J Clin Exp Hematopathol
Vol. 47, No. 2, Nov 2007

Original Article

Enhancement of Anti-tumor Cytotoxicity of Expanded γδ T Cells by Stimulation with Monocyte-derived Dendritic Cells

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In order to establish the method of generating powerful γδ T cells for anti-tumor immunotherapy, we investigated the effects of monocyte-derived dendritic cells (mo-DCs) on anti-tumor cytotoxicity of expanded γδ T cells. Activation of γδ T cells co-cultured for 2-3 days with immature or mature mo-DCs was evaluated by CD69 expression and anti-tumor cytotoxicity using two assays : the 5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester-based cytotoxicity assay and the calcein-AM-based Terascan assay. γδ T cells were used as effector cells and myeloma cell line (RPMI8226) or chronic myelogenous leukemia blastic crisis cell line (C2F8) were used as target cells. CD69 expression on γδ T cells was enhanced by co-culture with both immature and mature mo-DCs in a cell-number-dependent fashion. CD69 expression was enhanced after addition of mo-DCs of either autologous or allogeneic origin. Activation of γδ T cells with mo-DCs enhanced anti-tumor cytotoxicity of γδ T cells against RPMI8226 and C2F8 in an effector-to-target ratio-dependent manner. Activation of γδ T cells by mo-DCs was associated with the enhancement of anti-tumor cytotoxicity of γδ T cells. Potent γδ T cells activated by mo-DCs were considered to be applicable to an efficient γδ T cell-mediated immunotherapy for tumors. 〔*J Clin Exp Hematopathol 47(2) : 61-72, 2007*〕

Keywords: γδ T cells, monocyte-derived dendritic cells, CD69, anti-tumor cytotoxicity, cellular immunotherapy

INTRODUCTION

γδ T cells constitute a small proportion (1-5%) of human peripheral blood (PB) lymphocytes, and circulating γδ T cells are predominantly γ2δ2 T cells.[1] Human γ2δ2 T cells recognize various kinds of non-peptide antigens such as pyrophosphomonoesters,[2] alkyl amines[3] and nitrogen-containing bisphosphonates.[4] Recently, γδ T cells were demonstrated to expand from PB low-frequency γδ T cells after stimulation with non-peptide aminobisphosphonate compounds, which are currently used to treat hypercalcemia in cancer patients.[5] In addition, aminobisphosphonate-stimulated γδ T cells were demonstrated to produce a large amount of interferon (IFN)-γ and exhibit cytotoxicity against tumor cells.[6]

The γδ T cell-mediated anti-tumor effect relies on the active mevalonate pathway in tumor cells that is inactive in normal cells. Tumor cells contain an increased amount of intermediate metabolites such as phosphorylated non-peptide antigens [isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP)], which bind to the γδ T cell receptor (TCR) as potent antigens and lead to the proliferation of γδ T cells.[7] Elevated synthesis of phosphorylated non-peptide antigens in tumor cells is considered to be at least partly due to increased expression of hydroxy-methylglutaryl-CoA reductase (HMGR), the rate-limiting enzyme of the mevalonate pathway.[8,9] Increased presentation and secretion of phosphorylated antigens in certain tumor cells is believed to be a

Received : May 10, 2007
Revised : Jun 21, 2007
Accepted : Jul 9, 2007
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result of greater cytotoxicity of γδ T cells against tumor cells. In addition to binding antigenic molecules to γδ TCR, engagement of the NKG2D or CD6 molecule on γδ T cells with MICA/B[10] or CD166[11] on tumor cells has been demonstrated to play an important role in tumor cell-mediated γδ T cell activation and anti-tumor cytotoxicity of γδ T cells.

Antigen-presenting cells (APCs) like dendritic cells (DCs) do not seem to be critical for antigen-stimulated expansion of γδ T cells. However, the response of γδ T cells is enhanced by cell-to-cell interaction with APCs.[12,13] γδ T cells recognize aminobisphosphonates bound to the surface of APCs rather than free molecules.[14,15] Activation markers (such as CD69, CD25 and HLA-DR) of γδ T cells are up-regulated when PB-mononuclear cells (MNCs) are stimulated with poly (I:C) in the presence of IPP. IPP-expanded γδ T cells become activated due to type I IFN produced by $CD11c^+$ immature monocyte-derived DCs (mo-DCs) stimulated with poly (I : C).[16] Plasmacytoid DCs, which are the predominant cells producing type I IFN, also activate γδ T cells by toll-like receptor (TLR) 9-mediated production of type I IFN.[17] Meanwhile, pro-inflammatory cytokines (mainly IFN-γ) released by γδ T cells could induce the maturation of mo-DCs as evidenced by enhanced expression of CD83, CD86 and HLA-DR, and production of interleukin (IL)-12.[18]

Previous studies have suggested a reciprocal stimulation of γδ T cells and mo-DCs. Activation marker (CD25 or CD69) expression and production of cytokines [IFN-γ and tumor necrosis factor (TNF)-α] by γδ T cells have been demonstrated to increase after stimulation with mo-DCs. However, whether anti-tumor cytotoxic ability of γδ T cells is enhanced after stimulation with mo-DCs remains to be determined. In order to answer this question and explore the method of generating powerful γδ T cells for anti-tumor immunotherapy, we investigated the effects of mo-DCs on anti-tumor cytotoxicity of γδ T cells generated from PB-MNCs by *in vitro* stimulation with zoledronate.

MATERIALS AND METHODS

Expansion of human γδ T cells

MNCs were separated by Ficoll-Hypaque (Lymphoprep ; Nycomed, Oslo, Norway) density gradient centrifugation of drawn, heparinized PB. γδ T cells were expanded from PB-MNCs by culture at the cell concentration of 1×10^6/ml in 10% fetal bovine serum-containing RPMI-1640 (Kohjin Bio, Sakato, Saitama, Japan) with 1 μM zoledronate (donated by Novartis Pharmaceutical Co, Basel, Switzerland) and 50 U/ml IL-2 (donated by Shionogi Pharmaceutical Co, Osaka, Japan) for 1-2 weeks.[19]

Flow cytometry analysis

Cells were incubated with various FITC or PE-conjugated monoclonal antibodies at 4℃ for 30 min. A matched-isotype antibody was used as the negative control. The stained cells were analyzed by FACScan (BD Biosciences, Mountain View, CA), and the data were processed by CellQuest software (BD Biosciences) for positive cell percentages and mean fluorescence intensity, as described previously.[20] Monoclonal antibodies used in the present study are listed in Table 1.

