examined as a surrogate marker to determine if acquired immunity is induced by DC vaccination loaded with melanoma antigens [2,5,9,10]. The appearance of DTH reactions correlates with the clinical outcome [11–13], suggesting that evaluation of immune responses at the site of the DTH is important for understanding the immunological mechanism. Some previous studies reported histopathological analyses at sites of DTH following treatment with DC vaccines in melanoma patients, showing strong perivascular infiltration of $CD4^+$ and/or $CD8^+$ T cells [2,5,12,14]. Infiltration of Natural Killer (NK) cells and B cells has been looked at only once, but not found [12]. Furthermore information whether Th1 and/or Th2 responses are predominantly activated and by which cytokines is extremely important, but has never been reported to our knowledge. A histopathological assessment of infiltrating cells and cytokines associated with tumor immunity may allow tailoring the therapy regimen, including a determination of the therapeutic interval.

In this study, we performed a detailed analysis of DTH skin biopsies from 7 melanoma patients with acquired immunity due to treatment with a melanoma antigen-pulsed DC vaccine. We also histologically examined the time course of the DTH during a week and focused on the cellular infiltrate as well as cytokine production.

## 2. Patients and methods

### 2.1. Patients

Seven patients with stages III and IV melanoma were judged to be eligible for this study. Entry criteria included age over 18-year old, expression of HLA-A24 on PBMCs, and anticipated survival of greater than 3 months. The characteristics of the patients are listed in Table 1. The study protocol was approved by the Institutional Ethical Review Board of the Graduate School of Medical Science, Kyoto Prefectural University of Medicine. Informed consent was obtained from the patients before entry into the study.

### 2.2. Preparation of monocyte-derived peptide-pulsed DCs for vaccination

Preparation of monocyte-derived peptide-pulsed DCs and the vaccination protocol have been described previously [5]. In short: monocytes were isolated from peripheral blood, differentiated to DCs with IL-4 and GM-CSF for 7 days followed by maturation with TNF-α and Poly (I:C) for 3 days. Mature DCs were coincubated with a cocktail of MAGE peptides for 6 h. Patients were vaccinated with $10 \times 10^6$ cells per injection for 10 intradermal injections every week or every other week in the groin.

### 2.3. Evaluation of clinical outcome

The clinical outcome was evaluated based on analysis of whole body CT images before and after one course of treatment (10 vaccinations).

### 2.4. DTH response

Patients received intradermal injections of 10 μg of a HLA-A24 specific melanoma peptide (MAGE-2.156(9); Takara, Otsu, Japan) in 200 μl of PBS or of PBS only (as a control) at separate sites on the forearm. Forty-eight hours later, the DTH was assessed by determining the area of erythema and induration using two-dimensional measurements. In patient 1, an assessment of the time course of the DTH reaction was performed using measurements at 6, 24, 48, and 168 h after application. The DTH response was considered to be positive if the area of erythema and induration was greater than 10 mm.

### 2.5. Histology and immunohistochemistry

Punch biopsies of the skin at the DTH and control sites were performed 48 h after injection of MAGE-2 and PBS, respectively. Formaldehyde-fixed, paraffin-embedded specimens were stained with hematoxylin and eosin or immunostained with antibodies against CD4 (4B12, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), CD8 (1A5, Novocastra, USA), IFN-γ (n/a, Biogenesis Ltd. Oxford, UK), IL-10 (23738, DakoCytomation Co. Ltd. Kyoto, Japan),

**Table 1**
Characteristics of patients and status before DC vaccination, HLA type, DTH-related information, and clinical outcome evaluated after 1 treatment course (10 DC vaccinations)

| Patient | Age | Sex | History | Site of primary skin tumor | Tumor thickness (mm) | Previous therapy[a] | Stage | Site of metastasis | HLA-A allotype | Antigen pulsed to DC | | Clinical outcome | Total no of vaccines | Survival period[b] (months) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | MAGE-1, 2, 3 + tyrosinase peptides | MART-1 peptide + tumor lysate | | | |
| 1 | 61 | M | – | Cheek | >4.0 | S + C | IIIB | LN | A2, A24 | + | + | NED[c] | 76 | 44 |
| 2 | 51 | M | – | Scalp | Unknown | S | IIIB | LN | A2, A24 | + | – | NED[c] | 68 | 36 |
| 3 | 48 | M | – | Forearm | >4.0 | C | IV | Lung, LN | A3, A24 | + | – | PD | 14 | 5 |
| 4 | 45 | F | – | Scalp | Unknown | S + C | IV | Lung | A2, A24 | + | – | PD | 37 | 16 |
| 5 | 67 | F | – | Face | 1.7 | S + C + T | IV | Lung, adrenal gland | A11, A24 | + | – | SD | 16 | 9 |
| 6 | 54 | F | – | Sole | >4.0 | S + C | IV | Lung, bone, LN | A2, A24 | + | + | PD | 15 | 5 |
| 7 | 56 | F | – | Vulva | 3.0 | S + C | IV | Lung | A2, A24 | + | – | PD | 37 | 15 |

Mean follow-up period: 16.9 months; SD, stable disease; PD, progressive disease; NED, no evidence of disease; LN, lymph nodes.
[a] S = Surgery; C = Chemotherapy; T = Thermo-chemotherapy.
[b] This indicates the follow-up duration from the first DC vaccination to the patient's death.
[c] These patients had no evidence of disease from the time of entry into the study until completion of one course of treatment (10 vaccinations).