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Enhanced Immunotherapy against Lung Cancer Cells

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Lung cancer is one of the most lethal cancers. Lung cancer cells have the ability to undergo mutations allowing escape from an immune response so the cells can continue to grow. NK cells and γδ T cells are the first line of defense against bacteria and other infectious agents. We evaluated the ability of these innate immune responders to target lung tumor cells. Using enhanced NK cells, we tested the combination therapy with Cetuximab, an anti-EGFR antibody. We also evaluated combination therapy with γδ T cells and bisphosphonate. Treatment with Cetuximab and NK cells was 15 to 30% more effective against EGFR expressing cell lines compared to NK cell therapy alone. In contrast, lymphocyte activated killer cell (LAK) did not enhance cytotoxity induced by Cetuximab treatment. Next, we evaluated γδ T cell cytotoxicity in combination with Zoledronate, a bisphosphonate. Cytotoxicity by γδ T cells was enhanced against bisphosphonate treated tumor cell lines in vitro. Treatment with 50 μM Pamidronate or with 10 μM Zoledronate enhanced anti-tumor effects induced by γδ T cells. These two treatments may enhance immune responses in immunotherapy.

Key words: NK cell, Cetuximab, γδ T cell, Pamidronate, Zoledronate

Background

Lung cancer is one of the most lethal cancers. Lung cancer cells often mutate and become resistant to treatments such as chemotherapy and radiation therapy[1)2)]. Although dendritic cell-based immunotherapy is an effective treatment, lung cancer cells often lose expression of MHC class I and II in order to escape anti-tumor immune responses, leading to tolerance[3)~5)]. Immunotherapy with innate immune effectors may overcome this tolerance as natural killer (NK) cells and gamma-delta (γδ) T cells can respond to tumors in a MHC-independent manner. NK or γδ T cell-based immunotherapy has few side effects and therefore has an advantage over traditional chemotherapy[6)7)].

Many tumors express high levels of the Epidermal Growth Factor Receptor (EGFR) and HER2[8)9)]. Since EGFR is mainly expressed by tumors, it may be a good target for cancer therapy. Cetuximab (Erbitux®) is a chimeric antibody, combining a human FC chain with a murine Fab fragment that recognizes human EGFR[10)]. Cetuximab is a standard therapy for metastatic rectal carcinoma and is used in combination with chemotherapy[11)~13)]. In this study, we evaluated whether pretreatment of tumor cells with Cetuximab enhanced antibody-dependent cell cytotoxicity (ADCC) mediated by activated NK cells.

We also evaluated the activity of γδ T cells after treatment with bisphosphonates, including Pamidronate (Pam) and Zoledronate (Zol). Bisphosphonates (also called biphosphonates), are a class of drugs that prevent the loss of bone mass and are commonly used to treat osteoporosis and similar diseases[14)]. It has also been shown that these drugs can prevent bone metastasis of some cancers[15)~17)]. Zol is used to prevent skeletal fractures in patients with cancers such as multiple myeloma and pros-

tate cancer[18)~20)]. It is also used to treat malignancy-associated hypercalcemia and is helpful for reducing pain associated with bone metastases. Zol has also been shown to induce the proliferation of γδ T cells in vitro[21)22)]. In this study, we examined whether lung tumor cell lines pre-treated with bisphosphonates were more sensitive to γδ T cell killing.

Materials and Methods

Cell lines and reagents

Human lung adenocarcinoma (LK87), human small cell lung carcinoma[8)~10)23)~27)] and lung squamous cell carcinoma (EBC-1) were provided by the Surgery Laboratories Cell Bank (Tohoku University Hospital, Sendai, Japan). The human lung adenocarcinoma, A549 was kindly supplied by the Respiratory Medicine Department of Tokyo Women's Medical University (TWMU). MCF7 and K562 were provided by the Department of Surgery, Institute of Gastroenterology, TWMU. Each cell line was grown in RPMI or DMEM containing 10% FCS.

Cetuximab (Erbitax®) was purchased from Merck KGaA. Zoledonate (Zometa®) and Pamidronate (Aredia®) were purchased from Novartis Pharma AG (Basel, Switzerland) and Nippon Kayaku (Tokyo, Japan).

