

Dendritic cells cross-talk with tumour antigen-specific CD8⁺ T cells, V γ 9 γ δ T cells and V α 24NKT cells in patients with glioblastoma multiforme and in healthy donors

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Summary

The finding that dendritic cells (DCs) orchestrate innate and adaptive immune responses has stimulated research on harnessing DCs for developing more effective vaccines for DC therapy. The expression of cytomegalovirus (CMV) antigens in glioblastoma multiforme (GBM) presents a unique opportunity to target these viral proteins for tumour immunotherapy. Here, we demonstrate that V γ 9 γ δ T cells, innate immune cells activated by zoledronate (Z) and V α 24 natural killer (V α 24NK) cells, 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 glioblastoma multiforme (GBM) and healthy donors in a manner that can amplify the activation and proliferation of CMVpp65-specific CD8⁺ T cells. The IFN DCs derived from patients with GBM used in this study express lower levels of programmed cell death ligand (PD)-L1 and PD-L2 and higher levels of C-C receptor 7 (CCR7) than the most commonly used mature interleukin (IL)-4 DCs. The expression level of programmed cell death 1 (PD-1) on CD8⁺ T cells, including CMVpp65-specific CD8⁺ T cells, expanded by IFN DCs pulsed with the CMVpp65-peptide and Z plus G (IFN DCs/P+Z+G), was lower than that expanded by IFN DCs pulsed with the peptide alone (IFN DCs/P). Multifunctional T cells, including human leucocyte antigen (HLA)-A*0201-restricted CMVpp65-specific CD8⁺ T cells, V γ 9 γ δ T cells and V α 24NKT cells, efficiently kill the HLA-A*0201-positive GBM cell line expressing CMVpp65 protein (T98G). These findings indicate that DC therapy using IFN DCs/P+Z+G and/or CTL therapy using CMVpp65-specific CD8⁺ T cells expanded by IFN DCs/P+Z+G may lead to a good clinical outcome for patients with GBM.

Keywords: alpha-galactosylceramide, CMVpp65-specific CD8⁺ T cells, cytomegalovirus (CMV)pp65, glioblastoma multiforme, zoledronate

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Introduction

Dendritic cells (DCs) are central players in the immune system and operate at the interface of innate and adaptive immunities. DCs have attracted much interest during the past decades for their use in therapeutic vaccination against cancer, owing to their professional antigen-specific T cell immunity. Clinical trials have shown that the use of tumour antigen-loaded DCs in cancer patients is safe; however, their potential in inducing anti-tumour immunity to eradicate tumours is seen in only a minority of patients [1,2].

This underscores the necessity to redesign and optimize current procedures for DC vaccine manufacture.

For DC therapy that eradicates tumours successfully, it is crucial to marshal and orchestrate a vast array of effector cells, including adaptive immune cells such as tumour antigen-specific CD8⁺ T and CD4⁺ helper T cells, innate immune cells such as γ δ T and natural killer (NK) cells and innate/adaptive immune cells, V α 24NKT cells [3], in which DCs play a pivotal role in bridging innate and adaptive immunities. Our clinical study using DCs

pulsed with α -galactosylceramide (G), which is a ligand of V α 24NKT cells for patients with melanoma, has demonstrated that the V α 24NKT cells activated by G-pulsed DCs bridge innate and adaptive immunities to induce the activation and proliferation of interferon (IFN)- γ -producing CD8⁺T cells following the activation of V α 24NKT and NK cells *in vivo* [4]. Furthermore, an *in-vitro* study has shown that the induction of tumour antigen-specific CD8⁺T cells was amplified by DCs pulsed with a tumour antigen and zoledronate (Z), in which V γ 9 γ δ T cells expanded by Z function as T helper (Th) cells through the production of Th1 cytokines such as IFN- γ and tumour necrosis factor (TNF) α [5,6].

In this study, we aimed for a much stronger induction of tumour antigen-specific CD8⁺T cells. We speculated that DCs pulsed with a tumour antigen and Z+G may enhance the induction of tumour antigen-specific CD8⁺T cells through further expansion of not only V γ 9 γ δ T cells, but also V α 24NKT cells.

The outcome of DC therapy depends upon the characteristics of DCs infused. The most widely adopted method of generating DCs of clinical use involves a 1-week, two-step *in-vitro* culture. It requires incubation of monocytes with IL-4 and granulocyte/macrophage-colony stimulating factor (GM-CSF) to obtain immature IL-4-induced DCs (IL-4 DCs), followed by treatment with different maturation stimuli to obtain various mature IL-4-induced DCs (mIL-4 DCs) [7,8]. In another method of DC preparation, it has been shown that monocytes cultured with GM-CSF plus IFN- α can be induced towards the DC lineage, so-called IFN DCs, which highly express CD56 and CD14 molecules [9–11]. Our previous *in-vitro* study has shown that CD56^{high}+IFN DCs possessing HLA-A*0201 effectively induce melanoma-associated antigen recognized by T cells (Mart1)-modified melanoma peptide (A27L)-specific CD8⁺T cells in the presence of A27L and Z through preferential expansion of CD56⁺V γ 9 γ δ T cells, which are potent anti-tumour effectors more capable of killing tumour cells than CD56-V γ 9 γ δ T cells [12]. Taken together with these previous studies of DCs, V γ 9 γ δ T cells and V α 24NKT cells, we highly expected that IFN DCs pulsed with a tumour antigen and Z+G enhance the induction of tumour antigen-specific CD8⁺T cells through the expansion of V γ 9 γ δ T and V α 24NKT cells *in vitro*. Then, infusion of IFN DCs pulsed with a tumour antigen and Z plus G results in a better clinical outcome through the effective expansion of tumour antigen-specific CD8⁺T cells *in vivo*, in which V γ 9 γ δ T and V α 24NKT cells function as adjuvants to enhance the expansion of tumour antigen-specific CD8⁺T cells.

Despite aggressive surgery, radiation and chemotherapy, treatment of patients with glioblastoma multiforme (GBM)

is rarely curative, and conventional therapies are inherently non-specific and damage surrounding normal tissues [13,14]. Immunotherapies including DC therapy provide a promising alternative owing to the intrinsic specificity and potentially long-lasting effects of immune activation [15]. In this study we chose pp65, an antigen from a common herpesvirus called CMVpp65, in GBM as the tumour antigen [16]. Importantly, the cytomegalovirus (CMV) antigens have been detected in cancellous areas of histological sections, but not in surrounding healthy tissues [17,18].

Recently, various molecules that can modulate T cell receptor (TCR) signals have been identified. Multiple co-stimulatory and inhibitory interactions regulate T cell responses to antigen-presenting cells (APCs) such as DCs [19–21]. Many ligands bind to multiple receptors, some of which deliver co-stimulatory signals and others deliver inhibitory signals. In this study, we focused on the expression levels of programmed cell death ligand (PD)-L1 and PD-L2 on IFN DCs as well as the expression levels of programmed cell death (PD)-1 and cytotoxic T lymphocyte antigen (CTLA)-4 on CD8⁺T cells, which are expanded by IFN DCs pulsed with the CMVpp65-peptide alone (IFN DCs/P) and compared them with those on CD8⁺T cells, which are expanded by IFN DCs pulsed with the peptide and Z+G (IFN DCs/P+Z+G).

Immunoinhibition renders T cells dysfunctional in the tumour microenvironment (TME), in which different immunosuppressive cell populations such as regulatory T cells (T_{regs}) and myeloid-derived suppressor cells are involved [22]. In this study, we also examined the induction of CD4⁺CD25⁺forkhead box protein 3 (FoxP3)⁺T_{regs} [23] by IFN DCs/P *versus* IFN DCs/P+Z+G.