Isolation of monocytes from PB

$CD14^+$ monocytes were isolated from PB-MNCs by anti-CD14 microbeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Briefly, 5×10^7 MNCs were incubated with 50 μl CD14 microbeads in 200 μl buffer [phosphate buffered saline (PBS) with 0.5% human serum

Table 1. Monoclonal antibodies used in the present study

Monoclonal antibodies	Clone	Isotype	Conjugate	Supplier
CD1a	BL6	mouse IgG1	PE	IM
CD14	M5E2	mouse IgG2a, κ	PE	BD
CD80	L307.4	mouse IgG1, κ	PE	BD
CD86	2331 (FUN-1)	mouse IgG1, κ	PE	Ph
CD83	HB15a	mouse IgG2b	PE	IM
HLA-DR	G46-6 (L243)	mouse IgG2a, κ	PE	BD
CD4	RPA-T4	mouse IgG1, κ	FITC	BD
BDCA4	AD5-17F6	mouse IgG1	PE	MB
γδ TCR	11F2	mouse IgG1, κ	FITC	BD
CD69	FN50	mouse IgG1, κ	PE	Ph

IM : Immunotech, Marseille, France, BD : BD Biosciences, San Jose, CA, USA, Ph : BD Pharmingen, San Diego, CA, USA, MB : Miltenyi Biotec, Bergisch Gladbach, Germany

albumin and 0.6% ACD-A] at 4℃ for 15 min. Thereafter, the cells were washed once with buffer. The resuspended cells were loaded on a MS-MACS separation column (Miltenyi Biotec), and $CD14^+$ monocytes were isolated after the column was removed from a magnetic field. The purity of the $CD14^+$ cells was always in the range of 90%-95%, determined by flow cytometry

Generation of mo-DCs from monocytes

Immature mo-DCs were generated by culturing $CD14^+$ monocytes in RPMI-1640 [containing 50 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) and 5 ng/ml IL-4] in the presence of 10% fetal bovine serum in a fully humidified incubator at 5% CO_2 and 37℃ for 7 days. Mature mo-DCs were generated by culturing $CD14^+$ monocytes in GM-CSF and IL-4 for 6 days and then culturing them for an additional 24 hr with additions of TNF-α (5 ng/ml), IL-1α (1,000 U/ml), IL-6 (10 ng/ml), IFN-γ (1,000 U/ml) and prostaglandin E_2 (1 μg/ml). In some experiments, immature or mature mo-DCs were generated by culturing monocytes with the same cytokines for 14 days. Generation of immature mo-DCs was identified by the presence of CD1a, CD80, CD86 and HLA-DR and the absence of CD14 with dim positive CD83 on cultured cells. Induction of mature mo-DCs was ascertained by increased expression of CD80, CD86, CD83 and HLA-DR and decreased expression of CD1a with negative CD14 on cultured cells (Fig. 1).

Activation of γδ T cells by mo-DCs

γδ T cells that had been generated by culturing with zoledronate and IL-2 for 14 days were washed and cultured with immature or mature mo-DCs at the cell-to-cell ratios of 1:0, 1:0.1, 1:0.5, or 1:1 (γδ T cells : mo-DCs) in autologous or allogeneic settings for 2-3 days. Activation of γδ T cells was evaluated by flow cytometry analysis of increased expression of activation marker CD69.

Transwell culture of γδ T cells and mo-DCs

In order to investigate a cell-to-cell interaction between γδ T cells and mo-DCs, Transwell® (24 well, pore size : 8.0 μm, Corning, NY) systems were used. Autologous or allogeneic, immature or mature mo-DCs were placed in the lower compartment of Transwells containing 0.6 ml medium. Then a Transwell insert was added to the well and the plate was incubated for 1 hr (equilibrium period). Isolated γδ T cells, which were generated from PB-MNCs by culture with zoledronate and IL-2 for 7 days, were added to the Transwell insert (0.1 ml medium) and cultured for 48 hr. Activation of γδ T cells in the Transwell insert was evaluated by flow cytometry analysis of increased CD69 expression.

Isolation of γδ T cells

γδ T cells were purified from mixed cultures of γδ T cells and mo-DCs using an anti-γδ TCR microbeads kit (Miltenyi Biotec) following the manufacturer's instructions. Briefly, γδ T cell-containing cultured cells were incubated with an anti-γδ TCR hapten-antibody for 10 min and then treated with MACS anti-hapten microbeads-FITC for 15 min. The treated cell suspension was applied to a MS-MACS column placed in the magnetic field of a MACS separator. After washing the column, the magnetically labeled cells were flushed from the column, which had been removed from the magnetic field, by

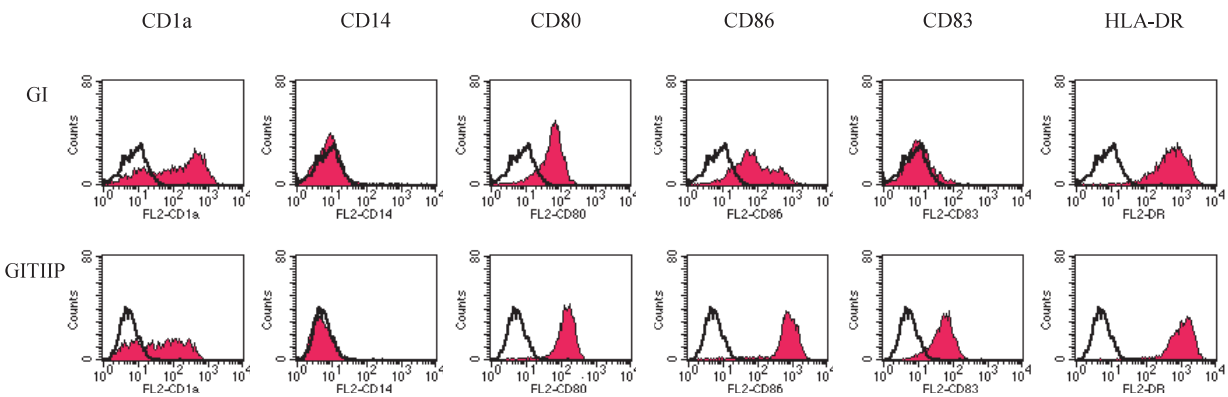


Fig. 1. Surface phenotypes of immature mo-DCs, which were generated from PB-$CD14^+$ monocytes by culture with GM-CSF and IL-4 for 6 days, and mature mo-DCs, which were induced from immature mo-DCs by culture with TNF-α, IL-1α, IL-6, IFN-γ and prostaglandin E_2 for additional 24 hr. Immature mo-DCs were demonstrated to be positive for CD1a, CD80, CD86 and HLA-DR and negative for CD14 with dim expression of CD83. Mature mo-DCs were characterized as increased expression of CD80, CD86, CD83 and HLA-DR and decreased expression of CD1a with no expression of CD14.

firm application of the plunger.