Phenotypic analysis of cells and flow cytometry

Antibodies recognizing human CD62L, CD56, CD57, CD86, CD25, CD4, CD45RA, CD45RO, CCR4, CCR7, CXCR3, CCR5, IFN-g and IL-4 were purchased from BD Biosciences (San Jose, CA, USA). Antibodies recognizing human CD11b, CD31, CD27 and CD28 were purchased from Invitrogen, Co. (Carlsbad, CA, USA). LAK and NK cells from PBMC were evaluated by three color immunofluoresence staining with PC-5 labeled CD3 mAb, PE labeled CD56 mAb, and FITC labeled CD16 mAb (all from Beckman Coulter Co., Marseille, France). The phenotype of γδ T cells and Alpha-beta T cells from PBMC were evaluated by flow cytometry with PE labeled anti-pan alpha-beta TCR mAb and PerCP labeled anti-pan gamma-delta TCR mAb (both from Beckman Coulter Co.). Flow cytometric analysis was performed on a FACScan cytofluorimeter (Becton Dickinson) and analyzed by using CELL QUEST software (Becton Dickinson).

Preparation of cells

PBMCs were collected from healthy donors (n = 8) and isolated by Ficoll-Paque (lymphoprep / Frensenius Kabi Norge AS, Norway) density gradient centrifugation. To generate LAK cells, whole PBMC were incubated in AIM-V medium (Invitrogen, Co.,) containing 10% FCS and 6,000 IU/ml IL-2 (Proleukin Chiron B.V., Amsterdam, The Netherlands) for 2 weeks. To generate NK cell cultures, NK cells were separated from PBMC by Rosette Sep Mixture NK Enrichment Kit (Stem Cell Technologies, Vancouver) and incubated for 2 weeks in Aim-V medium containing 10% FCS and 6,000 IU/ml IL-2. The phenotype of NK cells was verified by flow cytometric analysis and the purity of CD3 − / CD56 + cells was over 85%. To generate γδ T cells, PBMC were incubated in AIM-V medium containing 10% FCS and 200 IU/ml IL-2 supplemented with 5 μM Zol for 2 weeks. The phenotype of cultured γδ T cells was evaluated by flow cytometry by measuring CD3 (+)/TCR αβ (−)/TCR γδ (+) cells. Purity was over 85% in the cultured cells.

To evaluate γδ T cells, we compared it to Cytotoxicity activated T cells (CAT). CAT contains over 90% TCR αβ T cells. PBMC from healthy donors were cultured in AIM-V medium containing 10% FCS and 200 IU/ml IL-2 incubated in culture flasks coated with 2 μg/ml OKT-3 for 48 hours. Cells were then incubated in non-coated bags for 12 days.

Cytotoxicity assay

A Cr-51 release assay was used to measure cytotoxicity. For evaluation of LAK and NK cell killing, target cell lines were incubated with media or 5 μM cetuximab for 30 minutes. Targets were washed twice with PBS after incubation. To measure γδ T cell cytotoxicity, target cell lines were incubated with Zol or Pam at the indicated concentration and time overnight, then washed twice with PBS. After treatment, target cell lines were labeled with 100 μCi of Cr-51 for 2 hours. Cell lines were washed twice with PBS and co-cultured with effector cells. Target cells were cultured at 1×10^4 cells/well in Aim-V containing 1% FCS in 96 well round bottom plates. Effector cells were added at various effector

to target (E : T) ratios. Spontaneous and total release plates were incubated for 4 hours at 37℃ and 5% CO_2. The radioactivity of supernatant was measured on a gamma counter. The percentage of specific cytotoxity was calculated according to the following formula: % specific cytotoxity = (experimental cpm − spontaneous cpm) / (maximum cpm − spontaneous cpm) × 100.

MTT assay

LK87 cells were incubated with Pam for 1 week and cell proliferation was evaluated by MTT assay with the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Co. Madison, WI, USA). The percentage of proliferation was calculated according to the following formula: % proliferation = experimental value / spontaneous value × 100

Results

We first assessed the expression of EGFR on the surface of cell lines by flow cytometric analysis (Fig. 1a). The cell lines A549, 11-18 and EBC-1 expressed EGFR whereas MCF7 and K562 did not. Cell lines A549, 11-18 and EBC-1 also expressed HLA class I (data not shown). MCF7 expressed HLA class I by addition of low dose IFN-gamma (data not shown). We also measured the expression of CD3, CD16 and CD56 on LAK and NK effector cells. Figure 1b shows one representative sample. LAK are made up of activated T lymphocytes with 50% of the cells expressing CD3. LAK also contain 30-40% NK cells. Very few (0.19%) of the NK cells expressed CD3. Over 90% of NK cells expressed CD56 and CD16.