Human CMV (HCMV) is a ubiquitous opportunistic pathogen. Symptomatic HCMV infection occurs predominantly in immunocompromised hosts, such as patients after allogeneic haematopoietic stem cell transplantation (alloSCT), whereas symptomatic infection of healthy donors (HDs) is rare. Although inapparent CMV viraemia as a potential prestage of a manifest CMV system or an organ disease can be detected as early as 10–14 days after alloSCT and may last for several weeks, but usually resolves after an early pre-emptive treatment with nucleoside anti-viral agents such as ganciclovir [24], it is conceivable that infusions of CMV-specific CD8⁺T cells from allogeneic HDs may decrease relapse risk in the patients who had alloSCT. Thus, we also analysed the ability of HD-derived IFN DCs/P+Z+G.

The aims of this study were as follows:

1. To determine whether IFN DCs/P+Z+G derived from GBM patients can induce CMVpp65-specific CD8⁺T

cells most extensively, as well as expanded V γ 9 γ δ T and V α 24NKT cells, compared with IFN DCs/P, IFN DCs/P+Z or IFN DCs/P+G.

- To assess whether the expression level of PD-1 on CD8⁺ T cells, including CMVpp65-specific CD8⁺ T cells expanded by IFN DCs/P+Z+G, is lower than that on the CD8⁺ T cell-expanded IFN DCs/P.
- To determine whether effector cells including HLA-A*0201-restricted CMVpp65-specific CD8⁺ T, V γ 9 γ δ T and V α 24NKT cells efficiently kill the HLA-A*0201-positive T98G cell line expressing CMVpp65 protein that was established from a patient with GBM [25,26].

Materials and methods

Flow cytometry and reagents

Surface phenotypes were determined using an Epics XL MCL (Beckman Coulter, Brea, CA, USA). The following monoclonal antibodies (mAbs) were purchased from Beckman Coulter: anti-CD3, anti-CD4, anti-CD8, anti-V γ 9TCR, anti-CD14, anti-CD25, anti-CD45, anti-CD54, anti-CD56, anti-HLA-DR, anti-CD40, anti-CD80, anti-CD86, anti-CD11c, anti-CD36, mouse immunoglobulin (Ig)G1, mouse IgG2 and mouse IgG2b mAbs. Anti-HLA-class 1 and anti-CCR7 mAbs were purchased from Beckton Dickinson (San Jose, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Anti-TCR V α 24TCR and anti-TCR V β 11 mAbs were purchased from Beckman Coulter (Villepinte, France). Anti-human CD273 (PD-L2) and CD274 (PD-L1) mAbs were purchased from eBioscience (San Diego, CA, USA). Anti-human CD152 (CTLA-4) and CD279 (PD-1) mAbs were purchased from BioLegend (San Diego, CA, USA). Anti-FoxP3 mAb for intracellular staining was purchased from BD Biosciences (Tokyo, Japan). All mAbs were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), antigen-presenting cells (APC), extracellular domain (ECD), proprotein convertase (PC)5 or PC7. Z was purchased from Novartis Pharmaceuticals (Basel, Switzerland) and G from Funakoshi Co. Ltd (Tokyo, Japan).

Generation of IFN DCs

Peripheral blood mononuclear cells (PBMCs) were isolated from four patients with GBM and three HDs. HLA types of these donors are listed in Table 1a (patients) and Table 1b (HDs). All subjects provided their written informed consent to use their PBMCs for research purpose. For cell culture in this study, AIM-V medium (Invitrogen, Tokyo, Japan) containing 10% heat-inactivated human AB serum was used. PBMCs separated by density

Table 1. Human leucocyte antigen (HLA)-types of donors are listed from four patients with glioblastoma multiforme (GBM) (a) and three healthy donors (HDs) (b)

(a) HLA-type patients	
Patient 1	HLA-A*0201/A*0101
Patient 2	HLA-A*0201/A*3101
Patient 3	HLA-A*0101
Patient 4	HLA-A*2402/A*3002
(b) HLA-type healthy donors	
HD-1	HLA-A*0201/A*2402
HD-2	HLA-A*0201/A*2402
HD-3	HLA-A*0201

gradient centrifugation with Lymphoprep (Nycomed, Asker, Norway) were suspended in the medium and then incubated in a flask (Corning Incorporated, Tokyo, Japan) for 1 h at 37°C. After removing non-adherent cells containing CD8⁺ T cells, V γ 9 γ δ T cells and V α 24NKT cells, monocytes obtained as adherent cells were cultured in the medium containing GM-CSF (1000 U/ml; Primmune Inc., Kobe, Japan) and IFN- α (1000 U/ml; Intron, MSD K.K., Chiyda-ku, Japan) for 3 days to obtain IFN DCs. Alternatively, adherent cells were cultured for 5 days in the medium containing GM-CSF (1000 U/ml) and IL-4 (500 U/ml) to obtain IL-4 DCs. The IL-4 DCs were cultured for a further 2 days in the presence of TNF- α (10 ng/ml; Reptech Ltd, UK) to obtain mature IL-4DCs (mIL-4DCs).

Induction of tumour antigen-specific CD8⁺ T cells by IFN DCs

To investigate major histocompatibility complex (MHC)-restricted immune responses mediated by CD8⁺ T cells, HLA-A*0101-restricted, CMVpp65-modified 11-mer synthetic peptides (YSEHPTFTSQY), HLA-A*0201-restricted, CMVpp65-9-mer synthetic peptides (NLVPMVATV) and HLA-A*2402-restricted, CMVpp65-9-mer synthetic peptides (VYALPLKML) (Proimmune, Oxford, UK) were used. Lymphocytes (2×10^6) as a non-adherent cell fraction were cultured with autologous IFN DCs (2×10^5) under different conditions. IFN DCs were pulsed with the HLA-A*0101-, HLA-A*0201- or HLA-A*2402-restricted P (5 μ g/ml) for 2 h in serum-free medium and then cultured with the lymphocytes possessing HLA-A*0101, HLA-A*0201 or HLA-A*2402. In some experiments, Z (0.1 μ M) and/or G (100 ng/ml) was added during the culture. The cultures were supplemented with 50 U/ml IL-2 (Chiron Benelux B. V., Hengelo, the Netherlands) during culture. The percentage of CMVpp65-specific CD8⁺ cells was assessed using a PE-CMVpp65 pentamer (Proimmune) and FITC-anti-CD8 mAb (Beckton Dickinson).

Cytotoxicity assay

The T98G tumour cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Fluorochromasia cell-mediated cytotoxicity assay was carried out using Terascan VPC (Minerva Tech., Fukuoka, Japan), according to the manufacturer's instructions. Cells were labelled with calcein-AM solution (Dojindo Laboratories, Kumamoto, Japan) and used as target cells at E/T ratios of 0.6, 1.3, 2.5, 5, 10, 20 and 40 for 2 h and the cytotoxic activity was evaluated by the release of fluorochromasia into the medium [27].

Statistical analyses

The *P*-values for analysis of the expressions of PD-L1, PD-L2 and CCR7 on DCs were calculated using paired/two-tailed Student's *t*-test and considered highly significant at < 0.01 . In some cases, the *P*-values for analysis of the expression of CCR7 were calculated using Bonferroni's multiple-comparison test and considered highly significant at < 0.01 .