Isolation of plasmacytoid DCs (pDCs)

PB-MNCs were stained with a mixture of FITC-conjugated anti-CD4 and PE-conjugated anti-BDCA4 monoclonal antibodies and were exposed to 7-aminoactinomycin D (7AAD) to eliminate dead cells before sorting. The labeled cells were sorted on a FACSAria (BD Biosciences) to collect $CD4^+BDCA4^+7AAD^-$ pDCs. The purity of sorted pDCs was consistently higher than 98% by judging from the expression of $CD4^+BDCA4^+$.

IFN-α quantification of culture supernatant

Culture supernatants of immature mo-DCs, mature mo-DCs and pDCs, which were cultured with or without intact influenza virus (H1N1, generously donated by Dr. Reiko Saitoh, Department of Public Health, Niigata University, School of Medicine) were collected and assayed for the quantification of IFN-α by enzyme-linked immunoassay (ELISA : Hu-IFN-α ELISA Kit, PBL Biomedical Laboratories, Piscataway, NJ).

Cytotoxicity assay

Two kinds of cytotoxicity tests [5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE)-based cytotoxicity assay[21] and calcein-AM-based Terascan assay[22]] were performed to estimate the effect of mo-DCs on anti-tumor cytotoxicity of γδ T cells. Two kinds of tumor cells, which are myeloma cell line (RPMI8226) and a chronic myelogenous leukemia blastic crisis cell line (C2F8),[23] were used as target cells for the cytotoxicity test. Target cells were labeled with 10 μM CFSE (Molecular Probes, Eugene, OR) or 10 μg/ml calcein-AM (Kohjin Bio, Saitama, Japan) for each cytotoxicity assay. γδ T cells, which were generated from PB-MNCs by culture with zoledronate and IL-2 for 7 days and co-cultured with or without mo-DCs for 2 days, were used as effector cells after separation with γδ TCR microbeads. Labeled target cells were co-cultured with effector cells at 37℃ in a fully humidified 5% CO_2 atmosphere in tubes for the CFSE-based cytotoxicity assay and a 96-well-plate for the calcein-AM-based Terascan assay.

For the CFSE-based cytotoxicity assay, co-cultured cells (consisting of effector cells and target cells) were stained with 7AAD to identify dead cells, and a fixed amount (10,000 beads/tube) of FITC-labeled CaliBRITE beads (BD Biosciences) were added just prior to flow cytometry analysis for quantitative analysis of the cell population. Viable target cells ($CFSE^+7AAD^-$) and CaliBRITE beads were gated in FSC/SSC and FL-1/FSC dot plots, respectively, of target cells cultured without effector cells. For each sample tube containing target cells with effector cells at various effector-target ratios, 5,000 CaliBRITE beads were acquired, which made it possible to calculate the absolute numbers of viable target cells. When a fixed amount (5,000) of CaliBRITE beads were not able to be acquired, the absolute number of viable target cells was enumerated by calculating the ratio between the number of viable target cells and the number of CaliBRITE beads. Percent cytotoxicity of the assay was calculated by the following formula : % cytotoxicity = [(absolute No. of viable target cells in the tube containing target cells only − absolute No. of viable target cells in the sample tube containing target cells and effector cells)/absolute No. of viable target cells in the tube containing target cells only] × 100%.

For the calcein-AM-based Terascan assay, the plate was placed on microscope stage of the Terascan instrument (Minerva Tech, Tokyo, Japan), which serially measured the fluorescence emitted by intact target cells during co-culture of target cells and effector cells. Percent cytotoxicity of the assay was calculated by the following formula : % cytotoxicity = {1-[(average fluorescence of the sample wells-average fluorescence of the maximal release control wells)/(average fluorescence of the minimal release control wells-average fluorescence of the maximal release control wells)]} × 100%.

RESULTS

Activation of γδ T cells by mo-DCs

γδ T cells, which were expanded from normal PB-MNCs by culture with zoledronate and IL-2 for 14 days, were co-cultured with immature or mature mo-DCs generated from normal PB-$CD14^+$ cells by culture for 14 days in autologous and allogeneic settings. Fig. 2A depicts a representative experiment showing the increased activation marker (CD69) expression of γδ T cells after co-culture with autologous or allogeneic immature mo-DCs for 2 days. 45-48% of γδ T cells cultured without immature mo-DCs were $CD69^+$ cells. The proportion of $CD69^+$ cells was increased by co-culturing with immature mo-DCs in a γδ T cell-to-immature mo-DC ratio-dependent manner. In an autologous setting, co-culturing with immature mo-DCs at 10%, 50%, or 100% of γδ T cells increased the proportion of $CD69^+$ cells in γδ T cells up to 90%. In an allogeneic setting, co-culturing with the same ratio of immature mo-DCs increased the proportion of $CD69^+$ cells in γδ T cells up to 76%. Although the proportion of $CD69^+$ cells in γδ T cells cultured without added mo-DCs varied among the donors whose γδ T cells were used in the experiments, the proportion of $CD69^+$ cells was increased by co-culturing with immature mo-DCs for 48 hr in a γδ T cell-to-immature mo-DC ratio-dependent manner for both autologous and allogeneic settings (Fig. 2B). Fig. 3A depicts a representative experiment showing the increased CD69 ex-

