CD4 T Cell-Mediated Masking Effects of the Immunogenicity of Tumor-Associated Antigens are Qualitatively and Quantitatively Different Depending on the Individual Antigens

Okano, Shinji
Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciences, Kyushu University

Matsumoto, Yoshihiro
Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University

Yoshiya, Shohei
Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University

Yamashita, Yo-Ichi
Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University

他

https://doi.org/10.15017/26073
Original Article

CD4 T Cell–Mediated Masking Effects of the Immunogenicity of Tumor–Associated Antigens are Qualitatively and Quantitatively Different Depending on the Individual Antigens

Shinji Okano¹, Yoshihiro Matsumoto², Shohei Yoshiya², Yo-ichi Yamashita², Norifumi Harimoto³, Toru Ikekami³, Ken Shirabe³, Mamoru Harada³, Yasunobu Yoshikai⁴ and Yoshihiko Maehara²

¹Division of Pathophysiological and Experimental Pathology, Department of Pathology, Kyushu University, Fukuoka 812–8582, Japan
²Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812–8582, Japan
³Department of Immunology, Shimane University Faculty of Medicine, Izumo, Shimane 693–8501, Japan
⁴Department of Infection Control, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812–8582, Japan

Abstract

The use of cancer immunotherapy as part of multidisciplinary therapies for cancer is a promising strategy for the cure of advanced cancer patients. In cancer immunotherapy, the effective priming of tumor–associated antigen (TAA)–specific CD8⁺ T cells is essential, and therefore, the appropriate selection of the best peptide for targeting the cancer is a most important concern. One criticism in the selection of a TAA is the immunogenicity of the TAA, the vaccination of which effectively elicits clinical responses. However, the critical basic immunological factors that affect the differences in the immunogenicity of TAA remain to be elucidated. Here we found that CD4 T-cell responses suppressed the immunogenicity of the concomitant TAA in a murine melanoma model in which intratumoral activated dendritic therapy (ITADT) was used for treatment of the established cancer, and we observed that the antitumor effects were largely dependent on the CD8 T-cell response. CD4 T-cell depletion simply enhanced the tyrosinase–related protein (TRP) -2180–188 peptide–specific cytotoxic T-cell (CTL) responses, and CD4 T-cell depletion provided immunogenicity for mgp10025–33 peptide, to which a CTL response could not be detected at all in CD4 T-cell–intact mice in the early therapeutic phase. Further, the mgp10025–33 peptide–specific CTL response again became undetectable after the recovery of CD4 T cells in previously CD4-depleted tumor–eradicated mice, whereas the TRP-2180–188 peptide–specific CTL response was still much stronger in CD4-depleted mice than in CD4–intact mice. These findings suggest that the CD4 T cell–mediated masking effects of the immunogenicity of tumor–associated antigens are qualitatively and quantitatively different depending on the individual antigens.

Key words: Dendritic cells, Cancer vaccines, Tumor–associated antigens, regulatory T cells
**Introduction**

Cancer immunotherapy is the fourth most promising strategy for standard cancer therapies, after surgery, chemotherapy, and radiotherapy. The application of cancer immunotherapy to multidisciplinary therapies for cancer holds some promise for advanced cancer patients, because cancer immunotherapy may provide the long-lasting memory response that benefits cancer patients. Dendritic cells (DCs), the most potent professional antigen-presenting cells (pAPCs), play a central role in the presentation of antigens to naive T cells and in the induction of the primary immune response\(^1\). In DC-mediated cancer immunotherapy, effective priming of tumor-associated antigen (TAA)–specific CD8\(^{+}\) T cells is the most important concern, since the frequency of functional TAA–specific effector CD8\(^{+}\) T cells is positively correlated with the clinical response or survival\(^2\). Many immunogenic TAA s have been identified\(^3\), and the appropriate selection of TAA s for the best peptide for targeting a cancer is a most important concern\(^4\). TAA s prefer to be universal tumor antigens, as they are broadly expressed by most tumors, and clinical responses should be observed in the vaccinations using the TAA. Although it is important for the success of immunotherapy to identify the immunogenicity of a TAA to be used, the critical basic immunological factors, which exert an influence on the differences in the immunogenicity of TAA s, largely remain to be elucidated.

