

Induction of Cytomegalovirus pp65-specific CD8+T Cells *in vivo* Following Administration of Autologous Tumorlysate-pulsed Dendritic Cells, Coplused with Zoledronate and α -Galactosylceramide in a Patient with Glioblastoma multiforme

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ABSTRACT

V γ 9 γ δ T cells, innate immune cells activated by zoledronate (Z), and V α 24 natural killer T cells, and innate/adaptive immune cells activated by α -galactosylceramide (G) can link innate and adaptive immunities through cross talk with interferon (IFN) α -derived dendritic cells (DCs) (IFN-DCs) from patients with GBM in a way that can amplify the activation and proliferation of cytomegalovirus (CMV) pp65-specific CD8+T cells and that can reduce proliferation of CD4+CD25+FoxP3+regulatory T cells (Treg cells) *in vitro*. In this study, we demonstrate for a patient with glioblastoma multiforme (GBM) that multiple administrations of IFN-DCs pulsed with autologous tumor lysates and Z plus G to the patient result in the enhancement of CMV pp65-specific CD8+T cells and in the reduction of the Treg cells *in vivo*. This leads to the higher induction of CMV pp65-specific CD8+T cells by IFN-DCs pulsed with CMV pp65 peptide and Z plus G derived from peripheral blood mononuclear cells (PBMCs) after DC therapy, compared with that by IFN-DCs pulsed with the peptide and Z plus G derived from PBMCs before DC therapy *in vitro*. Our results indicate that administration of IFN-DCs pulsed with autologous tumor lysates and Z plus G may lead to a good clinical outcome for patients with GBM.

Key words: α -galactosylceramide, cytomegalovirus pp65-specific CD8+T cells, cytomegalovirus pp65, glioblastoma multiforme, zoledronate

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and malignant primary brain tumor in adults. Despite advancing treatments including resection, radiotherapy, and chemotherapy, most patients survive <1 year.^[1-3] Recently, an association has been discovered between human cytomegalovirus (CMV) and malignant gliomas *in vivo* and an extremely robust human CMV-specific CD8+T cell response to the CMV pp65 immunodominant epitope

was observed after one injection of autologous tumor lysate-pulsed dendritic cell (DC).^[4] This rapid expansion of an anti-CMV immune response was not associated with any systemic symptoms of viral illness possibly due to the selective tropism of human CMV for glial cells. It has been also reported that CMV pp65 is an optimal target in GBM as the tumor antigen as CMVpp65 have been detected in cancellous areas of histological sections but not in surrounding healthy tissues.^[5-7] We have recently reported that V γ 9 γ δ T cells, innate immune cells activated by zoledronate (Z), and V α 24

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natural killer T (NKT) cells, and innate/adaptive immune cells activated by α -galactosylceramide (G) can link innate and adaptive immunities through cross talk with interferon (IFN)-DCs from patients with GBM in a way that can amplify the activation and proliferation of CMV pp65-specific CD8+T cells *in vitro* and that can reduce the proliferation of CD4+CD25+FoxP3+regulatory T cells (Treg cells) *in vitro*.^[8]

Based on these observations *in vitro* together with a previous study using tumor lysates from a patient with GBM *in vivo*,^[4] we have done with DC therapy for patient 1 with GBM using IFN-DCs pulsed with autologous tumor lysates and Z plus G. The results showed that multiple administrations of IFN-DCs pulsed with autologous tumor lysates and Z plus G induced enhancement of CMV pp65-specific CD8+T cells and reduction of the Treg cells *in vivo*. This resulted in the higher induction of CMV pp65-specific CD8+T cells by IFN-DCs pulsed with CMV pp65 peptide and Z plus G using peripheral blood (PB) mononuclear cells (PBMCs) after DC therapy, compared with that by IFN-DCs pulsed with the peptide and Z plus G using PBMCs before DC therapy *in vitro*.