Next we evaluated the effect of LAK or NK cell killing on EGFR expressing tumor cells pre-treated with Cetuximab (Cetu). As shown in Figure 2a, LAK cells killed A549, 11-18, and EBC-1 cell lines and pre-treatment with Cetu did not enhance the effect. In contrast, pre-treatment with Cetu did enhance the killing of A549, 11-18, and EBC-1 cell lines by NK cells (Fig. 2b). Together, these studies indicate that NK cells demonstrate enhanced killing of EGFR expressing cells pre-treated with Cetu. This effect was not demonstrated by LAK cells. To verify that EGFR expression is necessary for the enhanced NK cell killing in response to Cetu, MCF-7 or K562, both non-EGFR expressing cell lines, were pretreated with or without Cetu and co-cultured with NK cells at various effector to target ratios. Pre-treatment with Cetu did not enhance cytotoxic killing by NK cells in either cell line (Fig. 2c). Interestingly, we found that when we induced MHC class I expression on MCF-7 by incubation in low dose IFN-γ (not shown), NK cells demonstrated less cytotoxicity (Fig. 2a). As expected, these results confirm that NK cells demonstrate enhanced cytotoxicity against Cetu pre-treated cell lines, only if the cells express EGFR. In addition, these results indicate that NK cell-based immunotherapy may be more effective against MHC class I deficient cells.

Next, we evaluated the effect of zoldronate (Zol) on γδ T cells and compared phenotypic markers between cytotoxicity activated T cells (CAT) and γδ T cells. CAT are activated non-specific αβ TCR-expressing T cells. It has been shown that non-specific antigen or Zol can selectively increase γδ T cell numbers in cultured PBMC[24)25)]. There is no difference between these two culture methods (data not shown). As shown in Figure 3a, T cells in CAT cultures express TCR αβ chain and have very few γδ T cells, whereas PBMC cultured with Zol express the γδ TCR and very few αβ T cells are present[26)]. In Figure 3b, we measured the expression of surface markers and intracellular cytokine production between CAT and γδ T cells. There were no differences in expression patterns of the adhesion molecules CD11b, CD62L and CD31 between CAT and γδ T cells. There were also no differences in the expression of the chemokine receptors CXCR3, CCR4, CCR5 and CCR7. In contrast, expression of CXCR3 and CCR7 were significantly increased in CAT compared to γδ T cells. CAT also demonstrated higher expression of the TNF receptor (CD27), CD28, a costimulatory molecule, and the naïve T cell marker CD45RA. In comparison to CAT, γδ T cells had higher expression of the co-stimulatory molecule CD86 and the T cell activation marker, CD45RO. These results indicate that γδ T cells have an activated phenotype. CAT demonstrated activation but also contained naïve T cells.