Although the CD4 T–cell response is important for the priming of CD8 effector T cells\(^10\)–\(^12\), patients with various cancers (including breast, colorectal, esophageal, and gastric cancer, hepatocellular carcinoma, leukemia, lung cancer, lymphoma, melanoma, ovarian cancer, and pancreatic cancer) have increased numbers of regulatory CD4 T cells (Tregs)\(^13\)\(^14\). The accumulation of Tregs in a tumor site and draining lymph nodes are thought to suppress T–cell immunity, especially the priming of TAA–specific T cells\(^15\) and the expansion of the primed T cells in the tumor sites\(^16\), and this suppression is an obstacle to successful immunotherapy and active vaccination\(^13\)\(^14\). Therefore, total CD4 T–cell responses may tend to mask the immunogenicity of the concomitant TAA in a cancer patient with abundant Tregs, rather than function as an immunostimulant. It has been not clarified whether the degree and quality of the CD4 T cell–mediated masking effects of immunogenicity differ depending on the individual TAA. Such information would be very useful for determining which TAA should be used in immunotherapy. Here, we report that intratumoral activated dendritic therapy (ITADT) for an established malignant melanoma can exert antitumor effects, resulting in eradication of the melanoma at a high rate. The antitumor effects of ITADT were dependent on CD8 T cells, but not on CD4 T cells. Interestingly, the CD4 T–cell depletion enhanced the tyrosinase–related protein (TRP)\(^{2180\text{–}188}\) peptide–specific cytotoxic T–cell (CTL) response in ITADT–treated mice, and elicited an mpg\(^{100\text{–}25\text{–}33}\) peptide–specific CTL response which could not be detected at all in ITADT–treated, CD4 T–cell–intact mice. In addition, in B16 melanoma rechallenge experiments, the mpg\(^{100\text{–}25\text{–}33}\) peptide–specific CTL response again became undetectable after the recovery of CD4 T cells in tumor–eradicated mice that had been administered a CD4–depleting mAb and treated with ITADT. On the other hands, the TRP\(^{2180\text{–}188}\) peptide–specific CTL response was still much stronger after the recovery of CD4 T cells in tumor–eradicated mice that had been administered a CD4–depleting mAb and treated with ITADT than in the tumor–eradicated mice that had been treated with ITADT under intact CD4 T–cell subsets.

These findings suggest that the CD4 T cell–mediated masking effects of immunogenicity of TAAs are qualitatively and quantitatively different depending on the individual antigens;
that is to say, the immunogenicity of an mgp100 peptide is completely masked by the CD4 T-cell response, but that of a TRP-2 peptide is only a partial quantitative effect.

Materials and Methods

Mice
Female C57BL/6 (BL6, H-2b) of Charles River grade were obtained from KBT Oriental (Tosu, Japan). All mice were maintained in specific pathogen-free facilities and were fed standard rodent chow and tap water. All mice were used at 6–12 weeks old. The animal experiments were reviewed by the Ethics Committees for Animal Experiments and Recombinant DNA Experiments, Kyushu University, and were carried out under the ‘Guidelines for Animal Experiments’ of Kyushu University.

Tumor cell lines
A murine malignant melanoma cell line, B16.F1 cells, and a T-cell lymphoma cell line, EL-4, both of which originate from C57BL/6 mice, were purchased from American Type Culture Collections (ATCC, Manassas, VA). These cell lines were maintained in complete medium (RPMI 1640, Sigma–Aldrich, St. Louis, MO) supplemented with 10% FCS (Gibco Life Technologies, Osaka, Japan), 100 IU/ml penicillin (Meiji Seika, Tokyo), and 100 mg/ml streptomycin (Meiji Seika) under a humidified atmosphere containing 5% CO2 at 37°C.

Cell preparation
Axillary and inguinal lymph nodes and spleens were collected and kept on ice in complete culture medium. The lymph nodes or spleens were disrupted by pressing between two glass slides. Cell suspensions were filtered through nylon mesh and washed twice with culture medium. Viable nucleated cells were counted using a standard trypan blue dye exclusion method.

Preparation of bone marrow-derived DCs (bmDCs)
Murine bone marrow–derived DCs (bmDCs) were generated as described with minor modifications17. Briefly, bone marrow cells were obtained and RBCs and lineage-positive cells (B220, CD5, CD11b, Gr-1, TER119, 7/4) were depleted using the SpinSep mouse hematopoietic progenitor enrichment kit (StemCell Technologies, Vancouver, BC, Canada) or BD™ IMag Hematopoietic Progenitor Cell Enrichment Set–DM (BD Biosciences, San Diego, CA). These lineage–negative cells (5–10 × 10^4 / 5 ml / well) were cultured in 50 ng/ml granulocyte–macrophage colony-stimulating factor (GM–CSF ; PeproTech, Hamburg, Germany) and 25 ng/ml interleukin (IL)–4 (PeproTech) in endotoxin–free complete medium in 6–well plates. On Day 3 of culture, half of the culture medium was replaced by fresh medium supplemented with GM–CSF and IL–4 at the same concentration. DCs were harvested on Day 6. The obtained DCs were incubated with 1 µg/ml of lipopolysaccharide (LPS, Sigma–Aldrich) for 8 hours, followed by incubation with 50 µg/ml of polymyxin B for 30 minutes at 37°C. Finally, the DCs were washed three times in endotoxin–free phosphate–buffered saline (PBS, Sigma–Aldrich) for use in subsequent experiments. The maturation state of the DC was confirmed by flow cytometric analysis, as described17.