MATERIALS AND METHODS

Flow cytometry and reagents

Surface phenotypes were determined using an Navios (Beckman Coulter, CA). The following monoclonal antibody (mAbs) was purchased from Beckman Coulter: anti-CD3, anti-CD4, anti-CD8, anti-V γ 9T cell receptor (TCR), anti-CD14, anti-CD25, anti-CD45, anti-CD56, anti-human leukocyte antigens (HLA) DR, anti-CD40, anti-CD80, anti-CD86, anti-CD11c, anti-CD36, mouse IgG1, mouse IgG2, and mouse IgG2b mAbs. Anti-HLA-Class 1 and anti-CCR7 mAbs were purchased from Becton Dickinson (San Jose, CA) and R and D system (Minneapolis, MN), respectively. Anti-TCR V α 24 mAbs and anti-TCR V β 11 mAbs were purchased from Beckman Coulter (France). Anti-FoxP3 mAb for intracellular staining was purchased from BD Biosciences (Japan). All mAbs were conjugated with fluorescein isothiocyanate (FITC), PE, APC, ECD, PC5, or PC7. Z was purchased from Novartis Pharmaceuticals (Switzerland) and G from Funakoshi Co., Ltd. (Japan).

Generation of IFN-DCs

PBMCs were isolated from HLA-A*0201 positive patient 1 with GBM. The subject provided their written informed consent to use their PBMCs for research purpose. For cell culture in this study, AIM-V medium (Invitrogen, Japan) containing 10% heat-inactivated fetal bovine serum for *in vitro* study or autologous plasma for *in vivo* study was used. PBMCs separated by density gradient centrifugation with Lymphoprep (Nycomed, Norway) were suspended in the medium and then incubated in a flask (Corning Incorporated, Japan) for 1h at 37°C. After removing non-adherent cells

containing CD8+T cells, V γ 9 δ T cells, and V α 24NKT cells, monocytes obtained as adherent cells were cultured in the medium containing granulocyte macrophage colony-stimulating factor (GM-CSF) (1000 U/mL; Primmune Inc., Japan) and IFN α (1000 U/mL; INTRON, MSD K.K., Japan) for 3 days to obtain IFN-DCs.

Preparation of tumor lysate-pulsed IFN-DCs

Tumor lysates were prepared by multiple freeze and thaw cycle method described previously.^[9] IFN-DCs were incubated with tumor lysates (100 μ g/ml) overnight and then washed with PBS twice to remove excess tumor lysates to obtain tumor lysate-pulsed IFN-DCs.

Induction of CMV pp65-specific CD8+T cells by IFN-DCs *in vitro* before and after DC therapy

To investigate MHC-restricted immune responses mediated by CD8+T cells before DC therapy and after DC therapy *in vitro*, HLA-A*0201 restricted, CMV pp65-9-mer synthetic peptides (NLVPMVATV) were used. Lymphocytes (2×10^6) as a non-adherent cell fraction were cultured with autologous IFN-DCs (2×10^5) under two different conditions. IFN-DCs were pulsed with the HLA-A*0201-restricted CMV pp65 peptide (5 μ g/mL) for 2h in serum-free medium and then cultured with the lymphocytes possessing HLA-A*0201. In some experiments, Z (0.1 μ M) and G (100 ng/mL) were added during the culture. The cultures were supplemented with 50 U/mL interleukin-2 (Chiron Benelux B. V., The Netherlands) during the culture. The percentage of CMV pp65-specific CD8+T cells was assessed using a PE-CMV pp65 pentamer (Proimmune) and FITC-anti-CD8 mAb. The percentage of V γ 9 δ T cells among lymphocytes was assessed by anti-V γ 9TCR and anti-CD3 mAbs. The percentage of V α 24NKT cells among lymphocytes expanded was assessed by anti-V α 24 and V β 11 mAbs. The percentage of FoxP3+cells among CD4+CD25+lymphocytes expanded was assessed by anti-FoxP3 mAbs previously described.^[10]

Treatment of patient

Patient 1 received seven intradermal (id) injections of 5×10^6 IFN-DCs pulsed with tumor lysates and Z plus G per vaccine at 1 week intervals.