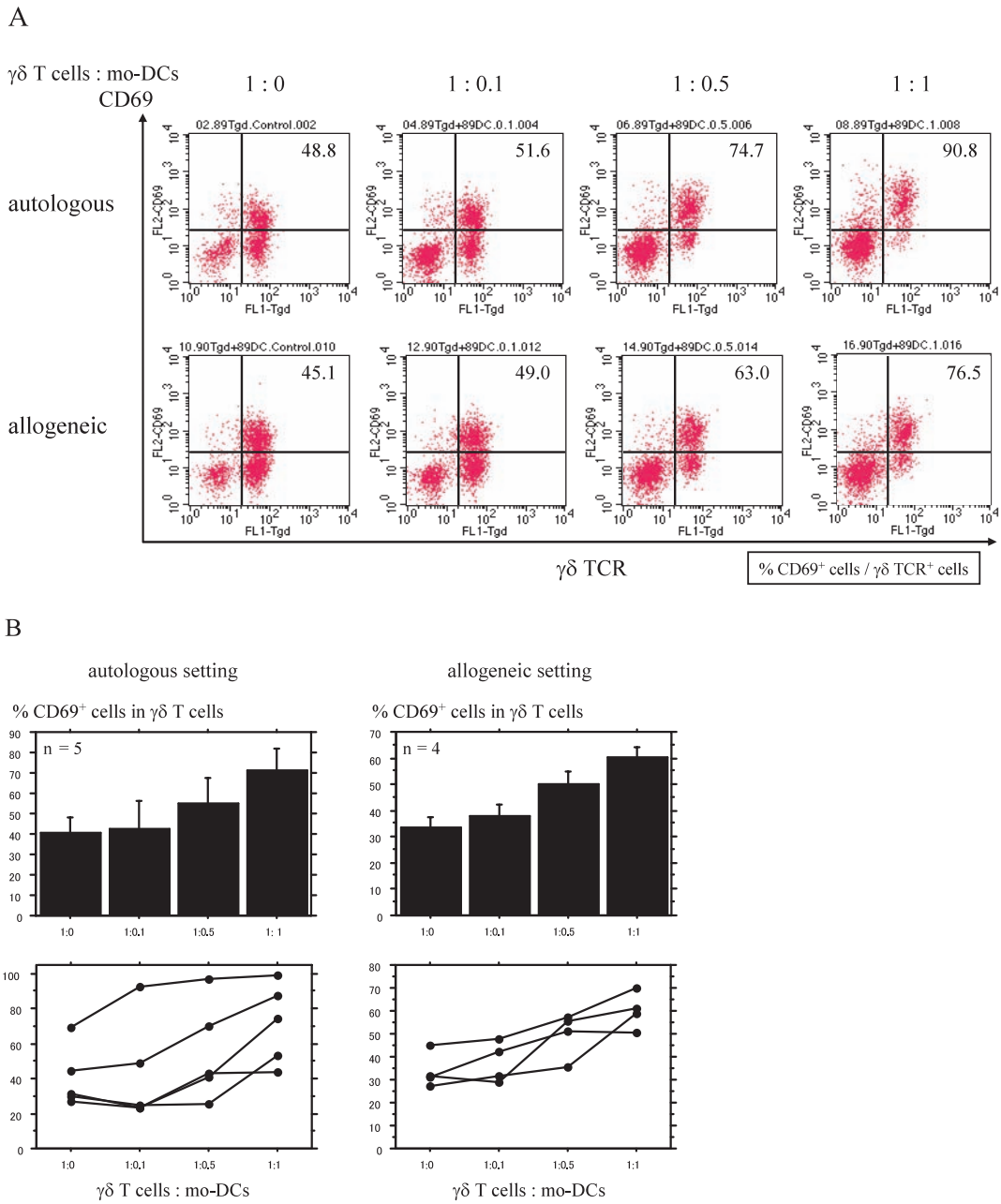


Fig. 2. Activation of γδ T cells by co-culture with autologous or allogeneic immature mo-DCs for 2 days. Activation of γδ T cells was evaluated by the increased expression of activation marker (CD69) of γδ T cells as shown in a representative experiment (*2A*). Although the proportion of $CD69^+$ cells in γδ T cells varied among blood donors, the proportion of $CD69^+$ cells was increased by co-culture with immature mo-DCs at γδ T cell-to-immature mo-DC ratio dependent manner in both autologous (n = 5) and allogeneic (n = 4) settings of added mo-DCs (*2B*).

pression of γδ T cells after co-culture with immature or mature mo-DCs for 3 days in autologous or allogeneic settings. Regardless of mo-DC maturity, the proportion of $CD69^+$ cells was increased by co-culture with mo-DCs in both autologous and allogeneic settings. In both autologous and allogeneic settings, co-culture with immature or mature mo-DCs at 10%, 50%, or 100% of γδ T cells increased the proportion of $CD69^+$ cells in γδ T cells up to 90% or more. Although the baseline proportion of $CD69^+$ cells in γδ T cells without mo-DCs varied among donors, the increase in $CD69^+$ cells was very similar between immature and mature mo-DCs, which were co-cultured with γδ T cells in both autologous and allogeneic