Intratumoral-activated DC therapy (ITADT) for established subcutaneous tumors
C57BL/6 mice were subcutaneously (s.c.) injected with 1.5 × 10^5 melanoma cells into the right flank on Day 0, and the established tumors were injected with 5 × 10^5 DC in 100 µl of PBS via an intratumoral (i.t.) injection route on the days specified in the Figures.

In all experiments, control groups received 100 µl of PBS alone, instead of DCs. The size of the tumors was assessed three times per week using
and the tumor volume was calculated using the following formula: (tumor volume \( \text{mm}^3 \)) = 0.5236 \times (\text{long axis}) \times (\text{short axis}) \times (\text{height})^{18}.

In vivo depletion of immune subsets

Anti–CD4 or anti–CD8 mAb, which was derived from GK1.5 or 53–6.72 hybridoma cells, respectively, was given intraperitoneally (i.p.; 250 \( \mu \text{g/dose} \)) for CD4+ T cells or CD8+ T cell depletion.\(^{19,20}\) Elimination of CD4 or CD8 cells in tumor-bearing mice was done by i.p. injection of mAbs on Days 5, 6, 7, 10, 13, 16, 19, 21, 24, 27, and 30 after the primary tumor inoculation. Flow cytometry confirmed a 98% depletion of the target cells.

Flow cytometry

Collected cells were centrifuged and incubated with 100 \( \mu \text{l} \) of the supernatant from a cultured hybridoma line producing anti–mouse CD16/32 mAb (2.4G2; ATCC), or with a commercial anti–mouse CD16/32 mAb (BioLegend Japan, Tokyo), for 30 minutes at 4°C (Fc–blocking). The cells were washed and then incubated with various combinations of mAb for 30 minutes at 4°C. The cells were then washed once. The labeled cells were analyzed using a FACSCalibur cytometer with CellQuest software (Becton Dickinson, San Jose, CA). Data were assessed using the FLOWJO program (TREE STAR, San Carlos, CA).

Analysis of Treg, CD4, and CD8 T-cell subsets in the tumor site

Tumor tissues were resected and minced into small pieces. The fragmented tissues were digested with 0.4 mg/ml Liberase Cl (Roche, Mannheim, Germany) and 1% (wt/vol) DNase I (Roche) for 30 minutes at 37°C before the digestion was terminated by the addition of ice-cold PBS supplemented with 10% FCS (Gibco) and 2 mM EDTA (Sigma–Aldrich). As for the analysis of Tregs in the tumor tissue, after Fc–blocking, the cells were stained with allophycocyanin (Apc)–conjugated CD4 mAb (RM4–5, eBioscience, San Diego, CA), fluorescein isothiocyanate (FTTC)–conjugated anti–CD3 mAb (145–2C11; BD Biosciences), and biotinylated anti–CD25 mAb (7D4; BD Biosciences) followed by staining with peridinin chlorophyll protein (PerCP)–streptavidin (BD Biosciences), and washed twice. The cells were fixed and permeabilized with Foxp3 fixation/permeabilization concentrate and diluent (eBioscience), washed with permeabilization buffer (eBioscience), and subsequently stained with a phycoerythrin (PE)–conjugated anti–FOXP3 mAb (FJK–16s, eBioscience) or a phycoerythrin (PE)–conjugated isotype control mAb (eBioscience). For the analysis of CD4 and CD8 T cells, cells were stained with PE-conjugated CD8 mAb (536.7; eBioscience). Apc–conjugated anti–CD3 mAb (145–2C11; BD Biosciences), and biotinylated anti–CD4 mAb (RM4–4; this mAb reactivity cannot be blocked by depleting anti–CD4 mAb, GK1.5; BD Biosciences) followed by staining with PerCP–streptavidin (BD Biosciences), and washed twice. Finally, 125 ng of propidium iodide was added to 250 \( \mu \text{l} \) of cell suspension immediately prior to its application onto the cytometer in order to detect and exclude dead cells from the analysis.