RESULTS AND DISCUSSION

Phenotypical analysis of IFN-DCs derived from monocytes as adherent cells in the presence of IFN α and GM-CSF

The cells defined as IFN-DCs in this study were CD56 $^{++}$, CD14 $^{+++}$, CD36 $^{+++}$, CCR7 $^{++}$, programmed death ligand (PDL)1 $^{++}$, and PDL2 $^{-}$ as shown in Figure 1. The IFN-DCs were also HLA-class I $^{+++}$, HLA-DR $^{+++}$, CD80 $^{+++}$, CD86 $^{+++}$, CD40 $^{+++}$, CD54 $^{+++}$, and CD11c $^{+++}$ (data not

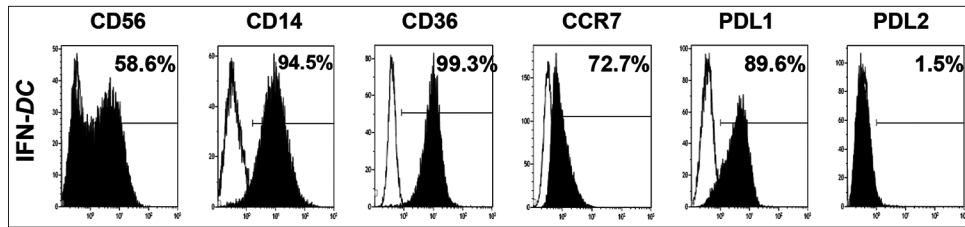


Figure 1: Representative data on phenotypic patterns of CD56, CD14, CD36, CCR7, PDL1, and PDL2 on IFN-DCs Pt. 1 with glioblastoma multiforme. The cells defined as IFN-DCs in this study were CD56⁺⁺, CD14⁺⁺⁺, CD36⁺⁺⁺, CCR7⁺⁺, PDL1⁺⁺⁺, and PDL2⁻

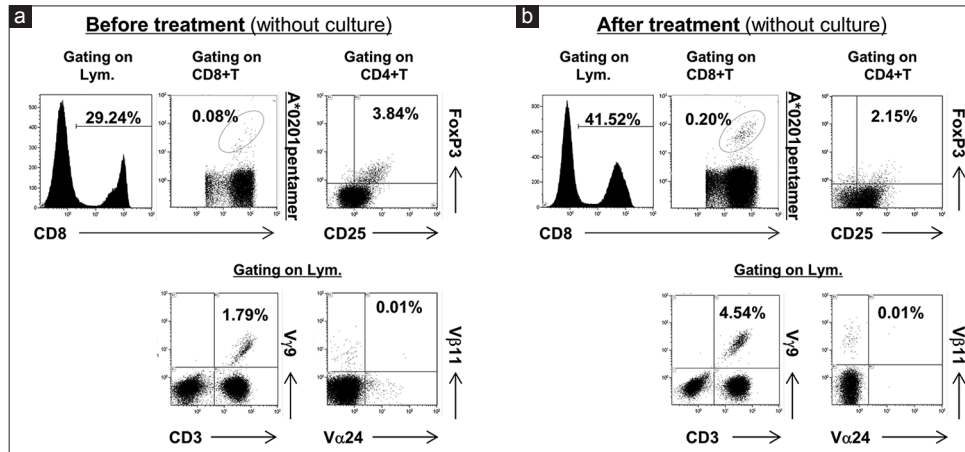


Figure 2: Immunological responses following DC therapy for Pt. 1 using IFN-DCs pulsed with tumor lysates and zoledronate (Z) plus alpha-galactosylceramide (G). (a) Before treatment, percentages of CD8⁺T cells, V γ 9 γ δ T cells, and V α 24 natural killer T cells among peripheral blood (PB) lymphocytes, percentage of HLA-A*0201-restricted cytomegalovirus (CMV) pentamer positive cells among CD8⁺T cells, and percentage of Treg cells (CD4⁺FoxP3⁺CD25⁺cells) among lymphocytes were shown. (b) After treatment, on day 30 following seven intradermal (id) injections of IFN-DCs pulsed with the autologous tumor lysates and Z plus G, percentages of CD8⁺T cells, V γ 9 γ δ T cells, and V α 24NKT cells among PB lymphocytes, percentage of HLA-A*0201-restricted CMV pp65-specific CD8⁺T cells among CD8⁺T cells, and percentage of Treg cells (CD4⁺CD25⁺FoxP3⁺cells) among PB lymphocytes were shown

shown). CD36 is an important molecule for cell processing^[11] and CCR7 is an important molecule for migration of DCs to lymph nodes where interact with lymphocytes.^[12] Following treatment with tumor lysates, expression of CCR7 on IFN-DCs increased (data not shown). Note that high expression of CD36 on IFN-DCs treated with tumor lysates is favorable for processing tumor lysates, resulted in the higher induction of CMV pp65-specific CD8⁺T cells which may be associated with a better clinical outcome.

