Cytotoxic function of gamma delta (γ/δ) T cells against pamidronate-treated cervical cancer cells

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The cytotoxic function of polyclonal expanded γδ T cells against pamidronate-treated cervical cancer cells in vitro and in vivo were determined. The γδ T cells were isolated and purified from PBMCs by using miniMACS and were later treated with 10 μM pamidronate. The expansion of γδ T cells was 15 times more than the non-stimulated cells. Among the expanded γδ T cells, 47% were Vγ9/Vδ2 T cells with a purity of 87%. Analyzing the cytotoxic function of γδ T cells against 3 cervical cancer cells in vitro by LDH cytotoxicity test revealed that the killing efficacy increased if the cervical cancer cells (HeLa, SiHa and CaSki) were pretreated with pamidronate. The presence of CD107 on γδ T cells indicated the degranulation of perforin and granzyme pathway is one of the mechanisms used by the γδ T cells to kill cancer cells. The killing ability of γδ T cells against cancer cells in vivo was preliminary assessed by using mouse baring HeLa cells. The results demonstrated that γδ T cells induce apoptosis in tumor cells. Our study supports the usefulness of γδ T cells in future development of immunotherapy for cervical cancer.

Keywords: Cervical cancer, Gamma-delta T cells, Pamidronate, Immunotherapy

Cervical cancer is the second most common cancer found in women worldwide, especially in developing countries such as Thailand. Epidemiological and molecular studies have clearly shown that the human papillomavirus (HPV), especially HPV-16 and 18, are the causative agents of this cancer1-4. The current standard therapeutic protocols for cervical cancer are surgical, radiotherapy, chemotherapy or combination of these protocols. Even though these protocols are effective in controlling the initial disease, in some patients, recurrent and metastasis can be seen after primary treatment and is generally associated with poor prognosis5. In order to improve clinical outcome of these patients, new forms of therapy for recurrent or metastatic cervical cancer are needed. For example, immunotherapy using CD8+ T cells has been used to treat malignancies such as renal cancer and breast cancer but its limitation is its antigen recognition system which is highly dependent on MHC class I molecule of the cancer cells6,7. Unfortunately, HPV positive cervical cancer cells downregulate MHC class I and therefore produce very low levels of the viral antigens8,9. As a result of this, immunotherapy using CD8+ T cells are ineffective in treating cervical cancer. Despite this, recently it has been shown that a minor subset population of T cells known as the gamma-delta (γ/δ) T cells is important in tumor surveillance and could kill various types of solid tumor such as colon cancer, prostate cancer and renal cancer10,11. The Vγ9/Vδ2 T cells, unlike α/β T cells, recognize non-MHC peptide antigens and hence are very effective in eliminating MHC downmodulated cancers. Furthermore, nitrogen bisphosphonate drugs (nBPs) can enhance cytotoxic activity of γ/δ T cells by interfering with the mevalonate pathway causing isopentenyl pyrophosphate (IPP), an intermediate product of mevalonate pathway and antigen located on all mammalian cells that is specifically recognized by Vγ9/Vδ2 T cells12, to accumulate in the tumor cells13. However, it is not known whether γδ T cells can effectively eliminate cervical cancer cells pre-treated with pamidronate or not. Therefore, in vitro
and in vivo studies have been undertaken to investigate the cytotoxic function of polyclonal expanded \(\gamma / \delta\) T cells against pamidronate-treated cervical cancer cells.

**Materials and Methods**

*Cell lines*—Three HPV-DNA positive cervical cell lines were used: HeLa, CaSki and SiHa. HeLa cells containing HPV-18 DNA were obtained from the Virology Division, Department of Microbiology, Faculty of Medicine, Chulalongkorn University. Both CaSki (CRL-1550 Lot No. 3794357) and SiHa cell lines (HTB-35 Lot No. 4031219) containing HPV-16 DNA were purchased from ATCC, USA. HPV negative fibroblast cell line (NTY) was isolated from a cervical cancer patient from the King Chulalongkorn Memorial Hospital. All cells were grown in MEM media supplemented with 10% fetal bovine serum (FBS, HyClone, USA), penicillin (50 unit/mL), streptomycin (50 \(\mu\)g/mL) and 2 mM L-glutamine (PAA, Austria).