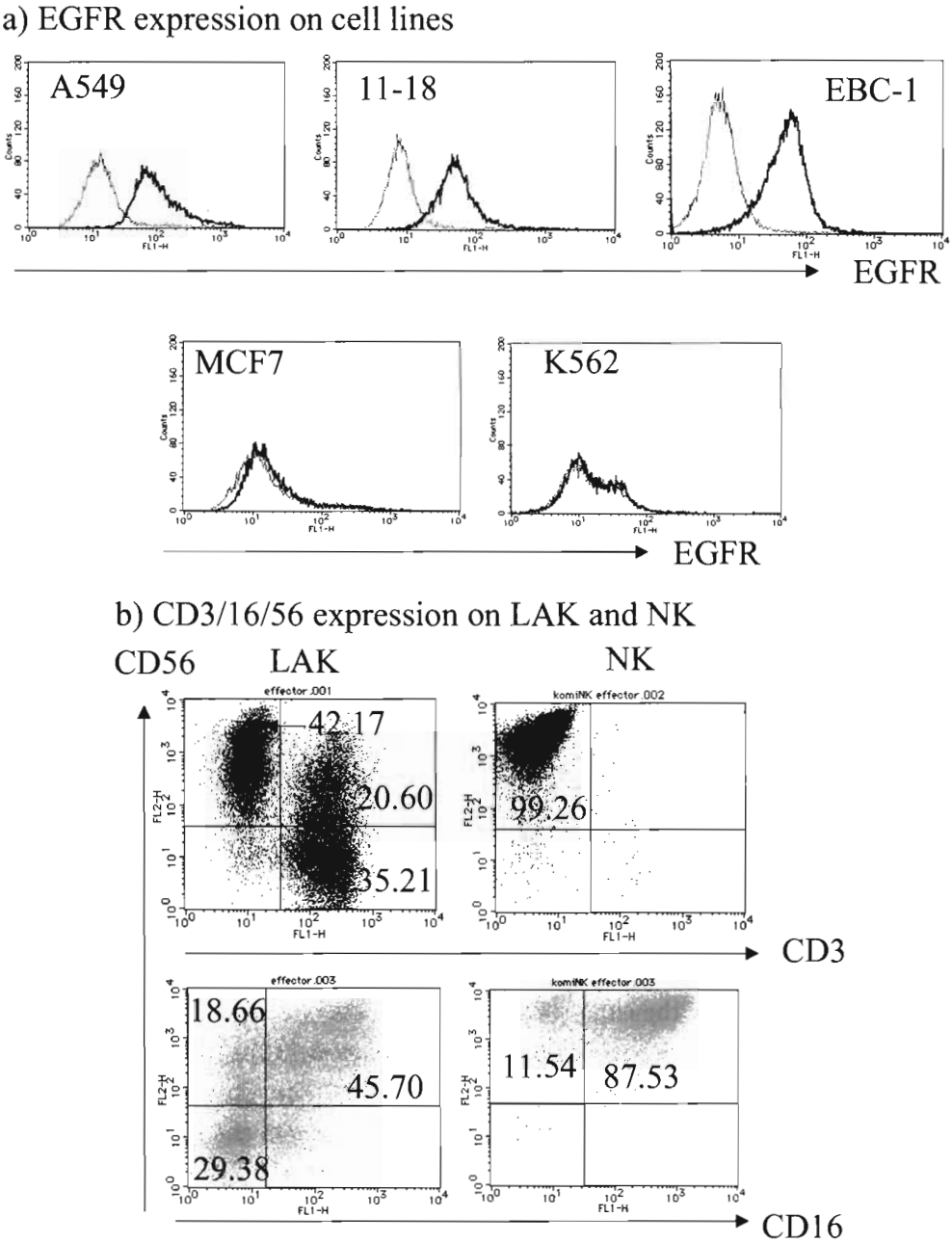


Fig. 1

a) EGFR expression on cell lines. A549, 11-18, EBC-1, MCF-7, and K562 cell lines were stained with mouse anti-human EGFR followed by FITC conjugated anti-mouse antibody (Dark line; EGFR, Light line; isotype control).

b) Phenotypic analysis of LAK and NK cells. PBMC or purified NK cells were cultured for 14 days and CD3, CD16 and CD56 expression was measured by flow cytometry.

Although CAT and γδ T cell are non-specific T cells, CAT looks more immature and may require additional activation by an antigen presenting cell, such as DC.

Next, we examined the effects of pre-treatment with Pamidronate (Pam) on tumor cells. LK87 cells were cultured alone or with Pam and proliferation was measured by a MTT assay at various time points. As shown in Figure 4a. at 50 μM, Pam treatment of LK87 led to a decrease in proliferation over time. Even at 10 μM Pam, cells experienced a decreased proliferation at 100 hours.

We next measured whether pre-treatment of tumor cells with Pam enhanced killing by γδ T cells.