\( ^{51}\)Cr release assay for cytolytic activity of cytotoxic T lymphocytes (CTLs)

Assays for CTL activity were performed as described with minor modifications.\(^{17,21}\) We obtained draining lymph nodes (inguinal and axillary lymph nodes) 7 days after the first immunization with DC, or spleens from the mice as specified in the Figure legend. Lymphocytes (4 \( \times \) 10\(^5\)) were cultured with 1 \( \mu \text{M} \) tyrosinase–related protein (TRP) \(^{2,17,18} \) synthetic peptide (H–2K\(^b\)–restricted antigenic epitope: SVYDFDVWL) or mhp10025–33 peptide (H–2T\(^a\)–restricted antigenic epitope: EGSRNQDWL). Two days later, 30 IU/ml human rIL–2 was added to the medium. After 5 days, the cultured cells were collected and used as CTL effector cells. To
detect B16 melanoma–specific CTL activity, we used TRP–2–peptide–pulsed, mgp100–peptide–pulsed EL–4 target cells, or EL–4 cells pulsed with lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP)34–41 peptide (H–2Db and H–2Kb restricted peptide : AVYNFATCGI), as a third–party control. All of the peptides (purity > 97% as measured by HPLC) were synthesized at Genenet Co. (Fukuoka, Japan). The target cells were labeled with 100 μCi Na2 51CrO4 for 1.5 hours, and a 51Cr release assay was performed as described.2225 The percentage of specific 51Cr release was calculated as follows : % cytotoxicity = [(Cr release of experimental medium – culture medium background) / (maximum Cr release – culture medium background)] × 100. Each data point was obtained from triplicate wells.

**Immunofluorescence for detection of Treg in the tumor tissue**

BL6 mice were inoculated with B16.F1 cells on Day 0, and the established melanomas were resected on day 17. The tumor tissues were embedded in TissueTek cutting medium (Sakura Finetek, Tokyo) using Cryomold (Sakura Finetek), and 6–μm frozen tissue sections were fixed with cold acetone, washed with PBS, and incubated with 5% skim milk in PBS for the blocking of nonspecific staining for 1 h. The sections were immunostained with PE–conjugated anti–FOXP3 mAb (FJK–16s, eBioscience) and Apc–conjugated CD4 mAb (RM4–5, eBioscience), counterstained with DAPI/anti–fade mounting medium (ProLong® Gold antifade reagent with DAPI, Life Technologies, Carlsbad, CA). Microscopy was performed with Biozero BZ–8000 (KEYENCE Japan, Osaka, Japan), and composite images were created using the attached software.

**Statistical analysis**

We used the Mann–Whitney U test for the statistical evaluations of numerical variables between different groups. A probability value of p < 0.05 was considered significant. All data were analyzed using GraphPad PRISM®4 software (version 4, GraphPad Software, San Diego, CA).

**Results**

**ITADT induces efficient antitumor effects against established B16 melanoma, and the antitumor effects are dependent on CD8 T cells, but not on CD4 T cells**

We and others previously reported that the i.t. injection of bone marrow–derived DCs without pulsation with tumor lysates could induce efficient antitumor responses to various cancers with TAA–specific CTL responses in murine subcutaneous tumor models.1724125 In the present study, we referred to this DC–based cancer immunotherapy as intratumoral–activated DC therapy (ITADT). In general, the antitumor effects of ITADT are dependent on the T cells, because the antitumor effects are canceled or diminished in antibody–mediated depletion studies of CD4 and/or CD8 T cells1725 and in experiments using T–cell receptor knockout mice or nude mice (unpublished data). Here, BL6 mice were subcutaneously injected with B16.F1, and an i.t. injection of DCs was given 7 days later, followed by two further injections at 1–week intervals. Consistent with previous reports1724, ITADT using bone marrow–derived DCs induced an efficient antitumor effect, resulting in significant suppression of tumor growth, with four of the five tumors being totally eradicated (Fig. 1).

To investigate which subsets of T cells were essential for the antitumor effects, we performed antibody–mediated depletion studies of CD4 or CD8 T cells before the intratumoral injection of DCs. The suppression of the tumor growth with ITADT was completely cancelled by depletion of CD8 T cells, resulting in outgrowth of all of the tumors (Fig. 1). On the other hand, CD4 T–cell depletion did not affect the tumor volume on day 24 (Fig. 1). Four of the five established tumors were eradicated in the ITADT–treated...
CD4 T-cell depletion enhances the TAA-specific CTL response in ITADT-treated mice with B16 melanomas

Effective priming of TAA–specific CD8+ T cells is one of most important concerns in DC-based immunotherapy\cite{34}. Moreover, TAA–specific CTL responses are essential for the antitumor effects of ITADT, because ITADT induced no effective antitumor response against B16 melanomas in T-cell–receptor β chain–deficient mice (data not shown) and CD8 T-cell–depleted mice (Fig. 1). It is interesting how TAA–specific CTL responses are affected by the CD4 T-cell depletion, and we assessed the CTL responses in the ITADT–treated mice, which had been administered a depleting CD4 mAb, or not.

We confirmed that the B16 melanoma cell line expressed mRNAs (RT–PCR) and proteins (western blotting for TRP2 and intracytoplasmic antigen detection using a flow cytometer for mgp100) of both TRP–2 and mgp100, and interfe-

mice in the absence of CD4 T cells (Fig. 1).