*Activation of peripheral blood (\(\gamma / \delta\)) T cells*—Peripheral blood mononuclear cells (PBMC), derived from buffy coat of healthy blood donors, were isolated by density gradient centrifugation using LeucoPREP (BD, USA). The buffy coat was 2-fold diluted with RPMI-1640 (PAA, Austria) and then 40 mL of diluted buffy coat was layered on top of 10 mL of LeucoPREP solution in a sterile 50 mL centrifuge tube. Layered blood was then centrifuged at 1,500 rpm for 30 min. The PBMC layer was transferred to a new 50 mL centrifuge tube. Layered blood was then centrifuged at 1,500 rpm for 30 min. The PBMC layer was transferred to a new 50 mL centrifuge tube and washed several times with 0.45% sodium acetate in phosphate buffered saline (PBS) until the supernatant was clear. Cultivation of PBMC and activation of \(\gamma / \delta\) T cells were modified from the methods previously described. Total PBMC were counted and resuspended in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, and 10 mM HEPES (R-10). Approximately \(10^8\) PBMC in 15 mL of R-10 medium were seeded into the 75 cm² tissue culture flask and then incubated at 37 °C in humidified atmosphere of 5% CO₂. PBMC were stimulated once with 10 \(\mu\)M of pamidronate (Novartis, USA) and 100 unit/mL of IL-2 (PeproTech, USA) on the first day of culture. \(\gamma / \delta\) T cells were obtained by restimulating the PBMC with 100 unit/mL of IL-2 every 3 days. On day 14, the PBMC were harvested and the \(\gamma / \delta\) T cells were isolated by positive selection using miniMACS (Miltenyi Biotech, Germany). After purification, cell number and viability were determined by using trypan blue. The purity of \(\gamma / \delta\) T cells was confirmed by flow cytometry (FACS Calibur flow cytometer; BD Bioscience, USA).

*Staining of \(\gamma / \delta\)/V\(\gamma / \delta\) T cells subpopulation—\(\gamma / \delta\)—*T cells were obtained as described above. After 14 days of ex vivo expansion, the cells were directly stained with anti-pan \(\gamma / \delta\) antibody FITC, anti-V\(\gamma / \delta\) antibody PE or anti-V\(\gamma / \delta\) antibody FITC (BD Bioscience, USA). The percentage of V\(\gamma / \delta\)/V\(\delta\) T cells subpopulation was analyzed by flow cytometry (FACS Calibur flow cytometer; BD Bioscience, USA).

*Cytotoxicity assay*—Purified \(\gamma / \delta\) T cells of at least 90% purity were used as effector T cells (E). After purification, the cells were left in R-10 for another 3 days to remove anti-FITC antibody from its surface. HeLa, CaSki and SiHa were used as target cells (T). NTY and phytohemagglutinin (PHA) autologous PBMC blast were used as controls. Active, proliferating target cells were seeded in to 96-well u-bottom plates at \(1 \times 10^3\) cells/well in R-10 with or without 10 \(\mu\)M of pamidronate and incubated at 37 °C. After 24 h, the target cells were washed twice with PBS and once with RPMI-1640. Fresh R-10 supplemented with low dose IL-2 (20 unit/mL) and efector cells at a ratio (E:T) of 20:1 or 40:1 were added and co-cultured for 5 h at 37 °C in humidified atmosphere of 5% CO₂. After that, cytotoxicity assay was performed by using LDH Cytotoxicity Detection Kit (Roche, USA). Low control in the system was target cells with 50 \(\mu\)L of assay medium. High control was target cells with 50 \(\mu\)L of assay medium and 5 \(\mu\)L of lysis solution which was added 15 min before the end of the incubation period. Medium alone was used as background control. All of the reagents were prepared according to the manufacturer’s protocol. The absorbance (OD) was read at 492 nm and the percentage of cytotoxicity was determined by the following equation:

\[
\text{Cytotoxicity} = \frac{(\text{experiment value} - \text{low control})}{\text{high control} - \text{low control}} \times 100
\]

The experiment was repeated using different purified \(\gamma / \delta\) T cells obtained from 5 healthy donors. Each experiment was done in duplicate.