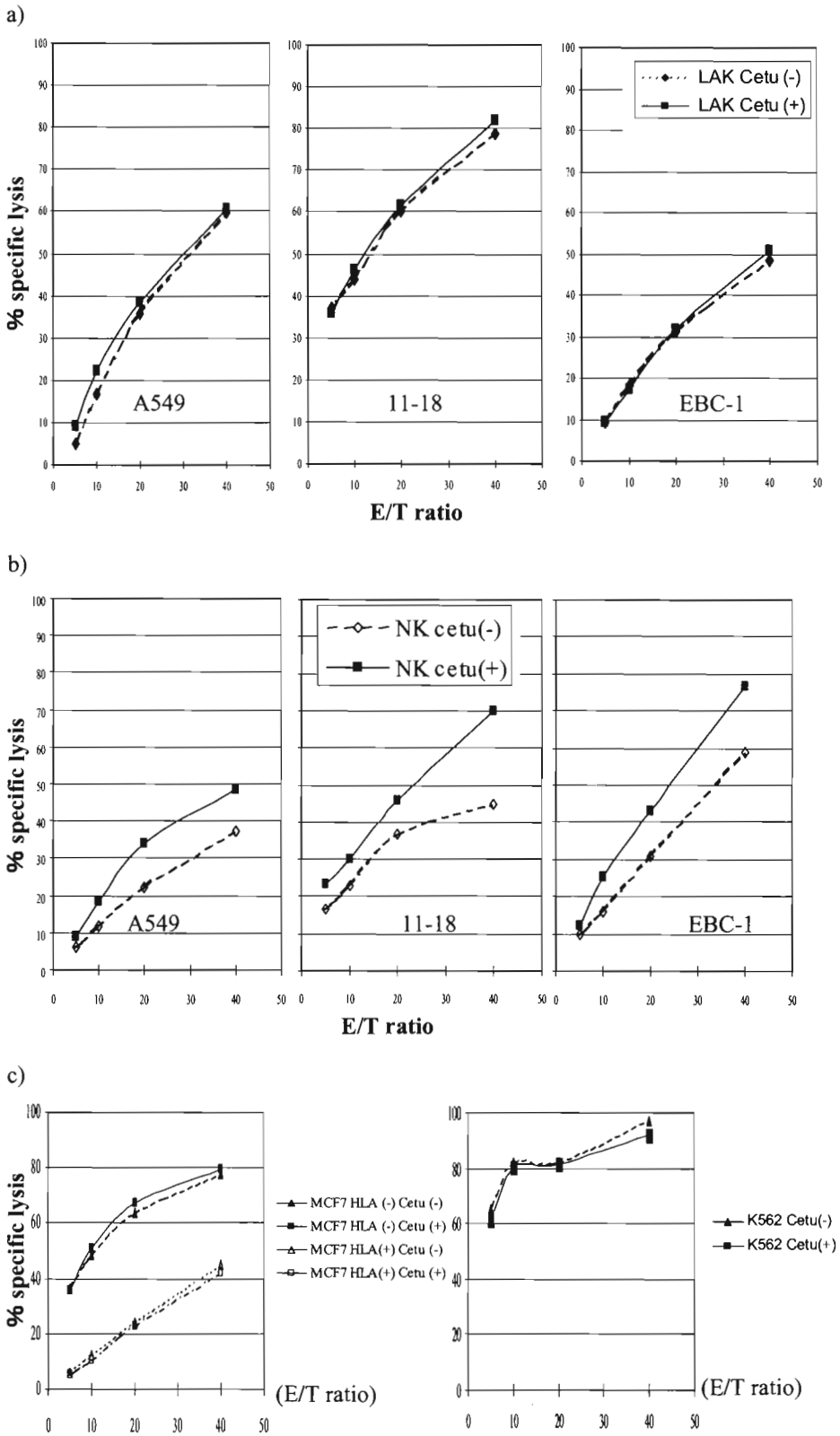


Fig. 2 Cell Cytotoxicity after pre-treatment with Cetuximab

Tumor cell targets were cultured with or without 50 μg/ml Cetuximab prior to culture with LAK or NK cells. Cytotoxicity was measured by a ^{51}Cr assay.

a) LAK cell killing of EGFR expressing tumor cells.

b) NK cell killing of EGFR expressing tumor cells.

c) NK cell killing of EGFR negative tumor cells. Percentage of specific lysis (Y-axis) at a given effector/target (E/T) ratio (X-axis) indicates the mean. These data show one of three independent experiments.

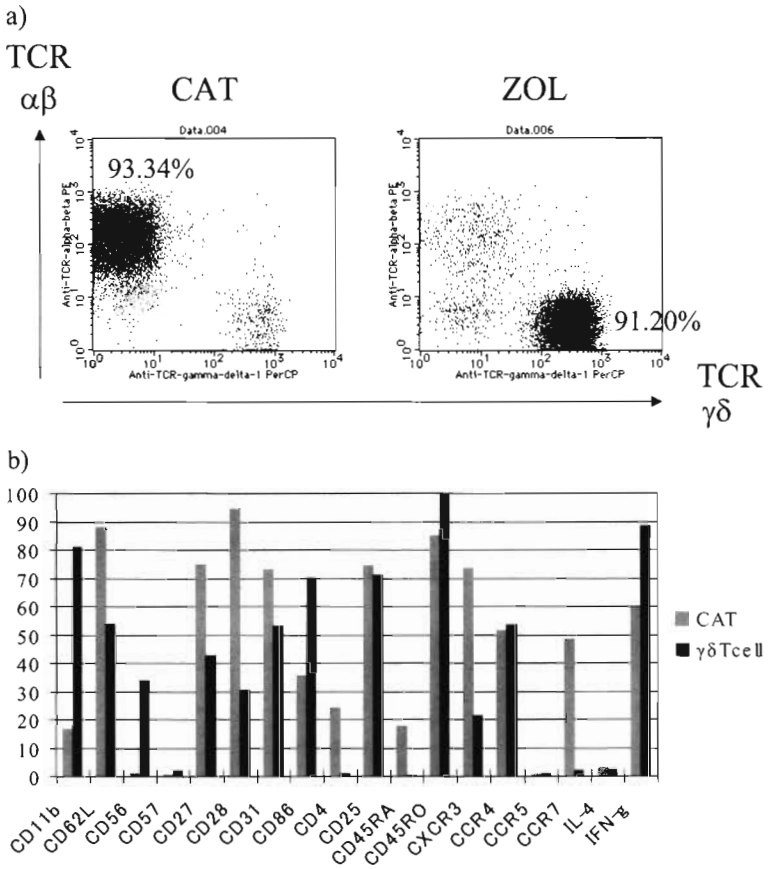


Fig. 3 Phenotypic analysis of CAT and γδ T cells

Cell surface molecule expression on PBMC cultured for 14 days. To generate CAT, PBMC were incubated with IL-2 and anti-CD3 antibody. To generate γδ T cells, PBMC were incubated with IL-2 and zoledronic acid.

a) TCR expression for confirmation of T cell populations.

b) Expression of cell surface markers on CAT and γδ T cells.