Results

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

To characterize surface antigens on IFN DCs, in comparison with those on mIL-4 DCs, IFN DCs and mIL-4 DCs were obtained from four patients with GBM and three HDs. As representative results from patient 1, the IFN DCs showed an almost identical phenotypical pattern to mIL-4 DCs expressing high levels of HLA class I, HLA-DR, CD80, CD86, CD40, CD54, CD11c and CD36 (data not shown), but not CD56, CD14, PD-L1, PD-L2 and CCR7 (Fig. 1a). The expression levels of CD56 and CD14 on IFN DCs from patients with GBM were significantly higher than those on mIL-4 DCs (data not shown), as reported previously in patients with various cancers [12,28]. Phenotypical patterns of IFN DCs from HDs are very similar to those from patients regarding

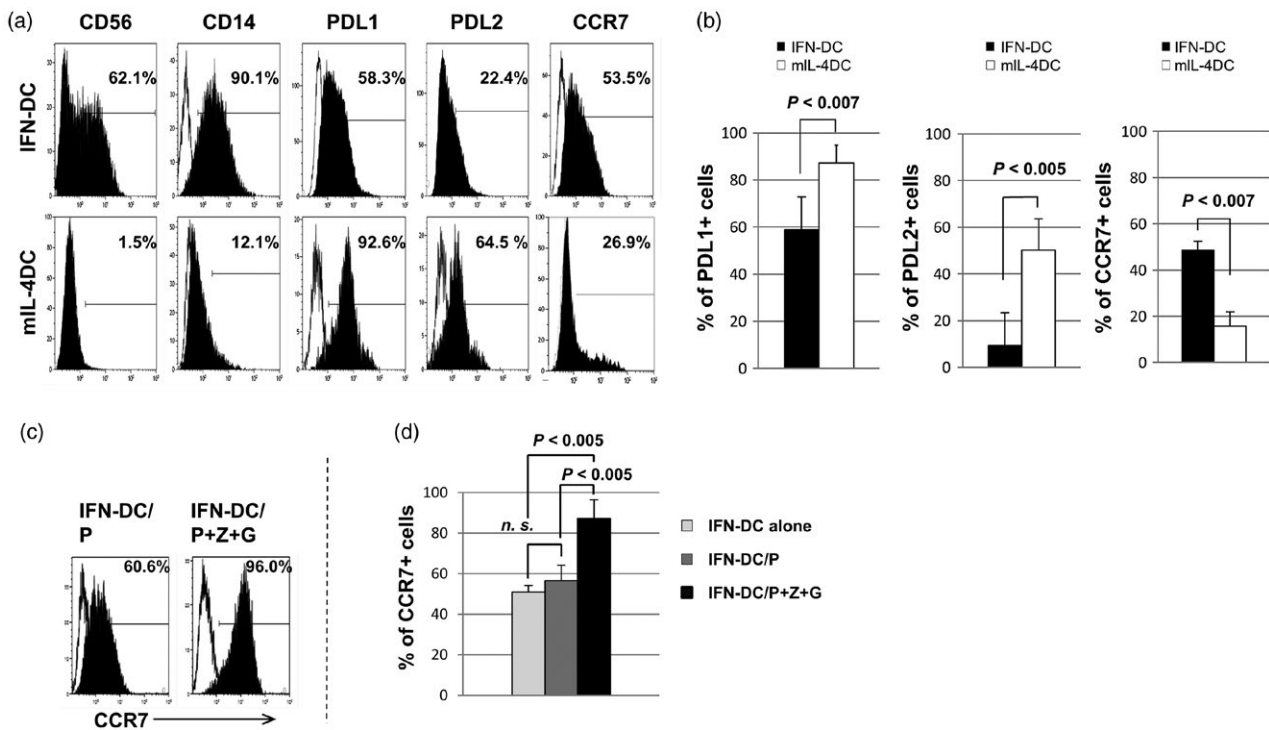


Fig. 1. Phenotypical patterns of interferon dendritic cells (IFN DCs) versus mature interleukin (mIL)-4 DCs. (a) Representative data on phenotypical patterns of CD56, CD14, programmed death ligand 1 (PD)-L1, PD-L2 and C-C receptor 7 (CCR7) on IFN DC and mIL-4 DCs from patient 1 with glioblastoma multiforme (GBM). (b) Statistical analysis using data from four patients with GBM using the paired/two-tailed Student's *t*-test showed that the percentages of PD-L1⁺ cells and PD-L2⁺ cells among IFN DCs were significantly lower than those among mIL-4 DCs, and the percentage of CCR7⁺ cells among IFN DCs was significantly higher than those among mIL-4 DCs. (c) The expression level of CCR7 on IFN DCs from patient 1 gradually increased following addition of the CMV66-peptide (P) alone, and the addition of the cytomegalovirus (CMV)pp65-peptide (P) and zoledronate (Z) plus α -galactosylceramide (G). (d) Statistical analysis of data from four patients with GBM using Bonferroni's multiple-comparison test showed that the percentage of CCR7⁺ cells among IFN DCs/P was not significantly (*n.s.*) higher than that among IFN DCs alone. The percentage of CCR7⁺ cells among IFN DCs/P+Z+G was significantly higher than that among IFN DCs alone ($P < 0.005$) and was significantly higher than that among IFN DCs/P ($P < 0.005$).

the expression levels of HLA-class I, HLA-DR, CD80, CD86, CD40, CD54, CD11c, CD36, CD56, CD14, PD-L1, PD-L2 and CCR7, as described previously [28].

The expression levels of PD-L1 and PD-L2, which are considered to mediate inhibitory signals predominantly towards T cells [29] on IFN DCs, were significantly lower than those on mIL-4 DCs (Fig. 1b), indicating that IFN DCs have a higher ability to expand T cells than mIL-4 DCs. Furthermore, the expression level of lymph node homing receptor CCR7 on IFN DCs was significantly higher than that on mIL-4 DCs (Fig. 1b). The expression level of CCR7 on IFN DCs/P+Z+G was significantly higher than that on IFN DCs/P (Fig. 1c,d). These results suggest that IFN DCs pulsed with a tumour antigen and Z+G have a higher ability to migrate to the lymph nodes, in comparison with IFN DCs pulsed with a tumour antigen.

Effective induction of CMVpp65-specific CD8⁺ T cells by IFN DCs/P+Z+G derived from patients with GBM

The induction of CMVpp65-specific CD8⁺ T cells by autologous IFN DCs was examined under four different conditions: (1) IFN DCs/P, (2) IFN DCs/P+Z, (3) IFN DCs/P+G and (4) IFN DCs/P+Z+G. The case of patient 1 possessing HLA-A*0201 and HLA-A*0101 before culture each population is shown in Fig. 2a. Before culture, percentages of HLA-A*0201-restricted CMVpp65-specific CD8⁺ T cells and HLA-A*0101-restricted CMVpp65-specific CD8⁺ T cells among CD8⁺ T cells were 0.62 and 0.17%, respectively, and percentages of CD8⁺ T cells, Vγ9γδT cells and Vα24NKT cells among lymphocytes were 30.00, 1.71 and 0.06%, respectively. After culture for 10 days, the results are shown in Fig. 2b. Remarkably, IFN DCs expanded most CMVpp65-specific CD8⁺ T cells extensively when pulsed with HLA-A*0201-restricted P and Z+G, compared with other different conditions mentioned above. In addition, using IFN DCs/P+Z+G, expansions of Vγ9γδT and Vα24NKT cells were also observed (Fig. 2b).

To verify our own pentamer results, we assessed the antigen-specific cytotoxic activity of lymphocytes containing 51.44% HLA-A*0201-restricted CMVpp65-specific CD8⁺ T cells, 12.12% Vγ9γδT cells and 0.35% Vα24NKT T cells with P+Z+G (Fig. 2b with P+Z+G) *versus* that of lymphocytes containing 39.46% HLA-A*0201-restricted CMVpp65-specific CD8⁺ T cells, 0.10% Vγ9γδT cells and 0.01% Vα24NKT cells (Fig. 2b with P). Expression levels of MHC class I, PD-L1, PD-L2, MHC class I-related chain A (MIC-A), which is a ligand for NKG2D on Vγ9γδT cells, and CD1d, which is a ligand for Vα24NKT cells, on the T98G tumour cell line used as target cells were 99.5, 11.5, 3.3, 0.3 and 0.1%, respectively (Fig. 2c).