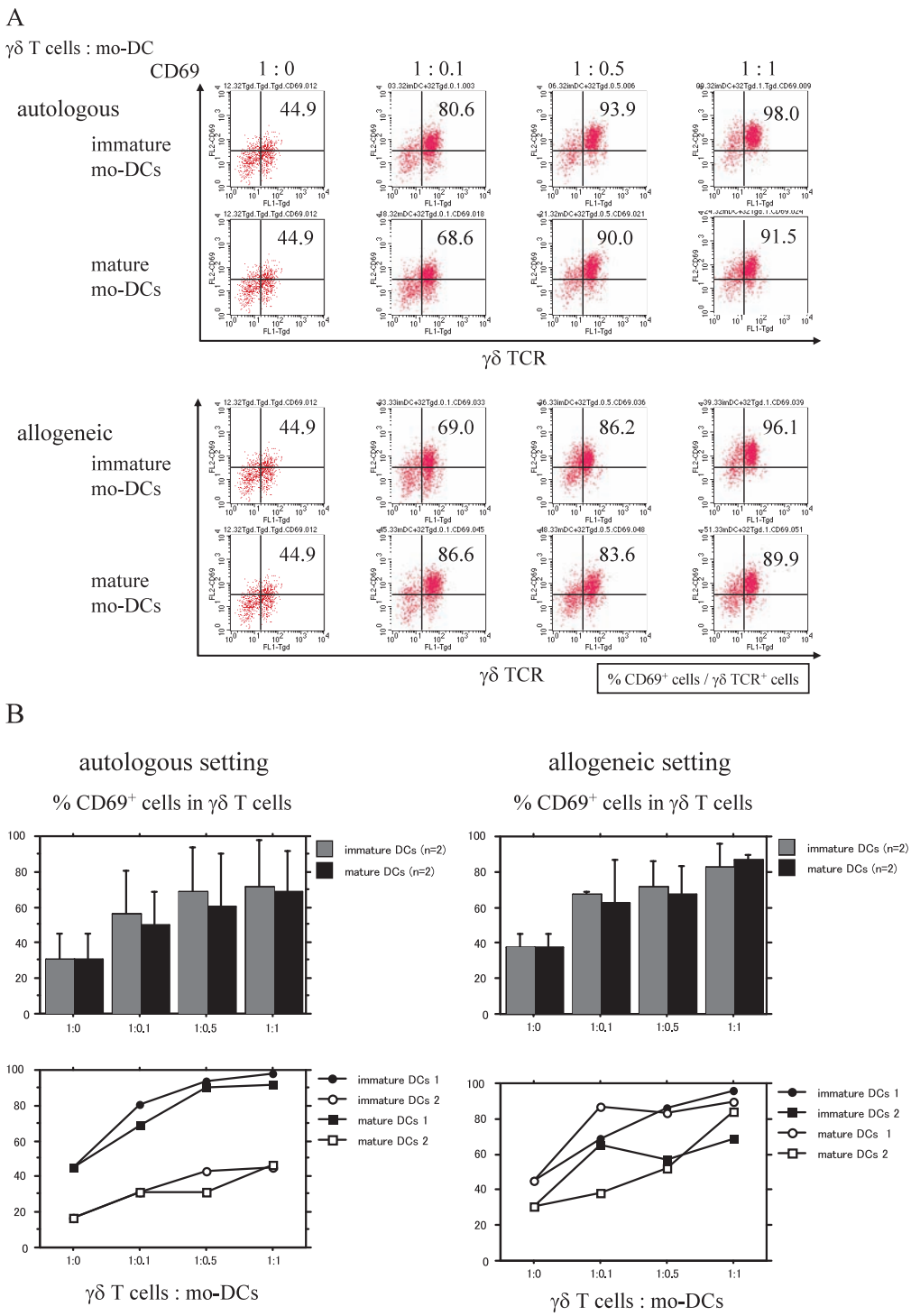


Fig. 3. Activation of γδ T cells by co-culture with immature or mature mo-DCs for 3 days in autologous or allogeneic settings. Activation of γδ T cells was evaluated by the increased activation marker (CD69) expression of γδ T cells as shown in a representative experiment (*3A*) chosen from 3 separate experiments. Regardless of mo-DC maturity, the proportion of $CD69^+$ cells was increased by co-culture with mo-DCs in both autologous and allogeneic settings (*3B*). Although the baseline proportion of $CD69^+$ cells in γδ T cells without mo-DCs varied among donors, the increase in $CD69^+$ cells was very similar between immature and mature mo-DCs, which were co-cultured with γδ T cells in both autologous and allogeneic settings.

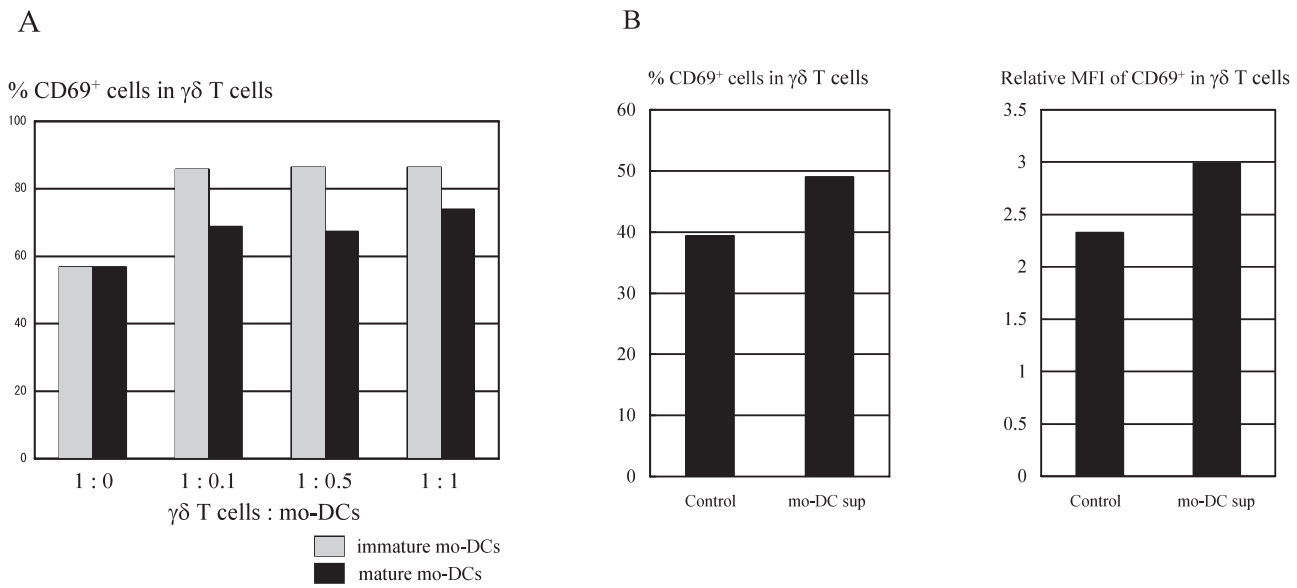


Fig. 4. Activation of γδ T cells by humoral factors secreted by mo-DCs. (*4A*) : Activation of γδ T cells in Transwells, cultured with immature or mature mo-DCs for 2 days in allogeneic setting. Isolated γδ T cells were placed in the Transwell insert and cultured with immature or mature mo-DCs placed in the lower compartment of the Transwell at various cell-to-cell ratios. Adding mo-DCs to the lower Transwell compartment increased the proportion of $CD69^+$ γδ T cells in the upper compartment in a γδ T cell-to-mo-DC ratio dependent manner. (*4B*) : Activation of γδ T cells by incubation for 2 days with supernatant from the immature mo-DC culture. Activation of γδ T cells was evaluated by the increased expression of activation marker (CD69) of γδ T cells. This figure depicts a representative experiment chosen from 2 separate experiments. MFI : mean fluorescence intensity.

settings (Fig. 3B).