Immunological responses following DC therapy using IFN-DCs pulsed with tumor lysates and Z plus G

Following DC therapy using IFN-DCs pulsed with tumor lysates and Z plus G for patient 1, increase of CMV pp65-specific CD8⁺T cells among PB CD8⁺T cells (from 0.08% to 0.20%) and decrease of CD4⁺CD25⁺FoxP3⁺Treg cells among PB lymphocytes (from 3.84% to 2.15%) were observed on day 30 following seven id injections of IFN-DCs pulsed with the autologous tumor lysates and Z plus G, as shown in Figures 2a and b. In addition, increase of V γ 9 γ δ T cells among

lymphocytes expanded was also observed (from 1.79% to 4.54%). However, the percentage of V α 24NKT cells among PB lymphocytes was not changed before and after DC therapy at least on day 30 after seven treatments using tumor lysate-pulsed IFN-DCs presumably due to the very low frequency of V α 24NKT cells among PB lymphocytes (0.01%) before treatment [Figures 2a]. Note that slightly decrease of CD4⁺T cells (from 38.40% to 29.65%) and slightly increase of CD3⁺CD56⁺NK cells among PB lymphocytes (from 19.88% to 22.8%) were observed on day 30 following seven treatments using tumor lysate-pulsed IFN-DCs copulsed with Z plus G. Taken together, these results show that administration of IFN-DCs pulsed with autologous tumor lysates copulsed with Z plus G for patient 1 induced the enhancements of CMV pp65-specific CD8⁺T cells and V γ 9 γ δ T cells and the reduction of Treg cells *in vivo*.

Furthermore, we have compared the expansion ability of CMV pp65-specific CD8⁺T cells by IFN-DCs pulsed with CMV pp65 peptide versus that by IFN-DCs pulsed with CMV pp65 peptide and Z plus G before DC therapy and