As shown in Fig. 2d, the effector cells including CD8⁺ T cells containing CMVpp65-specific CD8⁺ T, Vγ9γδT and Vα24NKT cells (Fig. 2b with P+Z+G) killed the T98G cell line more efficiently in a dose-dependent manner in comparison with the effector cells including mainly CD8⁺ T cells containing CMVpp65-specific CD8⁺ T cells (Fig. 2b with P). This killing activity was not enhanced in the presence of anti-PD-L1 and/or anti-PD-L2m Ab, owing presumably to the low expression levels of PD-L1 and PD-L2 on the T98G cell line, as shown in Fig. 2c.

Furthermore, we conducted experiments using IFN DCs from patient 1 pulsed with the HLA-A*0101-restricted P. The results were similar to those from patient 1 pulsed with HLA-A*0201-restricted P, showing that IFN DCs expanded CMVpp65-specific CD8⁺ T cells most extensively when pulsed with HLA-A*0101-restricted P and Z+G, in addition to the expansions of Vγ9γδT and Vα24NKT cells, compared with the other three different conditions mentioned above (Fig. 2e). We conducted further experiments using IFN DCs from another HLA-A*0201-positive patient 2 and observed that the percentage of HLA-A*0201-restricted CMVpp65-specific CD8⁺ T cells among the expanded CD8⁺ T cells expanded by IFN DCs pulsed with HLA-A*0201-restricted P+Z+G (40.37%) was higher than that expanded by IFN DCs pulsed with P (22.34%). In another HLA-A*0101-positive patient 3 using the HLA-A*0101-restricted P, the induction of CMVpp65-specific CD8⁺ T cells by IFN DCs/P+Z+G was also higher than that by IFN DCs/P (21.61% for IFN DCs/P+Z+G *versus* 15.43% for IFN DCs/P). We also tested HLA-A*2402-positive patient 4 using the HLA-A*2402-restricted peptide. The results showed that the induction of CMVpp65-specific CD8⁺ T cells by IFN DCs/P+Z+G was also higher than that by IFN DCs/P (24.30% for IFN DCs/P+Z+G *versus* 14.31% for IFN DCs/P). Taken together, the induction of CMVpp65-specific CD8⁺ T cells by IFN DCs/P+Z+G was higher than that by IFN DCs/P in all cases using HLA-A*0201-restricted, HLA-A*0101-restricted and HLA-A*2402-restricted P. Note that remarkable expansion of Vγ9γδT and Vα24NKT cells was observed in all patients using IFN DCs pulsed with P+Z+G.

For all patients, it was observed that the total number of lymphocytes expanded by IFN DCs/P+Z+G was larger than that expanded by IFN DCs/P (Table 2: experiments 1–5). This resulted in the higher induction of CMVpp65-specific CD8⁺ T cells using IFN DCs/P+Z+G, in comparison with the use of IFN DCs/P (Table 2: experiments 1–5). Note that in the cases of stimulation by IFN DCs/P+Z+G from patient 1, the total number of lymphocytes expanded was larger than those of stimulation by DCs under the other three conditions: with P, P+Z and P+G (Table 2: experiments 1 and 2). This provided the highest induction

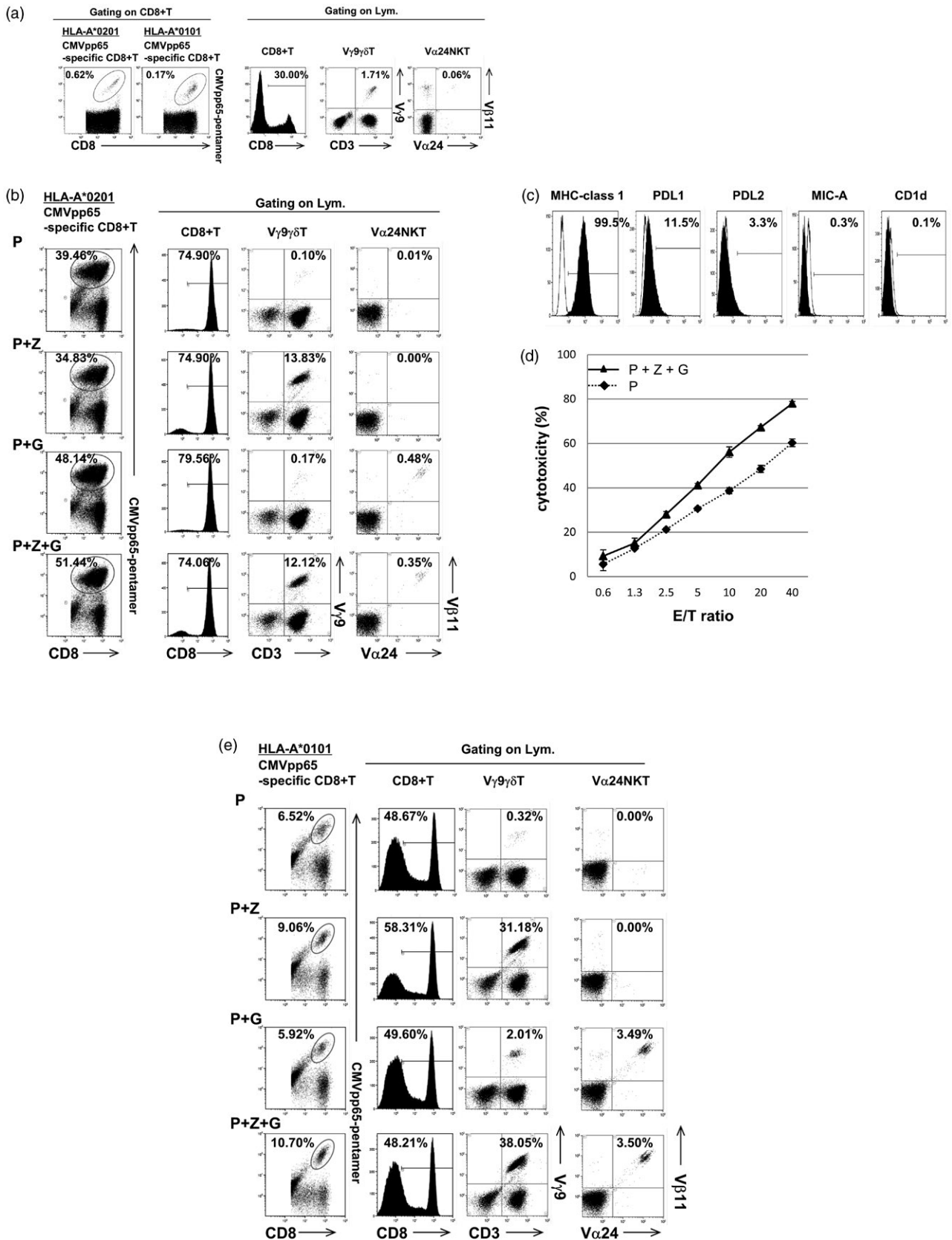


Fig. 2. Effective induction of cytomegalovirus (CMV)pp65-specific CD8⁺ T cells by interferon dendritic dells/peptide and zoledronate plus α -galactosylceramide (IFN DCs/P+Z+G) derived from patients with glioblastoma multiforme (GBM). (a) Each cell population was shown in the case of patient 1 possessing human leucocyte antigen (HLA)-A*0201 and HLA-A*0101, before culture. (b) After culture, IFN DCs from patient 1 induced CMVpp65-specific CD8⁺ T cells most extensively when pulsed with HLA-A*0201-restricted P and Z+G in addition to the expansion of V γ 9 γ δ T and V α 24NKT cells, in comparison with the other three different conditions (P, P+Z and P+G). (c) Expression levels of major histocompatibility complex (MHC) class I, programmed cell death ligand 1 (PD-L1), PD-L2, MHC class I-related chain A (MIC-A) and CD1d on the T98G tumour cell line established from a patient with GBM used as target cells are shown. (d) The effector cells including CD8⁺ T cells containing CMVpp65-specific CD8⁺ T, V γ 9 γ δ T and V α 24NKT cells (as shown in Fig. 2b with P+Z+G) killed the T98G tumour cell line more efficiently in a dose-dependent manner, in comparison with the effector cells including mainly CD8⁺ T cells containing CMVpp65-specific CD8⁺ T cells (as shown in Fig. 2b with P alone). (e) After culture, IFN DCs from patient 1 induced CMVpp65-specific CD8⁺ T cells most extensively when pulsed with HLA-A*0101-restricted P plus Z+G, in addition to the expansion of V γ 9 γ δ T and V α 24NKT cells, in comparison with the other three different conditions (P, P+Z and P+G).