*Quantification of cytokines secreted by \(\gamma / \delta\) T cells*—After 5 h of co-cultivation of effector and target cells, the supernatants were collected.
Quantification of IFN-γ in the supernatants was done by using commercially available ELISA detection kit (PeproTech, USA).

**CD107 cytotoxicity assay**—The CD107 assay was performed according to the methods described by Betts et al.\(^\text{15}\) Approximately 10\(^4\) cells of cervical cancer cell lines (HeLa, SiHa and CaSkI) were cultivated in 96-well plate in MEM with or without 10 \(\mu\)M of pamidronate at 37 \(^{\circ}\text{C}\) in 5% \(\text{CO}_2\) atmosphere for 12 h. After that, the culture medium was removed and the cells were washed once with PBS. A total of 2\(\times10^5\) γδ T cells was added to each well followed by 3 \(\mu\)L of anti-CD107a antibody FITC (BD Bioscience, USA). The plate was centrifuged and then incubated at 37 \(^{\circ}\text{C}\) for another 5 h. The cells were collected, washed once with PBS and stained with anti-human Vγ9 TCR antibody (BD Bioscience, USA) for 15 min. The cells were washed again with PBS and re-suspended in 200 \(\mu\)L of PBS and analyzed by flow cytometry (FACS Calibur flow cytometer; BD Bioscience, USA). The γδ T cells from three individuals were performed in three independent experiments using duplicate wells.

**Mouse bearing tumor cells**—Mouse bearing tumor cells were prepared as previously described\(^\text{16}\). In brief, female BALB/c-athymic nude mice from National Laboratory Animal Center of the Salaya Campus, Mahidol University, Bangkok, Thailand, aged 4-8 weeks and weighing 20–25 g were used. Pentobarbital sodium (50 mg/kg body weight) was intraperitoneally injected in to the mice. The dorsum skin-fold window chamber was fixed on the dorsal of each mouse and 1\(\times10^6\) HeLa cells were inoculated into each window chamber. Approximately 2 weeks after the development of the tumor, the mice were used for the in vivo study. Control (medium only) and experiment (HeLa cells) groups consisted of 3 mice each.

**Deposition of γδ T cells**—The purified γδ T cells were labeled with 25 \(\mu\)M of Cell Tracker green (5-chloromethyl fluorescein diacetate; CMFDA, Invitrogen, USA) in a dark chamber at 37 \(^{\circ}\text{C}\) for 45 min. After that, the labeled cells were washed once and re-suspended in RPMI-1640 and immediately used. Mice were anesthetized with pentobarbital sodium (40 mg/kg). The CMFDA labeled γδ T cells (3\(\times10^6\)) were injected into several positions in the tumor area. The control mice were injected with CMFDA labeled γδ T cells, a depleted fraction of PBMC flow-through. The trafficking of γδ T cells was observed and photographed by using in vivo imaging System (Kodak, USA). The mice were sacrificed 3 days after adoptive transfer of γδ T cells. Tumor tissues were collected for further analysis.

**Anti-CD3 staining and in situ apoptosis detection**—To observe the deposition of γδ T cells within the tumor areas, tissue section was stained by in situ immunohistochemistry technique. Approximately 5 \(\mu\)M thickness of the formalin-fixed, paraffin embedded tissue was subjected to antigen retrieval by high pressure cooker treatment for 10 min. The tissue was stained with 1:500 specific human anti-CD3 polyclonal antibody (Rabbit anti-Human CD3 A0452, DAKO, USA) diluted in citrate buffer with a pH of 6.0 (0.45% of sodium citrate in PBS). The secondary antibody from the Envision detection system (envision\textsuperscript{TM} system, HRP, DAKO, USA) was used to detect T cells. Staining process of the tissue was performed by using an automatic staining machine (Ventana, Benchmark LT, USA).

Apoptosis assay for the tissue was determined by using in situ apoptosis detection kit (ApopTag\textsuperscript{TM} Plus Fluorescein In Situ Apoptosis Detection Kit; CHEMICON International, USA). Detection procedures were followed according to the manufacturer’s instructions.