We also observed that the growth suppression of the tumor in the ITADT–treated mice tended to be enhanced by the CD4 T-cell depletion in experiments performed to obtain reproductive data, although the effects did not reach a significant difference (data not shown). These findings suggest that the antitumor effects of ITADT are dependent on the CD8 T cells, but not CD4 T cells, and reciprocally CD4 T cells have a negative impact on tumor suppression by ITADT in the B16 melanoma model.

**Fig. 1** ITADT has efficient antitumor effects against established B16 melanoma, and the antitumor effects are dependent on CD8 T cells, but not on CD4 T cells. On Day 0, BL6 female mice were subcutaneously inoculated with B16.F1 cells, and the subsequently established melanomas were treated with ITADT via intratumoral injection on Days 7, 14, and 21 using bmDCs (the second bar from the left : n = 5). Control groups received PBS alone (the first left bar : n = 4). Some of the ITADT–treated mice were i.p. administered with depleting CD4 (the second bar from the right : n = 5) or CD8 mAbs (the first right bar : n = 3) for the elimination of CD4 or CD8 T cells. Tumor volumes were calculated as described in Materials and Methods. The bar graphs show mean tumor volume ± SEM on Day 24 after tumor inoculation. The eradication rates are indicated above each bar graph, in parentheses. The reproductive data were obtained from three separate experiments. The statistical significances (p value) between the indicated groups are shown in the graph. n.s. = no significant difference between the indicated groups.

**Fig. 2** Assessment of CTL reactivity to the B16 melanoma–associated antigens TRP–2 and mgp100 in the therapeutic phase. Established B16 F1 on BL6 mice (down–pointing triangles) and CD4-depleted BL6 mice (crosses) were treated with ITADT using bmDCs on Day 7. The draining lymph nodes were collected on Day 14. The lymphocytes were isolated and restimulated with TRP–2 peptide (the left line graph) or mgp100 peptide (the right line graph) in vitro for 5 days, and a standard 51Cr–release assay was performed as described in Materials and Methods. Control groups received PBS alone (triangles). Little cytotoxic activity against LCMV peptide–pulsed EL–4 as a third–party target was detected (data not shown). The dot plot data indicate mean % specific lysis ± SEM from triplicate wells at the indicated ET ratio in each group. The data are representative of two independent experiments.
on-gamma (IFN-γ) stimulation induced the expression of MHC (major histocompatibility complex) class I antigens on the cells (data not shown). H-2Kb-restricted CTL responses recognizing a dominant epitope of TRP-2180-188 were detected in the draining lymph nodes of B16-melanoma-bearing mice treated with ITADT, but no CTL response to mglp10025-33 peptide was detected at all (Fig. 2). No detection of CTL response to the mglp10025-33 was observed in multiple independent experiments (data not shown).

Interestingly, the CTL response to TRP-2180-188 was enhanced by in vivo CD4 T-cell depletion, and the CTL response to mglp10025-33 became detected only in the CD4-depleted mice with ITADT-treated B16 melanoma (Fig. 2). We also reproduced the detection of CTL response to mglp10025-33 (data not shown).

These findings suggest that ITADT to B16 melanoma is able to efficiently induce a TRP-2180-188-specific CTL response, but not an mglp10025-33-specific CTL response. In addition, the CD4 T-cell response suppresses the former, and may completely mask the latter from CD8-cell immune responses.

Abundant Tregs accumulate in the tumor site and CD4 depletion enhances the relative number of infiltrating CD8 T cells to the total number of intratumoral cells

It has been reported that Tregs accumulate in the lymph nodes and spleen and suppress the TAA-specific immune response15162627. In addition, the Tregs accumulate in the tumor site and mask the tumor immunogenicity16. We investigated whether the reciprocal CD4 T cell-mediated negative impact of tumor growth is due to an abundant accumulation of Tregs. C57BL/6 mice were subcutaneously injected with B16 melanoma, and tumors were resected on day 17. Fluorescent immunohistochemical studies revealed abundant accumulations of CD4+ FOXP3+ Treg cells (Fig. 3). We also observed

![Fig. 3](https://example.com/fig3.png)