of CMVpp65-specific CD8⁺ T cells in the case of using IFN DCs/P+Z+G. Furthermore, using IFN DCs/P+Z+G for five experiments from four patients, the number of V γ 9 γ δ T cells among lymphocytes expanded was eight- to 198-fold larger than that in using IFN DCs/P (Table 3). Also, the number of V α 24NKT cell-expanded IFN DCs/P+Z+G was larger than that expanded by IFN DCs/P, although the number was small in any conditions (data not shown). Moreover, the percentage of CD4⁺CD25⁺FoxP3⁺ T_{regs} among lymphocytes following culture with IFN DCs was examined. The results show that the percentage of CD4⁺CD25⁺FoxP3⁺ T_{regs} among lymphocytes following culture with IFN DCs/P+Z+G was lower than that following culture with IFN DCs/P in patients 1, 2 and 4 tested so far (Fig. 3a). Statistical analysis showed that the CD4⁺CD25⁺FoxP3⁺ T_{regs} among

lymphocytes following culture with IFN DCs/P+Z+G was significantly lower than the percentage of the T_{regs} among lymphocytes following culture with IFN DCs/P ($4.22 \pm 2.00\%$ versus $5.05 \pm 1.93\%$, $P < 0.01$) (Fig. 3b).

Immune checkpoint blockade with monoclonal antibodies against the inhibitory immune receptors of CTLA-4 and PD-1 has emerged as a successful treatment approach for patients with advanced cancer [30]. Considering the use of a combination of immunotherapies such as DC and CTL therapy with monoclonal antibodies against CTLA-4 and PD-1, we determined the expression levels of PD-1 and CTLA-4 on CD8⁺ T cells including CMVpp65-specific CD8⁺ T cells using IFN DCs from patients 1, 2, 3 and 4. Before culture for patients, the expression levels of PD-1 for four patients and CTLA-4 for three patients on CD8⁺ T cells were lower than 1% (Fig. 4a). Following

Table 2. Numbers of total lymphocytes and cytomegalovirus (CMV)pp65-specific CD8⁺ T cells following culture with interferon dendritic dells (IFN DCs) derived from patients with GBM before and after culture under different conditions [with the CMVpp65-peptide alone (P), the peptide with Z (P+Z), the peptide with G (P+G) and the peptide and Z+G (P+Z+G)]

Patients	Peptide used	Culture	Agents	No. of total lym.	No. of CMV-specific CD8 ⁺ T		
					A*0201	A*0101	A*2402
Experiment 1 Patient 1	A*0201	Before	None	2.0×10^6	37.2×10^2	n.t.	n.t.
			P	14.0×10^6	41.5×10^5	n.t.	n.t.
		After	P+Z	11.2×10^6	29.2×10^5	n.t.	n.t.
			P+G	10.9×10^6	41.7×10^5	n.t.	n.t.
			P+Z+G	14.1×10^6	53.7×10^5	n.t.	n.t.
Experiment 2 Patient 1	A*0101	Before	None	2.0×10^6	n.t.	10.2×10^2	n.t.
			P	6.8×10^6	n.t.	21.5×10^4	n.t.
		After	P+Z	9.8×10^6	n.t.	51.7×10^4	n.t.
			P+G	6.8×10^6	n.t.	19.9×10^4	n.t.
			P+Z+G	11.5×10^6	n.t.	56.8×10^4	n.t.
Experiment 3 Patient 2	A*0201	Before	None	2.0×10^6	8.2×10^2	n.t.	n.t.
			P	9.8×10^6	5.1×10^5	n.t.	n.t.
		After	P+Z+G	12.4×10^6	22.7×10^5	n.t.	n.t.
Experiment 4 Patient 3	A*0101	Before	None	2.0×10^6	n.t.	4.1×10^2	n.t.
			P	7.2×10^6	n.t.	22.4×10^4	n.t.
		After	P+Z+G	10.6×10^6	n.t.	67.3×10^4	n.t.
Experiment 5 Patient 4	A*2402	Before	None	2.0×10^6	n.t.	n.t.	2.3×10^2
			P	10.1×10^6	n.t.	n.t.	35.7×10^4
		After	P+Z+G	12.8×10^6	n.t.	n.t.	80.5×10^4

Table 3. Numbers of total lymphocytes and Vγ9γδT cells following culture with interferon dendritic cells (IFN DCs) derived from patients with glioblastoma multiforme (GBM) before and after culture under different conditions [with the cytomegalovirus (CMV)pp65-peptide alone (P) and the peptide and Z+G (P+Z+G)]

Patients	Peptide used	Culture	Agents	No. of Vγ9γδT
Experiment 1 Patient 1	A*0201	Before	None	3.4 × 10 ⁴
		After	P	1.4 × 10 ⁴
			P+Z+G	170.9 × 10 ⁴
Experiment 2 Patient 1	A*0101	Before	None	3.4 × 10 ⁴
		After	P	2.2 × 10 ⁴
			P+Z+G	437.6 × 10 ⁴
Experiment 3 Patient 2	A*0201	Before	None	3.7 × 10 ⁴
		After	P	27.8 × 10 ⁴
			P+Z+G	232.0 × 10 ⁴
Experiment 4 Patient 3	A*0101	Before	None	4.1 × 10 ⁴
		After	P	6.1 × 10 ⁴
			P+Z+G	406.6 × 10 ⁴
Experiment Patient 4	A*2402	Before	None	4.1 × 10 ⁴
		After	P	7.5 × 10 ⁴
			P+Z+G	462.2 × 10 ⁴

culture with IFN DCs under two conditions (with P and with P+Z+G), the expression level of PD-1 on CD8⁺ T cells including CMVpp65-specific CD8⁺ T cells increased markedly, in which the expression level of PD-1 on CD8⁺ T cells following stimulation by IFN DCs/P was higher than that following stimulation by IFN DCs/P+Z+G (for

patient 1, 72.91 *versus* 65.78%; for patient 2, 63.61 *versus* 40.72%; for patient 3, 61.10 *versus* 54.74%; for patient 4, 77.21 *versus* 58.31%) (Fig. 4a). Statistical analysis showed that the expression level of PD-1 on CD8⁺ T cells following stimulation by IFN DCs/P+Z+G was statistically lower than that following stimulation by IFN DCs/P (54.89 ± 9.09% *versus* 68.71 ± 6.59%, *P* < 0.05), as shown in Fig. 4b. In contrast, the expression level of CTLA-4 on CD8⁺ T cells, including CMVpp65-specific CD8⁺ T cells, was less than 3% under both conditions (Fig. 4a). Taken together, these results suggest that among different conditions, IFN DCs/P+Z+G in combination with anti-PD-1 mAb may be able to induce a large number of PD-1-negative CD8⁺ T cells *in vitro* and *in vivo*.