**Results**

**Pamidronate induced expansion of γδ T cells and Vγ9Vδ2 T cells subpopulation**—To induce expansion of γδ T cells in vitro, the optimal concentration of pamidronate was evaluated. PBMCs from 3 healthy volunteers were cultured for 14 days in different concentrations of pamidronate (0.625-200 \(\mu\)M) and percentages of γδ T cells were assessed. Concentrations of pamidronate from 0.625 to 20 \(\mu\)M effectively induced the expansion of γδ T cells, with the peak expansion at 10 \(\mu\)M (Fig.1). At 10 \(\mu\)M of pamidronate, the expansion of γδ T cells (54%) was 15 times greater than the (3.6%) unstimulated PBMC (Fig. 2). Since it has been shown that Vγ9Vδ2 T cells, a subpopulation of γδ T cells, are produced after IPP antigen stimulation\(^\text{17-20}\), the percentages of Vγ9Vδ2 T cells were analyzed. From the expanded γδ T cells, 47% were Vγ9Vδ2T (Fig. 2). The purity of the Vγ9/Vδ2 T cells obtained was 87%.
Cervical cancer cells treated with pamidronate enhanced killing capacity of expanded γδT-cells—In order to assess the killing capacity of γδ T cells, the LDH assay was used. All cervical cancer cell lines (HeLa, SiHa and CaSki) were killed by γδ T cells especially those cells that were pre-treated with pamidronate. The % cytotoxicity at E:T=40:1 ratio was stronger than that at 20:1 ratio in all cell types. Moreover, significant differences between treated and non-treated cells were detected in all cell types at the ratio of 40:1 (Fig. 3B). When the cytotoxicity of γδ T cells at E:T=20:1 was analyzed against each cervical cancer cell line, it was shown that the killing was based on the sensitivity-dependent hierarchical of the cervical cancer cell lines. HeLa cell line was the most susceptible cell line followed by SiHa and CaSki cell lines (Fig. 3A). Some killing was detected in NTY whereas there were none in autologous PHA-activated PBMC.

Killing of cervical cancer cell lines is mediated by CD107—Integral membrane proteins localized within cytolytic granules, CD107, are transiently mobilized to the surface of the cell during degranulation activation. CD107 expression on T cells can be used to estimate the cytotoxic function of CD8+ T cells and Vγ9Vδ2 T cells.21,22 The average expressions of CD107 on the cell surface of γδ T cells exposed to pamidronate-treated HeLa, SiHa and CaSki cervical cancer cell lines were 6.95, 10.85 and 4.84 times respectively, significantly higher than the non-treated cervical cancer cell lines but when compared amongst each other, these differences were not significant (Fig. 4B). All pre-treated cervical cancer cell lines were able to activate the degranulation process of γδ T cells.

Production of IFN-γ from γδ T cells activated by cervical cancer cells—IFN-γ has an essential role in tumor immune response because it can activate many innate immune cell types, allowing these cells to exert their anti-tumor functions.23 Activation of γδ T cells can induce a variety of Th-1 cytokines including TNF-α and IFN-γ.11-12,14,24 Like T-cell mediated antiviral responses, the production of IFN-γ is likely to reflect the anti-tumor function of γδ T cells. The amount of IFN-γ production was analyzed by ELISA using the supernatant which was collected from the co-culture between γδ T cells and cervical cancer cell lines. The amount of IFN-γ released from γδ T cells co-cultured with pamidronate-treated target cells were
higher than the untreated target cells except for CaSki. At least 1.08 to 2.1 fold increase of IFN-γ were detected in these co-cultures when compared to untreated cells. Like the cytotoxicity results, HeLa was the most effective cervical cancer cell line in stimulating γδ T-cell response (Fig 5). These γδ T cells recognized all of the cervical cancer cells and allogeneic cells except for autologous PHA-activated PBMC.