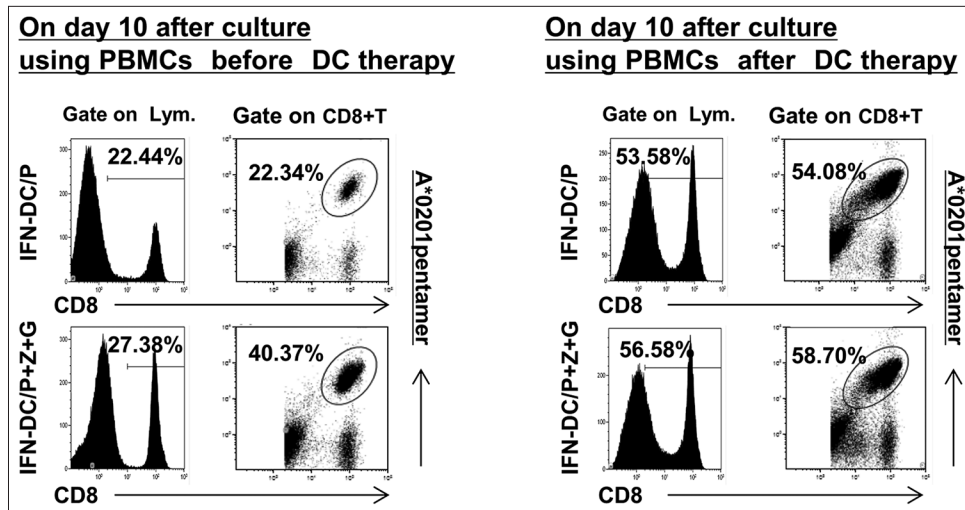


Figure 3: *In vitro* study, the expansion ability of cytomegalovirus (CMV) pp65-specific CD8+T cells by interferon (IFN)-dendritic cells (DCs) pulsed with CMV pp65 peptide and Z plus G versus that by IFN-DCs pulsed with CMV pp65 peptide alone. The expansion ability of CMV pp65-specific CD8+T cells by IFN-DCs pulsed with CMV pp65 peptide and Z plus G was higher than that by IFN-DCs pulsed with CMV pp65 peptide alone in both cases, before DC therapy (22.34% vs. 40.37%) and after DC therapy (54.08% vs. 58.70%) among which the expansion ability of CMV pp65-specific CD8+T cells by IFN-DCs pulsed with CMV pp65 peptide and Z plus G derived from peripheral blood mononuclear cells (PBMCs) after DC therapy was highest (i.e., 58.70%)

after DC therapy *in vitro* [Figure 3]. In the case of patient 1, HLA-A*0201pentermer positive cells among CD8+T cells expanded by IFN-DCs pulsed with CMV pp65 peptide using PBMCs before DC therapy versus that expanded by IFN-DCs pulsed with CMV pp65 peptide using PBMCs after DC therapy were 22.34% versus 54.08%. Further, HLA-A*0201 pentermer positive cells among CD8+T cells expanded by IFN-DCs pulsed with CMV pp65 peptide and Z plus G before DC therapy versus that expanded by IFN-DCs pulsed with CMV pp65 peptide and Z plus G after DC therapy were 40.37% versus 58.70%. These results indicate that the expansion ability of CMV pp65-specific CD8+T cells by IFN-DCs pulsed with CMV pp65 peptide and Z plus G was higher than that by IFN-DCs pulsed with CMV pp65 peptide alone in both cases, before DC therapy and after DC therapy among which the expansion ability of CMV pp65-specific CD8+T cells by IFN-DCs pulsed with CMV pp65 peptide and Z plus G using PBMCs after DC therapy was highest. Note that the total number of lymphocytes expanded by IFN-DCs/P+Z+G was larger than that expanded by IFN-DCs/P following 10-day culture (i.e., 2.0×10^6 on day 0 and 7.2×10^6 following culture with P alone versus 9.9×10^6 following culture with P+Z+G on day 10).

In humans, most of our knowledge on the specificity and biological role of $\gamma\delta$ T cells are derived from the analysis of a major peripheral subset referred to as $V\gamma9V\delta2$ T cells, comprising 5–10% of all circulating T cell in healthy donors (HDs).^[13] However, the percentage of $V\gamma9V\delta2$ T cells

among PB lymphocytes from patients with cancer is very low, <5%.^[14,15] In our own observation, the percentage of $V\gamma9V\delta2$ T cells among PB lymphocytes from patients with GBM was $1.78 \pm 0.16\%$ ($n = 4$). In this study, following administration of IFN-DCs pulsed with tumor lysates and Z plus G, the percentage of $V\gamma9\gamma\delta$ T cells increased from 1.79% to 4.54%, indicating that following infusions of IFN-DCs pulsed with Z and G, the percentage of $V\gamma9\gamma\delta$ T cells among PB lymphocytes from patient 1 closed to the percentage of $V\gamma9\gamma\delta$ T cells among PB lymphocytes from HDs. Further, it has been shown that $V\gamma9\gamma\delta$ T cells present tumor antigens to $\alpha\beta$ T cells.^[16,17] Thus, it is conceivable that $V\gamma9\gamma\delta$ T cells activated *in vivo* by infused IFN-DCs pulsed with tumor lysates and Z plus G might play a role as antigen presenting cells in the uptake of apoptotic bodies from tumor cells *in vivo*, resulted in the increase of CMV pp65-specific CD8+T cell induction. Taken together, administration of tumor lysate-pulsed IFN-DCs copulsed with Z and G is a promising immunotherapy for patients with GBM although more intensive investigations are warranted.

Authorship

M. Y. analyzed the data, wrote the manuscript, and prepared figures; H. T. designed the experiments; Y. E. performed the experiments, analyzed the data, and prepared figures; X. D. designed the experiments; and M. N. conceived the study, performed the experiments, analyzed the data, and wrote the manuscript.

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