Effective induction of CMVpp65-specific CD8⁺ T cells by IFN DCs/P+Z+G derived from HDs

We further tested the induction ability of CMV-specific CD8⁺ T cells using HLA-A*0201-positive IFN DCs from three HDs. The results are similar to those from the patients; that is, the ability of IFN DCs/P+Z+G to induce CMV-specific CD8⁺ T cells was higher than that of IFN DCs/P [Fig. 5a (HD1) and Fig. 5b (HD2)]. In the case of HD3, no induction of CMV-specific CD8⁺ T cells by IFN DCs under any conditions was observed, but the induction level of Vγ9γδT and Vα24NKT cells increased

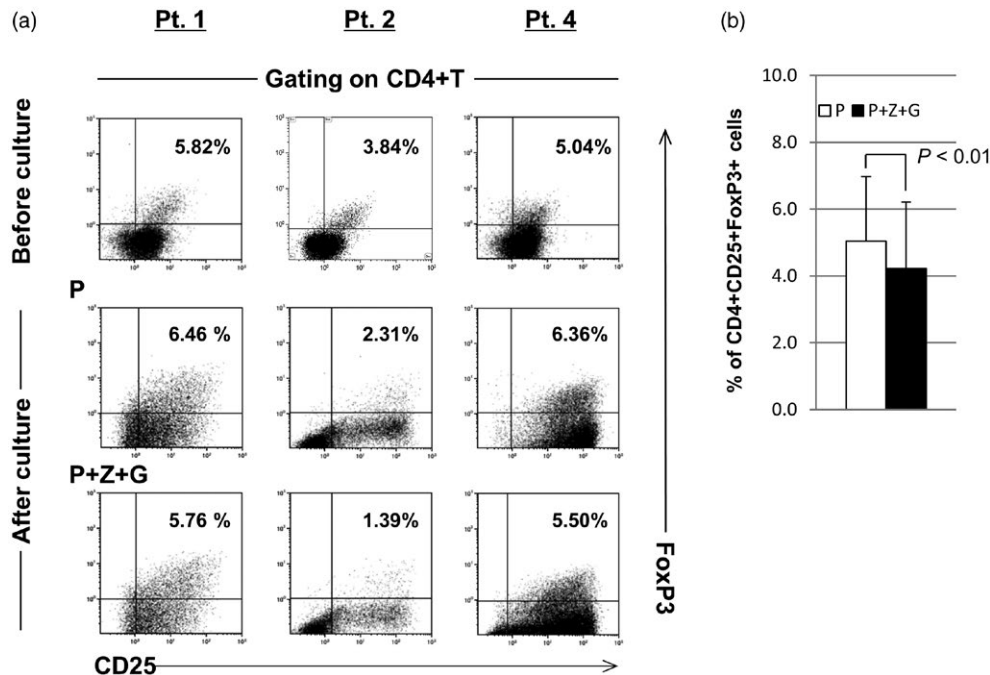


Fig. 3. (a) The percentage of CD4⁺CD25⁺forkhead box protein 3 (FoxP3)⁺ regulatory T cells (T_{regs}) among lymphocytes following culture with interferon dendritic cells/peptide and zoledronate plus α-galactosylceramide (IFN DCs/P+Z+G) from patients (patients 1, 2 and 4) was lower than that following culture with IFN DCs/P. (b) Statistical analysis showed that the percentage of CD4⁺CD25⁺FoxP3⁺ T_{regs} among lymphocytes following culture with IFN DCs/P+Z+G from patients was significantly lower than the percentage of CD4⁺CD25⁺FoxP3⁺ T_{regs} among lymphocytes following culture with IFN DCs/P (4.22±2.00% *versus* 5.05±1.93%, *P* < 0.01).

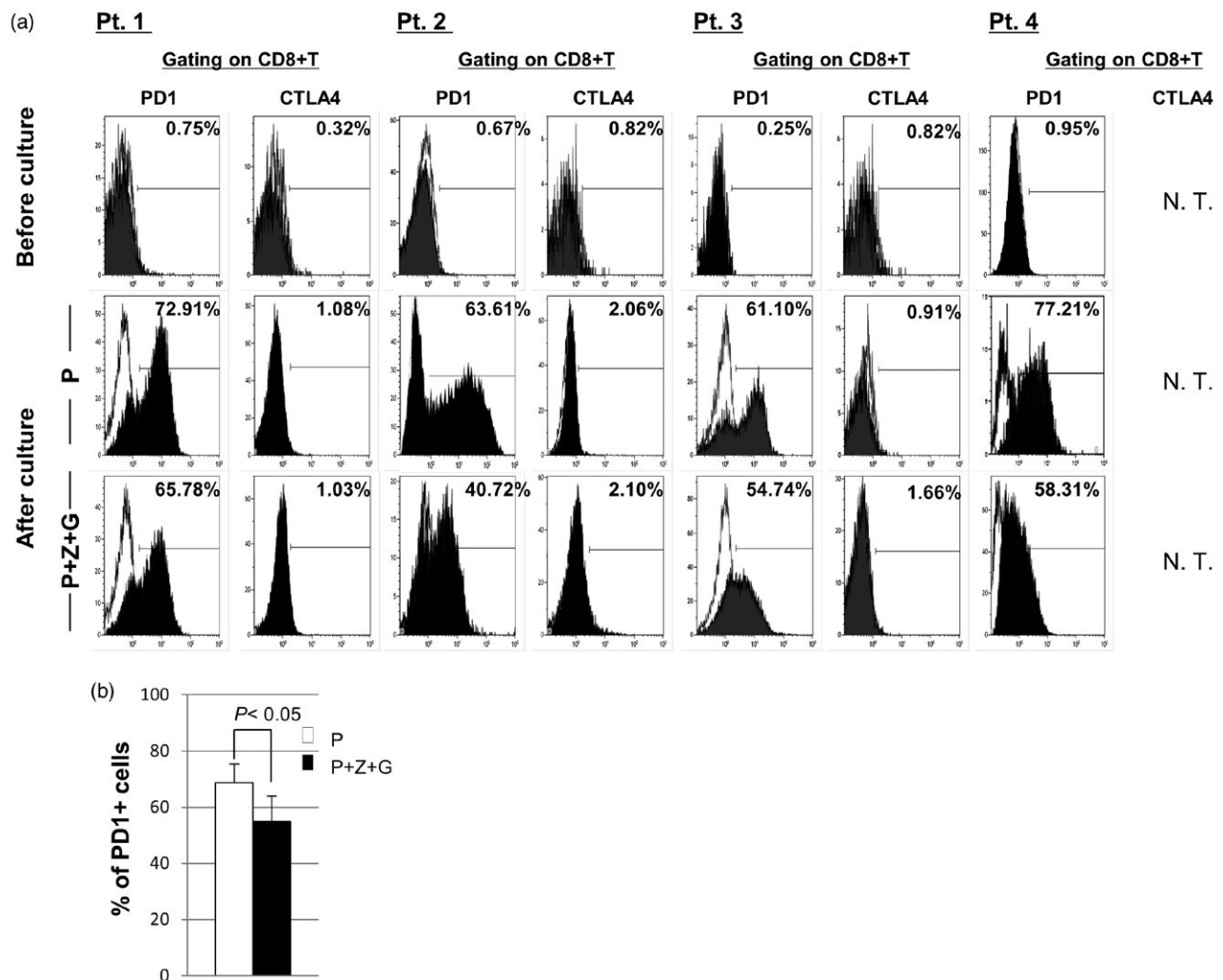


Fig. 4. The expression levels of programmed cell death 1 (PD)-1 and cytotoxic T lymphocyte (CTLA)-4 on CD8⁺ T cells before and after culture with patient (patients 1, 2, 3 and 4)-derived interferon dendritic cells/peptide (IFN DCs/P) versus patient-derived IFN DCs/P+Z+G. (a) The expression levels of PD-1 and CTLA-4 on CD8⁺ T cells including cytomegalovirus (CMV)pp65-specific CD8⁺ T cells before culture were less than 1%. After culture, the expression level of PD-1 on CD8⁺ T cells increased intensively, but the expression levels of CTLA-4 were less than 2.1% under both conditions (IFN DCs/P and IFN DCs/P+Z+G). (b) The expression level of PD-1 on CD8⁺ T cells following stimulation by IFN DCs/P+Z+G was significantly lower than that following stimulation by IFN DCs/P (54.89±9.09% for IFN DCs/P+Z+G versus 68.71±6.59% for IFN DCs/P, $P < 0.05$).

in using IFN DCs/P+Z+G (data not shown). It is important to note that for three HDs, the percentage of CD4⁺CD25⁺FoxP3⁺ T_{regs} among lymphocytes following their co-culture with IFN DCs/P+Z+G was lower than that following their co-culture with IFN DCs/P (Fig. 5c). Similar to the results for patients as shown in Table 2, the number of CMV-specific CD8⁺ T cells expanded by HD-derived IFN DCs/P+Z+G was larger than those expanded by IFN DCs/P (data not shown).