**Fig. 3** Accumulation of Tregs in the tumor sites. BL6 mice were inoculated with B16.F1 cells on Day 0, and the established melanomas were resected and prepared for frozen sections or analysis with flow cytometry on Day 17. (A) The frozen sections were stained with PE-conjugated anti-FOX3 mAb and Apc-conjugated CD4 mAb, counterstained with DAPI. The photographs (original magnification x200) indicate CD4 staining (upper left), FOX3 staining (upper right), DAPI (lower left), and a merged image of the three single images (lower right). (B) The tumors were resected and digested as described in Materials and Methods. Cells were then stained with Apc-conjugated CD4 mAb, FITC-conjugated anti-CD3 mAb, and biotinylated anti-CD25 mAb followed by staining with PerCP-streptavidin, and washed twice. The cells were fixed and permeabilized, and subsequently stained with PE-conjugated anti-FOX3 mAb (the left dot plot) or PE-conjugated isotype control mAb (the right dot plot). Dot plots show the expression of CD25 and/or FOX3 in the CD3+CD4+ gated population. The percentages are indicated within each quadrant. The data are representative of three independent experiments.
CD4 depletion enhances the relative number of infiltrating CD8 T cells to the total number of intratumoral cells. The established B16 melanomas on the BL6 mice (ITADT, CD4 depletion −, n = 3) or CD4-depleted BL6 mice (ITADT, CD4 depletion +, n = 3) had been treated with ITADT, and the tumors were resected on Day 16 (2 days after the second i.t. injection of DCs). Control groups received PBS alone (PBS ; n = 4). The intratumoral cells were stained with PE-conjugated CD8 mAb, Apc-conjugated anti-CD3 mAb, and biotinylated anti-CD4 mAb, followed by staining with PerCP-streptavidin. The percentages of CD3+ CD4+ (the left bar graph) or CD3+ CD8+ cells (the right bar graph) relative to the total intratumoral cells were evaluated. The bar graphs show mean percentages of indicated lymphocytes ± SEM. The data are representative of two separate experiments.

that CD4+ T cells infiltrated in the tumor site and that half of the CD4 T cells were CD3+ CD4+ CD25+ FOXP3+ Treg (Fig. 3). The established B16 melanomas were treated with ITADT, and infiltrating CD4 and CD8 T cells in the tumors were evaluated on Day 16 (2 days after the second i.t. injection of DCs). ITADT enhanced the infiltration of both CD4 and CD8 T cells in the tumor site (Fig. 4). When the ITADT−treated mice had been administered the depleting CD4 mAb, few CD4 T cells were detected in the tumor site on Day 16, indicative of highly efficient depletion of CD4 T cells even in the tumor sites (Fig. 4). Interestingly, the relative number of infiltrating CD8 T cells to the total number of intratumoral cells in the CD4−depleted mice was higher than that in the CD4−intact mice during ITADT. These findings are consistent with earlier reports, and the Tregs suppress the proliferation of effector CD8 T cells in the tumor site and masks the immunogenicity of the TAA of the melanoma.

The mgp10025-33−specific CTL response became undetectable after the recovery of CD4 T cells in previously CD4 T-cell-depleted, tumor-eradicated mice, whereas the TRP-2180-188−specific CTL response was still enhanced in the memory phase

If there is a difference in the type of suppression by CD4 T cells between the mgp10025-33−specific CTL response and the TRP-2180-188−specific CTL response, it is interesting how each CTL response changes in the experiments using the rechallenge of B16 melanoma in the memory phase after the recovery of CD4 T cells from the thymus of mice that had been administered CD4-depleting mAb and treated with ITADT. Fortunately, the antitumor effects were largely dependent on the CD8 T−cell response, and the established melanoma could be eradicated even in the CD4 T cell−depleted mice, which survive for a long term in this model (Fig. 1).

Established B16 melanoma in BL6 mice and CD4 T cell−depleted BL6 mice were treated with ITADT using bmDCs on Days 7, 14, and 21. In some of these mice, the established tumors were completely eradicated on around Day 24, and the administration of depleting anti-CD4 mAb was stopped on Day 30. We confirmed that the depleted CD4 T cells were completely recovered on Day 30 after the cessation of the administration of CD4 mAb using some of the tumor−eradicated mice. We also confirmed that the depleted CD4 T cells had been completely recovered in all the tumor−eradicated mice used for the assessment of CTL response on Day 104 after tumor inoculation when CTL response was evaluated. On Day 90, the mice were rechallenged with an inoculation of B16. F1, and 14 days later the spleens were obtained and the CTL assay was performed. For controls, we used age−matched untreated mice and the untreated, age−matched mice inoculated with B16. F1 14 days earlier.
In the memory response experiment, the TRP-2\textsubscript{180-188} -specific CTL response was still much stronger in the tumor-eradicated mice that had been administered CD4-depleting mAb and treated with ITADT and in which the CD4 T cells had already recovered, compared to that in the tumor-eradicated mice in which CD4 T-cell depletion had not been performed (Fig. 5). Surprisingly, the mgp100\textsubscript{25-33} -specific CTL response, which we had been able to detect in the therapeutic phase, became undetectable after recovery of the CD4 T cells in the previously CD4-depleted tumor-eradicated mice (Fig. 5). The mgp100\textsubscript{25-33} -specific CTL response was still undetectable in the memory phase in the tumor-eradicated mice, which had had intact CD4 T cells throughout this experiment (Fig. 5). Neither CTL responses to all peptides in the control mice nor CTL responses to third-party LCMV GP\textsubscript{34-41} peptide in the effector T cells of all the experimental mice could be detected.