Effects of humoral factors produced by mo-DCs on activation of γδ T cells

Isolated γδ T cells, which were generated from PBMNCs by culture with zoledronate and IL-2, were placed in a Transwell insert and cultured for 2 days with allogeneic, immature or mature mo-DCs placed in the lower compartment of a Transwell at the cell-to-cell ratios of 1:0, 1:0.1, 1:0.5, or 1:1 (γδ T cells : mo-DCs). Adding mo-DCs to the lower Transwell compartment increased the proportion of $CD69^+$ γδ T cells in the upper compartment in a γδ T cell-to-mo-DC ratio-dependent fashion for both immature and mature mo-DC environments (Fig. 4A). In addition, the proportion of $CD69^+$ γδ T cells and relative mean fluorescence intensity of CD69 expression in γδ T cells increased after a 2-day incubation with the supernatant from an immature mo-DC culture (Fig. 4B). Since we had previously demonstrated that γδ T cells could be activated by the addition of type I IFN, supernatants from immature and mature mo-DC cultures were assayed for IFN-α using ELISA. Although pDCs stimulated with influenza virus secreted a high amount of IFN-α, we could not detect a measurable amount of IFN-α in the culture supernatant of immature mo-DCs, mature mo-DCs, or unstimulated pDCs (Fig. 5).

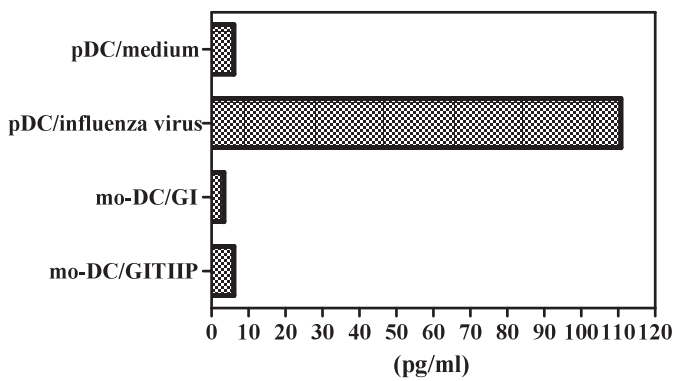
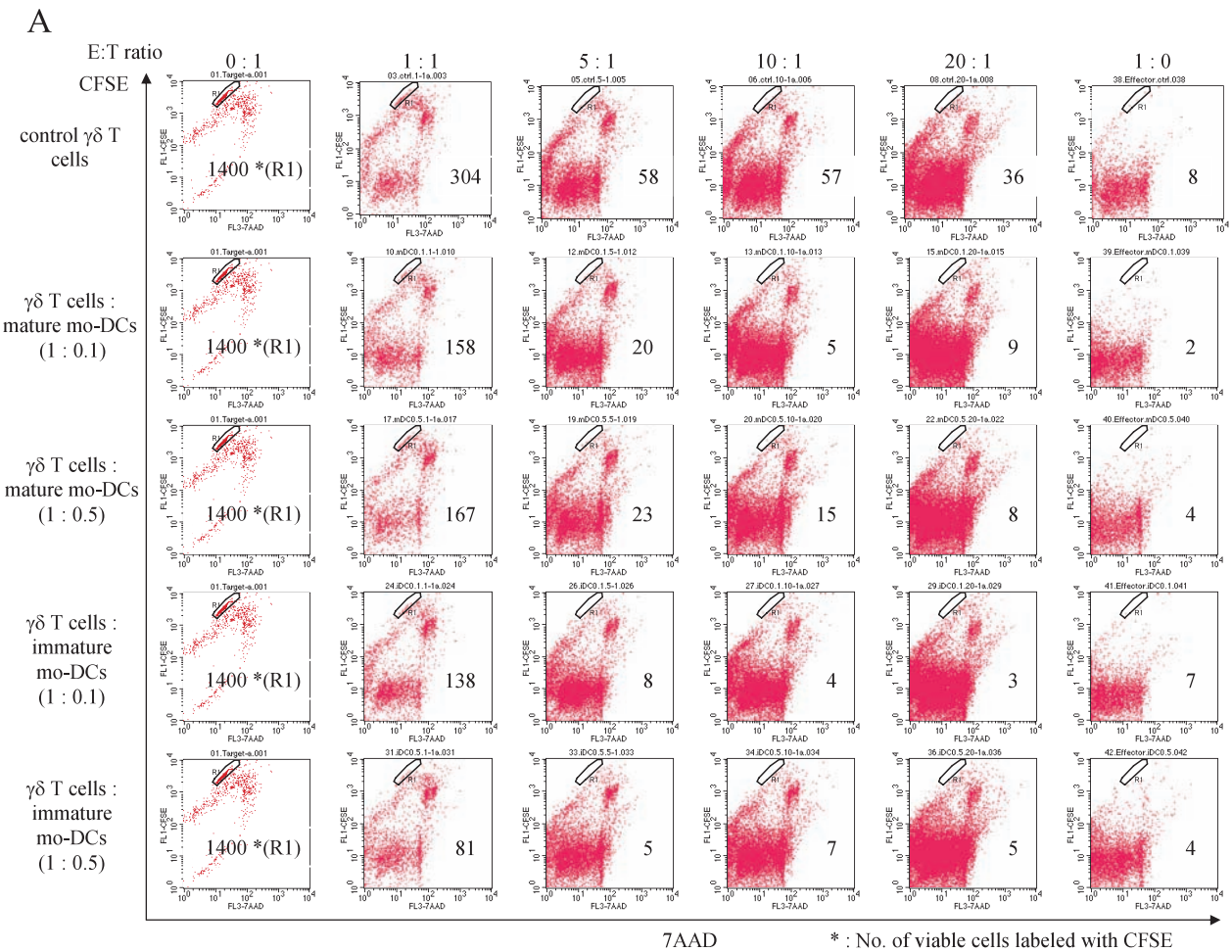


Fig. 5. Although pDCs stimulated with influenza virus secreted a high amount of IFN-α, a measurable amount of IFN-α was not detected in the culture supernatant of immature and mature mo-DCs as well as pDCs without stimulation.