Discussion

In this study, we demonstrate that IFN DCs/P+Z+G from patients with GBM and HD cross-talk with

CMVpp65-specific CD8⁺ T cells, Z-activated Vγ9γδT cells and G-activated Vα24NKT cells in a manner that amplifies the activation and proliferation of CMVpp65-specific CD8⁺ T cells. This has a number of important implications, some of which may have a significant impact on the use of Z and G in the setting of cancer therapy and represents a best-case scenario for immunotherapies, such as DC and CTL therapies, because a large number of tumour antigen-specific T cells may then be available *in vitro* and *in vivo*. As noted, the expansion ability of CMVpp65-specific CD8⁺ T cells by IFN DCs pulsed with HLA-A*0201-restricted P and Z+G was stronger than that by IFN DCs pulsed with HLA-A*0101-restricted P and Z+G, due presumably to a higher frequency of HLA-A*0201-restricted

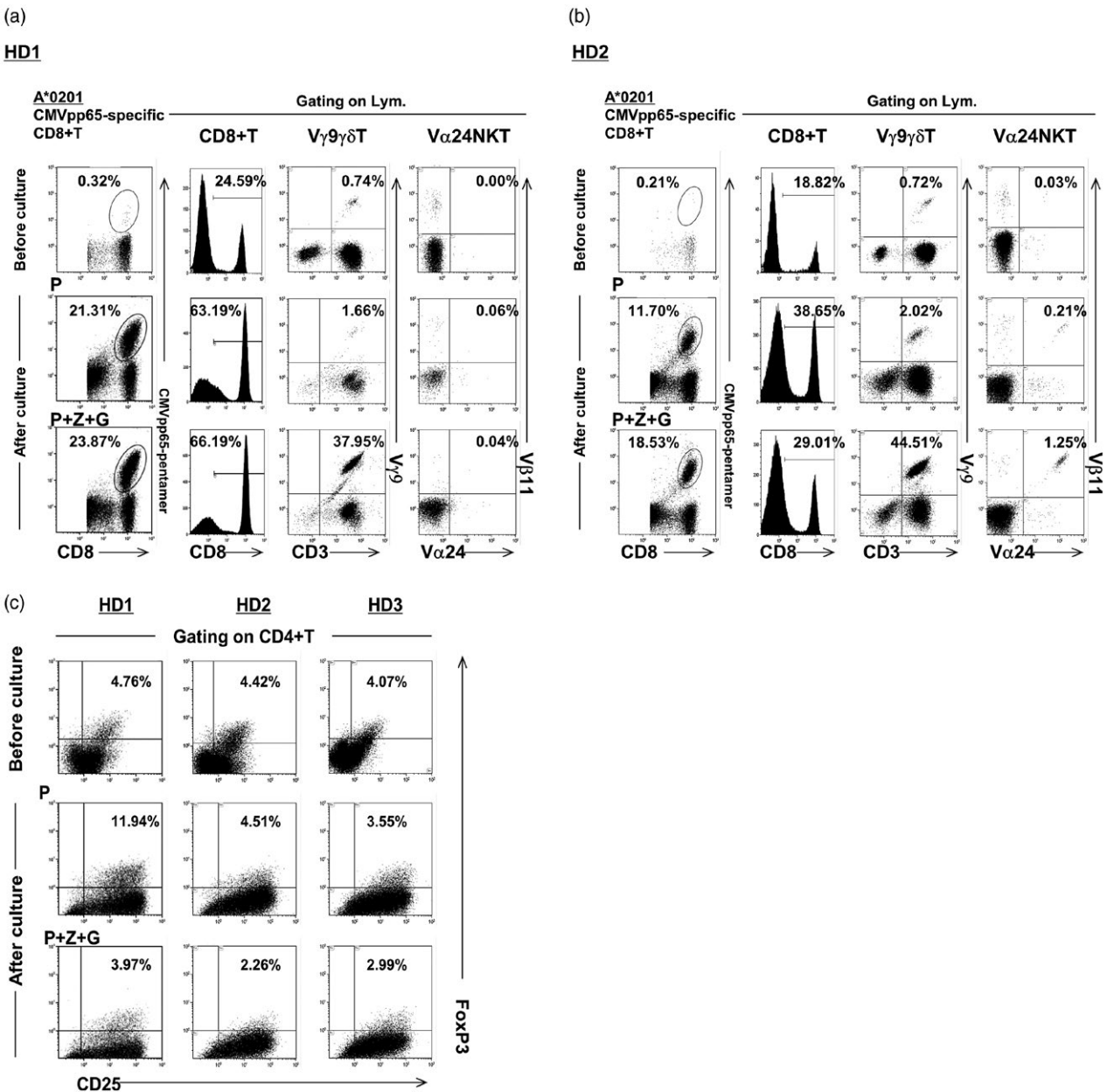


Fig. 5. Effective induction of cytomegalovirus (CMV)pp65-specific CD8⁺ T cells by healthy donor (HD)-derived interferon dendritic cells/peptide and zoledronate plus α -galactosylceramide (IFN DCs/P+Z+G). (a) The ability of IFN DCs from human leucocyte antigen (HLA)-A*0201-positive HD-1 pulsed with HLA-A*0201-restricted P and Z+G to induce CMV-specific CD8⁺ T cells was higher than that of IFN DCs/P. Using IFN DCs/P+Z+G, the expansions of V γ 9 γ δ T and V α 24NKT cells were also observed. (b) The ability of IFN DCs from HLA-A*0201-positive HD2 pulsed with HLA-A*0201-restricted P+Z+G to induce CMV-specific CD8⁺ T cells was higher than that of IFN DCs/P. Using IFN DCs/P+Z+G, expansions of V γ 9 γ δ T and V α 24NKT cells were also observed. (c) The percentage of CD4⁺CD25⁺forkhead box protein 3 (FoxP3)⁺regulatory T cells (T_{regs}) among lymphocytes from three HLA-A*0201-positive HDs (HD-1, H-2 and HD-3) following co-culture with autologous IFN DCs pulsed with HLA-A*0201-restricted P+Z+G) was lower than that following co-culture with autologous IFN DCs/P.

CMVpp65-specific CD8⁺ T cells before culture (0.62% for HLA-A*0201-restricted *versus* 0.17% for HLA-A*0101-restricted peptide), as shown in Fig. 2a.

The use of IFN DCs in this study had some advantages compared with mIL-4 DCs. Our results showed that the expression levels of PD-L1 and PD-L2 on IFN

DCs were significantly lower than those on mIL-4 DCs (Fig. 1b). This indicates that inhibitory signals from IFN DCs to T cells are weaker than those from mIL-4 DCs, which may be associated with the effective induction of tumour antigen-specific CD8⁺ T cells by IFN DCs *in vitro* and *in vivo*.