LK87 cells were pre-treated with various concentrations of Pam for 1-16 hours. To measure cytotoxicity, γδ T cells were added to pre-treated cells at an effector to target ratio of 20 : 1 and incubated for four hours.

Cytotoxicity was measured in a Cr^{51} release assay. Figure 4b shows that γδ T cells can effectively lyse LK87 cells pretreated for 10-16 hours with 50 μM Pam. In Figure 4c, EBC-1, A549, 11-18, and LK87 cells were treated with 50 μM of Pam for 12 hours prior to culture with γδ T cells. For all of the cell lines, pre-treatment with Pam enhanced cell lysis by γδ T cells.

Next, we examined the drug Zol as both a γδ T cell enrichment agent and a pre-treatment agent for tumor cell lines. While Pam was an effective agent to enhance γδ T cell killing of pre-treated cell lines, the required 50 μM concentration is too high for human use[17)28)] and would not be able to be translated into a clinical trial. To test Zol, we pre-treated A549 lung tumor cells with 10 μM of Zol. Cells were then cultured with γδ T cells at various effectors to target ratios for four hours. Figure 5a shows that γδ T cells had the most effective killing against A549 pre-treated with Zol for 12 hours. Next, we pre-treated A549 cells with various concentrations of Zol for 12 hours. As shown in Figure 5b, γδ T cell cytotoxity was enhanced when A549 cells were treated with 5, 10 or 20 μM of Zol. These results demonstrate that Zol is as effective as Pam for the pre-treatment of tumor cells to enhance γδ T cell cytotoxicity.