These findings strongly suggest that there are differences in the modality of suppression by CD4 T cells between the mgp100\textsubscript{25-33} -specific CTL response and the TRP-2\textsubscript{180-188} -specific CTL response.

**Discussion**

The ideal TAA should meet certain criteria\textsuperscript{9}, namely therapeutic function, immunogenicity, role of the antigen in oncogenicity, specificity, expression level and percent of antigen-positive cells, stem cell expression, number of patients with antigen-positive cancers, number of antigenic epitopes, and the cellular location of antigen expression. Among these criteria, therapeutic function and immunogenicity are the most important for the selection of the a TAA. However, we infer whether a selected TAA has the potential to exert therapeutic function or is immunogenic, just from the results of actual immunotherapies using a selected TAA. Since, in the relevant studies, the therapeutic protocols vary and the immunological statuses of patients are diverse, the lack of superb results could be due to multiple factors, including inadequate trial design or patient selection and inadequate vaccine formulation or regimens\textsuperscript{9}. These deficiencies of information for determination of therapeutic function and immunogenicity of a TAA may be complemented by more intelligent basic immuno-
logical assessments for the common characteristics of the TAA. However, nowadays, there is too little information regarding the basic immunological characteristics of the immunogenicity of TAA, and it is thus difficult to assess the precise immunogenicity in cancer immunotherapy.

It is well known that there is a significant correlation between immunogenicity and peptide–binding affinity to MHC class I molecules for peptides and altered peptide that improve the binding properties of low–affinity peptides, are useful for cancer immunotherapy. This characterization of TAA peptides elucidates a critical aspect of positive effector T-cell responses. However, it remains to be elucidated whether there are differences among individual TAA regarding their regulatory effects by accumulating immunosuppressive components in cancer patients. Here we present the first report that the CD4 T cell–mediated masking effects of the immunogenicity of TAA are qualitatively and quantitatively different depending on the individual antigens. Our findings shed light on a previously undiscovered characteristic difference in regulatory effects that depends on the individual TAA.

The effects of cancer immunotherapy are limited by various adverse microenvironments for immune responses including the down-regulation of MHC in tumor and tumor–associated stromal cells, the increase of regulatory T cells, the prevention of CD8+ T–cell recognition by immature myeloid cells, and immature microvessel formation preventing effective T–cell infiltration. Among these, regulatory T cells are a most important concern in the elucidation of the TAA–specific T–cell response, because Tregs accumulate in the tumor site and draining lymph nodes in cancer patients and then directly suppress the immunological response. CD4 T cells consist of various types of effector or regulatory T cells, such as Th1, Th2, Th17, Th9, Trl, and Treg, and the deviation of the CD4 T–cell subsets in a cancer patient may finally determine whether the positive or negative response of CD4 T cells to a TAA is induced. In the a murine melanoma model that underwent ITADT, we found that the CD4 T–cell depletion augmented the TAA–specific CD8 T–cell response in the lymph nodes in the therapeutic phase and increased the relative number of the CD8 T cells to the total intratumoral cells. These effects may be due to inducible or naturally occurring Tregs, because abundant Tregs accumulated in the tumor sites on Day 17, in consistent with the previous report .

Yu et al. also reported that the accumulated Tregs most likely suppressed the re–expansion of the infiltrated CD8 T cells in the tumor site, rather than priming or infiltration of the CD8 T cells.