The expression of CCR7 on DCs is essentially required for DC trafficking from peripheral tissues to draining lymph nodes, constituting a central determinant of DC migration and function [31]. In this study, IFN DCs/P+Z+G expressed CCR7 highly (Fig. 1c,d). This indicates a high trafficking activity of IFN DCs/P+Z+G across endothelial cells to lymph nodes, where they interact with T cells, including antigen-specific CD8⁺ T, V γ 9 δ T and V α 24NKT cells.

Current immunotherapies focus on promising a strong anti-tumour T cell response by altering the T cells themselves, either by increasing tumour-specific T cell frequency by adoptive cell transfer [32] with or without DCs, increasing the reactivity of responding cells by engineering of high-avidity TCRs specific for tumours [33], or by eliciting more potent T cells by blockade of inhibitory co-stimulatory molecules such as CTLA-4 or PD-1 [30]. Ipilimumab (anti-CTLA-4 mAb) is the first anti-cancer agent associated with a documented improved overall survival benefit in patients with advanced melanoma. Many tumour responses achieved by PD-1 and PD-L1 inhibition were durable and were seen in a higher proportion of patients with melanoma than observed typically with ipilimumab [34]. Nivolumab was the first mAb targeting PD-1 to show significant clinical activity in unresectable or metastatic melanoma, non-small-cell lung carcinoma and metastatic renal cell carcinoma [35].

In this study, we confirmed that the expression level of PD-1 on CD8⁺ T cells expanded by IFN DCs/P+Z+G was significantly lower than that on CD8⁺ T cells expanded by IFN DCs/P and that the expression level of CTLA-4 on CD8⁺ T cells was very low under both conditions (Fig. 4a,b). Under consideration of DC therapy together with an immuncheckpoint blockade, our results suggest that in the case of DC therapy using IFN DCs/P+Z+G, administration of a small amount of anti-PD-1 mAb such as nivolumab, not anti-CTL-A4 mAb such as ipilimumab, may result in higher induction of PD-1-negative CMV-specific CD8⁺ T cells *in vivo*.

In a previous study, CMV-specific T cells were expanded using PBMCs from patients with GBM and infused into the patients [36]. T cells, including CMV-specific T cells, were prepared by culturing PBMC with autologous PBMCs presensitized with the CMV-peptide and IL-2. Then, 25 to 40 $\times 10^6$ autologous CMV-specific T cells/infusion were infused. This previous study suggests that combination therapy with autologous CMV-specific T cells and chemotherapy is a safe novel treatment and may provide clinical benefits to patients with recurrent GBM. Considering CTL therapy using CMVpp65-specific CD8⁺ T cells expanded by IFN DCs/P+Z+G, more than 30 $\times 10^6$ of CMVpp65-specific CD8⁺ T cells will be harvested easily when

20–30 $\times 10^6$ lymphocytes are cultured with IFN DCs/P+Z+G. Usually, 36.0 $\pm 10.3 \times 10^6$ lymphocytes are collected from 50 ml of peripheral blood (our own observation; $n = 71$). Therefore, it is not difficult to prepare a sufficient number of CMV-specific CD8⁺ T cells per injection for patients with GBM when lymphocytes are cultured with IFN DCs/P+Z+G. Furthermore, in our study using IFN DCs/P+Z+G, a 500–2700-fold expansion of CMVpp65-specific T cells was completed (Table 2), which is much higher than using DCs transfected with CMVpp65-RNA, as reported previously [37].

A recent study provides strong evidence supporting the potential of combinatorial targeted and/or immunotherapeutic regimens in patients with GBM that may change patient outcome [38,39]. Our results suggest that a combination of immune checkpoint inhibitors, particularly anti-PD-1 mAb, and DC therapy as well as CTL therapy, leads to a better clinical outcome for patients with GBM. In future, the therapeutic pillar of checkpoint blockade will probably keep its central role in cancer immunotherapy. Therefore, adaptive immunotherapies such as DC and CTL therapy combined with checkpoint blockade will play important roles in treatment of cancer patients, including GBM patients.

Identification of the molecular drivers of T cell dysfunction is essential for continued progress of cancer research and therapy. Among different immunosuppressive cell populations in the TME, T_{regs} accumulate frequently in tumour microenvironment and even represent the major populations of infiltrating CD4⁺ T cells [22]. Depletion of T_{regs} or disruption of T_{reg} differentiation may restore anti-tumour T cell responses and immune surveillance against cancer. In this study, the induction of CD4⁺CD25⁺FoxP3⁺ T_{regs} among the lymphocytes expanded by IFN DCs/P+Z+G decreased following stimulation by IFN DCs/P (Fig. 3a,b). These results indicate that the adjuvant effect of V α 24NKT and V γ 9 δ T cells may be associated partially with the decrease in the percentage of CD4⁺CD25⁺FoxP3⁺ T_{regs} among the lymphocytes expanded. Taken together, our results suggest that DC therapy using DCs pulsed with tumour antigen and Z+G is a promising approach which results in the increase in the number of tumour antigen-specific CD8⁺ T cells and in the decrease in that of CD4⁺CD25⁺FoxP3⁺ T_{regs}, although the more precise phenotypical and functional analysis of T_{regs} are warranted, as shown in the previous study [40].

In this study, the results of HDs are very similar to those of patients, showing that a large number of CMVpp65-specific CD8⁺ T cells from HDs were harvested following activation of lymphocytes with autologous IFN DCs pulsed with the CMVpp65-peptide and Z+G *in vitro*.

Thus, infusion of the CMVpp65-specific CD8⁺ T cells expanded by IFN DCs derived from allogeneic HDs to patients with haematological malignancy who received alloSCT may decrease the relapse risk, although side effects should be considered carefully.

The adjuvant effects of a combination of Z-activated V γ 9 γ δ T cells with G-activated V α 24NKT cells on antigen-specific CD8⁺ T cells were similar when stimulator IFN DCs were washed to remove extracellular tumour antigens and Z+G, as when lymphocytes were cultured with IFN DCs pulsed with the tumour antigen and Z+G. These observations support the possible therapeutic applications of IFN DCs pulsed with a tumour antigen and Z+G. The observed favourable *in-vitro* expansion of CMVpp65-specific CD8⁺ T cells in response to DCs with IL-2 indicates that additional, small amounts of exogenous IL-2 may be required to maximize the immunotherapeutic benefits of DCs pulsed with a tumour antigen and Z+G in clinical settings.

Furthermore, it has been shown recently that, similarly to DCs, V γ 9 γ δ T cells present antigens to $\alpha\beta$ T cells [41,42]. It is conceivable the V γ 9 γ δ T cells activated *in vivo*, for example, by infused DCs pulsed with a tumour antigen and Z+G, might play a role as APCs in the uptake of apoptotic bodies from tumour cells. They subsequently induce tumour antigen-specific CD8⁺ T cells *in vivo* in which V α 24NKT cells may function as helper T cells producing large amounts of IFN- γ , as shown in our previous clinical study [4]. Note that the concurrent activation and proliferation of V γ 9 γ δ T cells by Z is MHC-unrestricted [43] and that V α 24NKT cells recognize G presented by non-polymorphic CD1d molecules [3]. Therefore, the adjuvant effect of the combination of Z and G is theoretically applicable to all cancer patients.

In summary, our study demonstrates the adjuvant effect of a combination of Z and G, resulting in the expansions of V γ 9 γ δ T and V α 24NKT cells upon induction of antigen-specific CD8⁺ T cells using IFN DCs/P+Z+G. This effect may be a physiologically and therapeutically important bridge between innate and adaptive immunities through DCs. Our results are consistent with accumulating evidence from clinical and laboratory studies, indicating that heterogeneous activation of lymphocytes shapes the responses to vaccination. Furthermore, our observations are anticipated to facilitate the design of more effective immune therapy strategies, particularly DC therapy combined with immuncheckpoint blockades such as anti-PD1 mAbs for patients with GBM.

Disclosure

The authors have declared no conflicting interests.

Author contributions

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

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