Discussion

In this study, we have shown two methods to en-

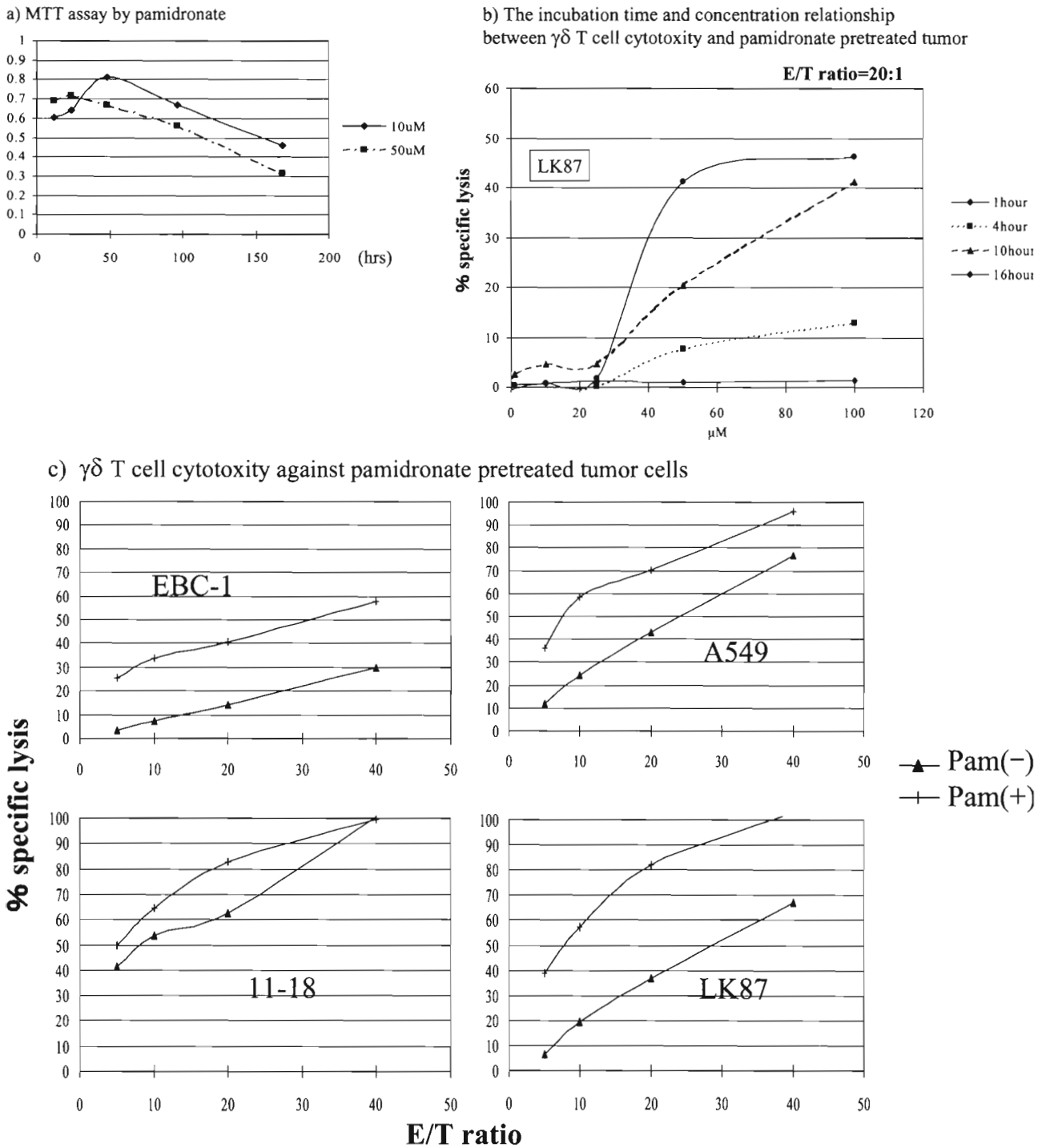


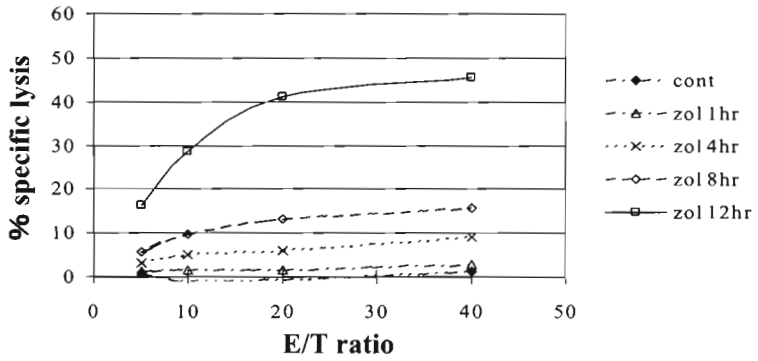
Fig. 4 Effect of pamidronate on LK87 cells

a) Pamidronate (Pam) was added to LK87 cells at 10 μM or 50 μM and cultured for various time points. Untreated cells were used as a control at each time point. Proliferation was measured by the MTT assay. Y-axis = pamidronate treated cell/untreated (Non-added) cell.

b) LK87 cells were pre-treated with Pam for various times and concentrations as indicated. Cells were co-cultured with γδ T cells at an E/T ratio of 20 : 1. Cytotoxicity was evaluated in a 4-hour ^{51}Cr assay.

c) Tumor cell lines were pre-treated with 50 μM of Pam for 12 hours and then incubated with γδ T cells. Cytotoxicity was measured in a 4-hour ^{51}Cr assay. Percentage of specific lysis (Y-axis) at a given effector/target (E/T) ratio (X-axis) indicates the mean. These data show one of three independent experiments.

a) cell cytotoxity and pretreatment time under zol 10uM.



b) cell cytotoxity and pretreatment concentration under 12 hrs pretreatment

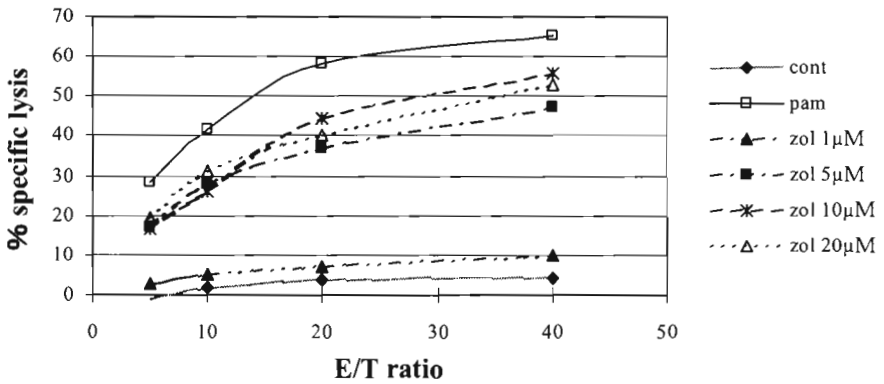


Fig. 5 Cell cytotoxicity induced by γδ T cells against cells pretreated with zoledronate (Zol)
The lung tumor cell line, A549, was pretreated with zoledronate and co-cultured with γδ T cells. Cytotoxity was measured in a 4-hour ^{51}Cr assay.
a) A549 cells were treated for various times with 10 μM Zol.
b) A549 cells were pretreated with various concentrations of Zol.