Deposition of γδ T cells in to the tumor area induced tumor apoptosis in vivo—As previously shown, the activated γδ T cells could secrete anti-tumor cytokines and kill pamidronate-treated cervical cancer cell lines in vitro. However, it is not known whether these activated γδ T cells can kill cervical cancer cells in vivo. Two weeks after the tumor was established, the tumor-bearing mice were injected with $3 \times 10^6$ γδ T cells labeled with CMFDA around the tumor site (n=3). The control mice were injected with γδ T-cell-depleted PBMC. Three days after adoptive transfer of the labeled cells, the mice were sacrificed and the tumor was collected for histopathology. The anti-human CD3 staining of the tumor tissues indicated that the γδ T cells (dark brown stained cells) were localized in the tumor areas (Fig. 6). The apoptosis of the surrounding tumor cells were analyzed to see if these infiltrated γδ T cells

![Fig. 3—Cytotoxicity of γδ T cells against pamidronate treated (T) and non-treated (N) cervical cancer cell lines at E:T ratio of 20:1 (a) and 40:1 (b) NTY cell and PHA-activated PBMC are shown. The results are the average from 5 individual of healthy blood donors. Each bar represents the mean ± SD of the percentage of γδ T cell cytotoxicity. A: * HeLa T : HeLa N, NTY T, NTY N and PHA activated PBMC (P=0.002, 0.007, 0.0006, and 0.0008, respectively). ** SiHa T : PHA activated PBMC (P=0.0084). *** CaSki T : PHA activated PBMC (P=0.039). B. HeLa-T : HeLa-N (**P=0.05) SiHa-T : SiHa-N (**P=0.019), CaSki-T : CaSki-N (**P=0.023) and NTY-T : NTY-N (**P=0.008).](image1)

![Fig. 4—Results from the CD 107 cytotoxicity assay. A: Results represent from one experiment, B: Statistical analysis of mobilization of CD107 on the cell surface between each treated and non-treated cervical cancer cell line. Each bar represents the mean ± SD of the percentage of CD107 positive cells. The results showed statistically significant difference for HeLa-T : HeLa-N (*P=0.185), SiHa-T : SiHa-N (**P=0.098) and CaSki-T : CaSki-N (**P=0.008). There is no statistically significant difference of CD107a between each of treated cancer cell lines. The data is the average from 3 independent experiments.](image2)

![Fig. 5—IFN-γ production from activated γδ cells. The results are from 5 independent experiments from 5 different healthy blood donors which were combined together and shown as mean± SD (pg/mL).](image3)
were functional or not. This was done by using an \textit{in situ} apoptosis detection system. The mice that had adoptive transfer of $\gamma\delta$ T cells showed higher degree of apoptosis when compared to the controls (Fig. 7).

**Discussion**

Recently, there is an increased interest in the role of $\gamma\delta$ T cells, especially $V\gamma9/V\delta2$ T cells. Several documents demonstrated that these cells were involved in host defense against a large range of tumor cell types such as myeloma, colon carcinoma, renal cell carcinoma and hepatocarcinoma\textsuperscript{17,19,25,26}. It has been shown that Isopentenyl pyrophosphate (IPP), intermediates of mevalonate pathway and antigen specific for $V\gamma9/V\delta2$ T cells\textsuperscript{12}, can enhance cytotoxic killing of these cells. As a result of this, nitrogen bisphosphonate drugs (nBps drugs) are used to induce the expression of IPP for the treatment of cancer\textsuperscript{27,29}.

Further, it has been suggested that adoptive transfer of $V\gamma9/V\delta2$ T cells in conjunction with nBP drugs may be an alternative approach in treating cancer.