Enhancement of γδ T cell-mediated cytotoxicity by stimulation with mo-DCs

CFSE-based cytotoxicity assays using RPMI8226 as target cells showed that γδ T cells by themselves (as effector cells) possessed a potent cytotoxic activity against RPMI8226. In addition, co-culturing γδ T cells with immature or mature mo-DCs markedly enhanced the cytotoxic activity of γδ T cells (Fig. 6A). Viable target cells with $CFSE^+/7AAD^-$ (R1 gate), which predominantly existed in the



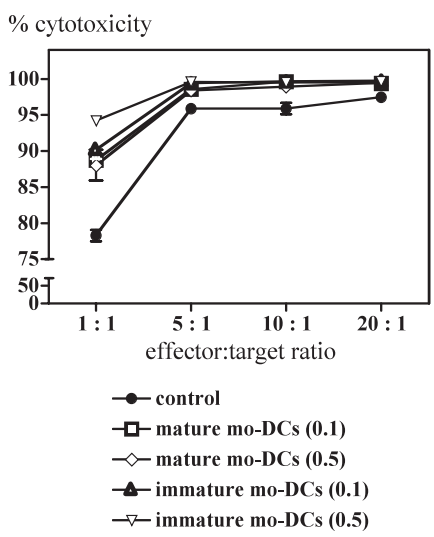


Fig. 6. Enhancement of γδ T cells' cytotoxicity against tumor cells after mo-DC stimulation. (*6A*) : CFSE-based cytotoxicity assay using RPMI8226 as target cells showed that viable target cells with $CFSE^{+}/7AAD^{-}$ (R1 gate) were damaged to be shifted into the dead cell target area ($CFSE^{+}/7AAD^{+}$) by culturing target cells with γδ T cells. Co-culturing γδ T cells with immature or mature mo-DCs for 2 days markedly enhanced the cytotoxic activity of γδ T cells in an effector-to-target ratio dependent manner. This is a representative result among three separate experiments. (*6B*) : The graph implied that although both immature mo- DCs and mature mo-DCs enhanced the cytotoxicity of γδ T cells, immature mo-DCs tended to enhance γδ T cells' cytotoxicity more than mature mo-DCs. The ratio 1:0.5 of γδ T cells to immature mo-DCs in the co-culture was more effective than 1:0.1 for enhancing γδ T cells' cytotoxicity.

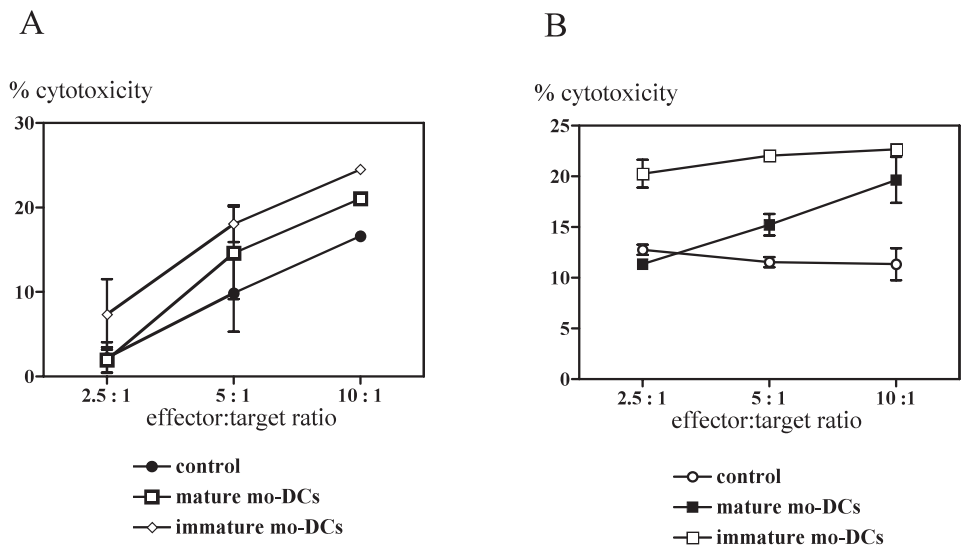


Fig. 7. Enhancement of γδ T cells' cytotoxicity against a chronic myelogenous leukemia blastic crisis cell line, C2F8, after co-culturing them with immature or mature mo-DCs. The γδ T cells' cytotoxic activity against C2F8 was enhanced by co-culture with mo-DCs. Immature mo-DCs had greater enhancing ability compared with mature mo-DCs. This is a representative result of two separate experiments. The similar results were obtained by CFSE-based cytotoxicity assay (*7A*) and Calcein-AM-based Terascan assay (*7B*).

tube containing target cells cultured without γδ T cells, were damaged to be shifted into the dead cell target area ($CFSE^+$/$7AAD^+$) by co-culturing target cells with γδ T cells previously incubated with mo-DCs for 2 days. Although both immature mo-DCs and mature mo-DCs were demonstrated to enhance the cytotoxicity of γδ T cells, immature mo-DCs tended to have a greater ability to enhance γδ T cells' cytotoxicity than mature mo-DCs. The ratio 1:0.5 of γδ T cells to immature mo-DCs in co-cultures was more effective than 1:0.1 for enhancing γδ T cells' cytotoxicity (Fig. 6B). CFSE-based cytotoxicity assays using C2F8 as target cells also showed that γδ T cells' cytotoxic activity was enhanced by co-culture with mo-DCs (Fig. 7A). Furthermore, immature mo-DCs had greater enhancing ability than mature mo-DCs (Fig. 7A). Calcein-AM-based Terascan assays using C2F8 as target cells showed similar results as those obtained by CFSE-based cytotoxicity assay, and also demonstrated that immature mo-DCs enhance γδ T cells' cytotoxicity more than mature mo-DCs (Fig. 7B).

DISCUSSION

In the previous study, we demonstrated that type I IFN could activate γδ T cells expanded from PB-MNCs by stimulation with zoledronate and IL-2, and it could enhance γδ T cell-mediated anti-leukemic cytotoxicity.[19] In the present study, we demonstrated that co-culture with mo-DCs increases activation marker (CD69) expression on expanded γδ T cells and mo-DC stimulation provides γδ T cells with greater anti-tumor cytotoxic activity than unstimulated γδ T cells. The enhancing activity of mo-DCs on the γδ T cells was demonstrated in both autologous and allogeneic settings using both immature and mature mo-DCs. The γδ T cell activation by mo-DCs was believed to be at least partly due to humoral factor(s) secreted from mo-DCs. Although IFN-α was not detected in the supernatant of unstimulated mo-DCs, mo-DCs were presumed to secrete various cytokines including IFN-α as a result of interacting with γδ T cells.