In the present study, we saw that the TRP–2180–188–specific CTL response was enhanced in CD4–depleted, ITADT–treated mice compared to that of the CD4 T cell–intact mice in the therapeutic phase (Fig. 2), and the enhanced response was long–lasting in the memory phase (Fig. 5). We also observed that the number of IFNγ–producing TRP–2180–188–specific CD8 T cells (short restimulation in an intracytoplasmic cytokine analysis using a flow cytometer) increased in the lymph nodes and/or spleens (data not shown), suggesting the augmentation of priming of oligoclonal TRP–2180–188–specific CTL repertoires in the lymph nodes and spleens of the CD4–depleted mice. Although the TRP–2180–188–specific CTL response seems to be quantitatively controlled by CD4 T cells, the CD4 T cell–mediated regulatory effects on the mgp10025–33–peptide–specific CTL response qualitatively differed from those of the TRP–2180–188 peptide–specific CTL response. The CTL response to mgp10025–33–peptide was completely masked in the CD4–intact mice during ITADT, and the response was detected only when there were no CD4 T cells in the therapeutic phase (Fig. 2). The CTL response to mgp10025–33 also became masked from immunological response by the recovery of CD4 T cells in the memory phase (Fig.
5. This phenomenon is very interesting, but the detailed mechanisms remain to be elucidated. It is unlikely that the inducible (or primed) regulatory CD4 T cells exert the masking effect on mgp100, because the recovering CD4 T cells after the depletion of CD4 T cells are newly emigrant from the thymus in the completely tumor-eradicated mice. It is therefore very likely that the essential immunological component is naturally occurring Tregs. Autoantigen-specific Tregs are continuously capacitated by responding to autoantigens in normal regional lymph nodes, and control effector autoimmune T cell response. Accordingly, in this experimental situation, mgp100-specific naturally occurring Tregs may be able to exert complete masking effects on the mgp100 antigen to CD8 T-cell response in the therapeutic phase, and the recovered mgp100-specific naturally occurring Tregs may control both the priming and the memory response of mgp100-specific CD8 T cells after CD4 T-cell recovery in the memory phase in previously CD4-depleted, tumor-eradicated mice. It is noteworthy that there is a difference in the immunoregulatory behavior of TRP-2 and mgp100, although they are both melanoma-associated autoantigens. This difference may be due to a qualitative or quantitative difference in the control of autoantigen expression in the thymus or autoantigen-specific Treg repertoire. The essential mechanisms—including the dominancy of Tregs in the CD4 T cell-mediated attenuation and masking effects of the immunogenicity of the melanoma-associated antigens and the detailed qualitative and quantitative assessment of immunological responses—warrant further investigations in future studies.

In conclusion, the immunobiological characteristics of individual TAAs differ in an aspect of the immunoregulatory response, especially the CD4 T-cell response, and the qualitative and quantitative differences affect the final TAA-specific T-cell responses in vivo, which are essential for cancer immunotherapy. This study emphasizes the importance of investigating the basic immunological characteristics of individual TAAs prior to their inclusion in vaccination and immunotherapy strategies, and such information is very useful for the selection of TAAs for successful cancer immunotherapy.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

We thank Katsuo Sueishi for advice and cooperation on the research direction and Chie Arimatsu for the animal care.

References

8) Stauss HJ, Thomas S, Cesco-Gaspere M, Hart...


28 Denkberg G, Klecheklov E and Reiter Y: Modification of a tumor–derived peptide at an


(Received for publication December 10, 2012)
CD4T 細胞による腫瘍関連抗原の免疫原性の抑制効果
個人の抗原に依存して、質的量的な違いがある

1) 九州大学大学院医学研究院 病理病態学分野
2) 九州大学大学院医学研究院 消化器・総合外科
3) 島根大学医学部 微生物・免疫学講座
4) 生体防御医学研究所 附属感染ネットワーク研究センター

岡野慎士 1), 松本佳大 2), 吉屋匠平 2), 山下洋市 2), 播本憲史 2),
池上徹 2), 調憲 2), 原田守 3), 吉開泰信 4), 前原喜彦 2)

がん免疫療法の集学的治療は進行がん患者の治癒が期待できる効果的治療である。がん免疫療法では腫瘍関連抗原特異的 CD8T 細胞応答が必要であり、これを、標的とする腫瘍に最も効果的な腫瘍関連抗原を選択することが重要である。その上で、臨床的な腫瘍を発症する腫瘍の特徴ある腫瘍関連抗原の選択において、考慮すべき重要な因子の一つ、その免疫原性である。しかし、現在のところ、腫瘍関連抗原の免疫原性の違いを特徴づけるための基礎免疫学的知見の解明は不十分である。我々はここでは、生着腫瘍に活性化樹状細胞を腫瘍内に投与する免疫療法を施行するマウスメラノーマモデル（これは CD8T 細胞依存性に抗腫瘍効果を発揮する）において、CD4T 細胞が腫瘍抗原の免疫原性を抑制することを見出した。その治療相において、CD4T 細胞の除去により、Tyrosinase-related protein (TRP)-2180-188 に対する CD8T 細胞応答は単純に増強されるのみであったが、CD4 が正常なマウスでは検出されない mgp10025-33 ペプチドに対する CD8T 細胞応答に関してはその検出が可能となった。更にメモリー相において、mgp10025-33 ペプチドに対する CD8T 細胞応答は CD4T 細胞除去後、CD4T 細胞が回復すると再び mgp10025-33 ペプチドに対する CD8T 細胞応答が検出されなくなった。一方で、TRP-2180-188 ペプチド応答は CD4T 細胞を除去し、治療によって腫瘍を拒絶したマウスの体内に CD4T 細胞が回復してきても、CD4T 細胞が終始正常なマウスで検出される応答よりも増強したままの状態であった。これらのことは、CD4T 細胞による免疫原性のマスキング効果は個々の腫瘍関連抗原に依存して、質的量的な違いを認めることが示唆された。