In this study, it was sought to test this theory out \textit{in vitro} and \textit{in vivo}. First, the expansion of peripheral
blood γδ T cells from healthy donors was explored by using pamidronate and IL-2. Large expansions of γδ T cells were seen when grown ex vivo with pamidronate; most of the expanded cells were Vγ9/Vδ2 T cells subpopulation (Fig. 2). This result is consistent with a previous study that showed pamidronate and other aminobisphosphonates (alendronate and ibandronate) were able to induce a significant expansion of γδ T cells in primary PBMC culture from all healthy donors19. Another study using bromohydrin pyrophosphate (BrHPP), a synthetic pyrophosphate antigen, also had the same result26. This is because the pyrophosphate antigens do not require antigen entry and processing so it can be presented directly to the Vγ9/Vδ2 T cells. On the other hand, PMBC treated with nBP drugs have a stronger amplification of Vγ9/Vδ2 T cells due to the accumulations of IPP within the treated cells when the drug inhibits the farnesyl pyrophosphate synthase (FPPS), a downstream enzyme used to convert IPP to other intermediate products. As a result of this, polyclonal expanded γδ T cells obtained from 5 individuals showed considerable cytotoxic function against pamidronate-activated cervical cancer cell lines. Each amplified γδ T cells had a higher cytotoxic potential against all 3 pamidronate-treated cervical cancer cells when compared to non-treated cells (Fig. 3). Even though the magnitude of killing at E:T=20:1 was different for each cervical cancer cell line, no differences were shown at E:T=40:1. The present results also showed NTY cells, a normal cell, were killed by γδ T cells. The reason for this killing is because the NTY cells were not autologous as that of the γδ T cells. Along the same lines, the killing of autologous PBMC PHA blast was low as expected. PHA-activated PBMCs pre-treated with pamidronate were not performed in this study however the killing effect of the pre-treated cells should be more than the non-treated cells. The present findings are consistent with the previous observation that purified Vγ9/Vδ2 T cells from colon cancer patient displayed different degrees of selective lytic potential toward autologous and allogologous colon cancer cell lines18.

The killing mechanism used by the γδ T cells was also investigated. It has been shown that CD107 expression on the cell’s surface can be utilized as a precise method to characterize the response of T cells. The expression of CD107 on γδ T cells supports the results obtained from the cell lysis assay. Differences in the levels of CD107 expressions were detected after the γδ T cells were exposed to pamidronate-treated cervical cancer cells but this was not significant. Similar observation was also detected when γδ T cells were exposed to non-treated cells (Fig. 4). The expression of CD107 was higher when exposed to pamidronate-treated cancer cells compared to the non-treated cancer cells, but this expression was not present on all of the cells. This implied that there were other pathways or co-stimulatory molecules involved in the killing of the cancer cells. For example, intraepithelial γδ T cells with FasL on its cell surface can induce apoptosis in Fas-expressing cancer cell lines32. Similarly, NKG2D and MICA/B receptors can induce killing of breast and renal cancer cells. Blocking of NKG2D receptor on γδ T cell will reduce lysis of target cells32. Other interactions with surface markers such as CD6 and CD166 on cancer cell lines can also enhance activation of γδ T cells. The expression level of CD6 and CD166 in cancer cell lines can also affect the function of γδ T cells because CD6 receptor is a scavenger receptor of cysteine-rich superfamily found to be associated with the Lck, Fyn and Zap-70 families34.

Several cytokines such as GM-CSF, MIP-1 α/β, RANTES, TNF-α and IFN-γ can be secreted from γδ T cells after activation with IPP35. Among these cytokines, IFN-γ is the cytokine that is secreted early during the activation of γδ T cells. Early secretion of IFN-γ and TNF-α during activation of γδ T cells are essential in tumor immunity as the function of the T cells’ response against tumor are regulated24,36. It has been observed that IFN-γ is released within 2-24 h after stimulation37. This information is in parallel with present results which showed the production of IFN-γ approximately 5 h after exposure to cancer cells. The results of the present study showed that the production of IFN-γ was higher after the γδ T cells were stimulated with pamidronate treated cervical cancer cells but this did not reach any statistical significance. It is possible that the use of each individual blood donor for each experiment may have contributed to the wide range of standard deviations. Therefore it is recommended that for future studies, the preparation of γδ T cells should come from pooled PBMC blood donors as previously described33.

Aside from the in vitro study, it was also demonstrated that when γδ T cells were deposited in the tumor site, apoptosis of tumor cells were induced.
(Fig. 7). This finding is similar to the results from a prostate cancer study which showed successful localization of the size of the tumor and growth modulation of the established tumor after adoptive transfer of syngenic mouse γδ T cells. Likewise, Zheng et al. showed that peripheral γδ T cells derived from healthy donors were able to kill nasopharyngeal cancer cells in mice. To our knowledge, the present study is the first of its kind to describe the role of γδ T cells, especially Vγ9/Vδ2 T cells, against cervical cancer cells. The present preliminary in vitro and in vivo data support the use of γδ T cells in active or passive immunotherapy for the treatment of recurrent or metastasis cervical cancer.

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