Some previous investigations have studied the interaction between mo-DCs and γδ T cells. Conti *et al*. reported the reciprocal activating interaction between mo-DCs and γδ T cells.[24] Co-culture of immature mo-DCs with γδ T cells in the presence of aminobisphosphonate (pamidronate) led to a significant up-modulation of CD86 and mo-DCs' MHC class I molecules. Activation of mo-DCs by pamidronate-stimulated γδ T cells required cell-to-cell contact. Reciprocally, activation (expression of surface CD25/CD69 and intracellular TNF-α/IFN-γ) of γδ T cells by pamidronate was dependent on cell contact with immature mo-DCs, being mediated in part by CD86 molecules. Shrestha *et al*. implied the regulation of acquired immunity by γδ T cell/mo-DC interactions.[25] They demonstrated that TLR-2-stimulated mo-DCs enhanced IFN-γ production by γδ T cells. Conversely, activated γδ T cells were shown to enhance TLR-2-induced DC maturation via soluble factors including IFN-γ, which stimulated IL-12 secretion by mo-DCs. At the same time, exposure of mo-DCs to activated γδ T cells was revealed to be critical for Th1 T-cell priming when TLR-2 stimulation was limiting.

Devilder *et al*. reported that immature DCs, and to a lesser extent mature DCs, potentiated the production of cytokines such as TNF-α, IFN-γ and IL-4 by established γ2δ2 T cell clones and γ2δ2 T cells cultured with bromohydrin pyrophosphate (BrHPP), but DCs had limited or no effect on γδ T cells' cytotoxicity and proliferation.[26] As to their study of γδ T cells' cytotoxicity using ^{51}Cr-release assay, γ2δ2 T cell clone was used as effector cells and immature or mature DCs were used as both stimulator cells and target cells simultaneously in the presence of high or low dose of BrHPP. Since the cytotoxicity of γ2δ2 T cell clone incubated with either immature or mature DCs were similar between the BrHPP concentrations of 10 nM and 500 nM, they concluded that DCs have little effect on the cytotoxicity of γ2δ2 T cells. Thereafter, they performed similar experiments using immature DCs infected with *Mycobacterium bovis* BCG (antigen for γδ T cells) instead of adding BrHPP to the culture of ^{51}Cr-release assay and demonstrated that the cytotoxic ability of γδ T cells was limited against BCG-infected immature DCs. Evaluating the effects of mo-DCs on γδ T cells' cytotoxicity in their study is difficult because they used a one-step procedure that simultaneously stimulated γδ T cells by mo-DCs and killed mo-DCs (target cells) with γδ T cells (effector cells). Martino *et al*. showed the complementary function of γδ T cells and mo-DCs in response to IPP and lipopolysaccharide antigens.[27] Activated γδ T cells increased the maturational state of lipopolysaccharide-stimulated mo-DCs, increasing the expression of co-stimulatory and MHC class I and II molecules. Mo-DCs' IL-12 production was strongly amplified in the presence of activated γδ T cells and the Th1 polarization of naive $CD4^+$ T cells was significantly increased. At the same time, mo-DCs were demonstrated to enhance γδ T cell functions induced by IPP and promote their IL-2 independent proliferation through CD86 contact. As mentioned above, several previous studies demonstrated that mo-DCs could enhance the activation markers of γδ T cells and cytokine production by γδ T cells. However, studies before this paper have not reported that γδ T cells' anti-tumor cytotoxicity is actually enhanced by interacting with mo-DCs.

Several reports exist concerning γδ T cell-mediated cellular immunotherapy for tumors. Wilhelm *et al*. showed the usefulness of *in vivo* combined administration of pamidronate and IL-2 for patients with myeloma and lymphoma who showed *in vitro* amplification of γδ T cells in culture with pamidronate and IL-2.[28] Treatment with pamidronate demonstrated elevated IFN-γ, TNF-α and IL-6 secretion in these patients. Tumor stabilization or even partial regression occurred in myeloma patients whose γδ T cells responded to stimulation with pamidronate and IL-2. Sicard *et al*. demonstrated that efficient *in vivo* expansion and activation of γ2δ2 T cells after injection of a synthetic phosphoantigen and IL-2 were followed by exhaustion of γ2δ2 T cell responses upon repeated treatments in monkeys.[29] Casseti *et al*. have found that intravenous (IV) administration of nitrogen-containing bisphosphonate or pyrophosphomonoester drugs combined with subcutaneous (SC) low-dose IL-2 induced an expansion of γ2δ2 T cells in cynomolgus monkeys' PB.[30] The administration of these drugs in the absence of IL-2 was substantially less effective, indicating the importance of additional exogenous co-stimuli. Animals receiving the stimulatory drug plus IL-2 exhibited up to a 100-fold increase in PB γ2δ2 T cell counts. Moreover, the expanded γ2δ2 T cells were potent Th1 effectors capable of releasing large amounts of IFN-γ. Kunzmann *et al*. initiated a clinical trial of administering zoledronate (IV) and low-dose IL-2 (SC) for patients with low-grade B cell malignant lymphoma, multiple myeloma, RAEB/ALL, renal cell carcinoma, or melanoma, who initially responded to an *in vitro* γδ T cell stimulation.[31] *In vivo* proliferation of γδ T cells and elevated serum concentrations of IFN-γ were observed in patients treated with zoledronate and IL-2. The treated patients developed no serious adverse effects in the observation periods. In addition to γδ T cell-mediated immunotherapy by *in vivo* administration with bisphosphonate and IL-2, clinical trials of administering *in vitro*-expanded γδ T cells were initiated to treat γδ T cell-responsive tumors such as myeloma and lymphoma. In order to establish an efficient γδ T cell-mediated cellular immunotherapy, an effective method is needed for generating large amounts of γδ T cells with potent anti-tumor cytotoxicity.

Although reciprocal activating interaction between mo-DCs and γδ T cells has been extensively investigated, whether γδ T cells activated by mo-DCs possess more potent anti-tumor cytotoxic activity compared with non-activated γδ T cells remains to be elucidated. In the present study, we show that activation of γδ T cells by mo-DCs enhances anti-tumor cytotoxicity of γδ T cells in both autologous and allogeneic settings between mo-DCs and γδ T cells and in both immature and mature mo-DCs. These findings suggest that activation of γδ T cells by mo-DCs could be used to generate more potent γδ T cells for establishing an efficient anti-tumor γδ T cell-mediated immunotherapy.

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