hance immunotherapies that target innate effectors. First, we demonstrated that NK cells could synergize with Cetuximab to enhance cytotoxicity against EGFR expressing cancer cells. NK cell therapy is known as a non-specific immunotherapy. When NK cell recognizes and kills allogeneic cells or autologous cells expressing foreign antigens, this mechanism involves MHC class I and II and the HLA Cw locus. While tumor cells should express the Cw locus, many tumors become deficient to escape the immune response or to become more malignant. NK cells can efficiently lyse tumors lacking Cw expression. Cetu is a chimeric antibody combining a human FC chain with a murine Fab fragment that recognizes human EGFR[10]. We demonstrated that combination therapy with Cetu enhanced NK cell mediated ADCC against tumor cells. It may be possible to translate this therapy to patients with EGFR-expressing tumors. No severe side effects have been reported with either Cetu or NK therapy. One limitation for clinical use is how to expand NK cells from patients. The protocol we used isolated NK cells and the cells had great responses against tumor cells. But this protocol needed a lot of blood for treatment. A new protocol for isolating NK cells selectively and easily may be needed.

Our data indicates that NK cells respond to cancer cell lines pre-treated with Cetu in a MHC independent manner. We are not sure the reason why NK cells were effective in our model but LAK were not. It may be that NK cells do not need specific antigen whereas LAK may require an antigen to respond. In addition, NK cells cultured in vitro may be capable of enhanced cytotoxicity against target cell lines. In our trial, Cetu bound EGFR expressing tumor cells and NK cells could attack these antibody-

coated tumor cells efficiently.

Second, we have shown that bisphosphonate treatment of tumors can enhance cell cytotoxicity mediated by γδ T cells. γδ T cells are innate immune effectors that respond to bacteria and other infectious agents[27)29)]. Our studies show that the cytotoxicity induced by γδ T cells results in delayed tumor growth but is not enough to eliminate tumor completely. We have also shown that γδ T cell activity is enhanced when tumor cells are pre-treated with bisphosphonates. Pam is a second generation bisphosphonate that is effective to prevent bone loss and osteoporosis[30)]. Side effects of Pam treatment include tetany because of low calcium level in the blood[31)32)]. In contrast, a third generation bisphosphonate, Zol is also effective for the prevention of bone loss and osteoporosis but side effects do not include a dangerous drop in calcium levels. Zol does induce a slight decrease in calcium levels which recovers rapidly allowing Zol to be used as a repeated therapy. Moreover, Zol is used as a treatment and prevention drug for cancer bone metastasis in Japanese patients. Zol has an additional benefit of increasing γδ T cells whereas Pam does not have this effect (data not shown). The Zol concentration for treatment is much lower and has been shown to be safe for patients, making Zol a more desirable drug for clinical trials. Our data shows that treatment of tumor cells with low dose Zol was as effective as Pam. In the clinic, we must check the efficiency and quality of life for patients, although they may choose the benefits of immunotherapy and other therapies like chemotherapy. While we didn't research all types of tumors, treatment with Zol may be better for other tumor cell types[33)]. Treatment by Zol has been used in patients and has demonstrated safety. Zol can be combined with other therapies, allowing patients to save time and money. In conclusion, we consider Zol better than Pam for pretreatment in a clinical setting.

Little is known about the activation of γδ T cells in response to immunotherapy[34)35)]. If we can control and regulate the enhanced immune response from innate cells, γδ T cell will be an established treatment in the future[36)]. Combination immunotherapies that activate innate immune effectors, such as γδ T cells, and enhance adaptive immunity, such as DC based vaccines, may lead to clinical responses in lung cancer patients.

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肺癌細胞株に対する養子培養免疫細胞療法の検討

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肺癌は高致死率の癌の一つである．肺癌は増殖の途中，たびたび変異を起こし化学療法などの治療や体内の免疫応答から回避し増殖する．肺癌細胞はたびたび抗腫瘍免疫応答を回避し免疫寛容に導くためにMHC class I, IIの発現を欠損させる．我々はMHC(主要組織適合遺伝子複合体)の制約を受けない生来の免疫応答細胞であるNK細胞やγδT細胞によるがん細胞に対する抗腫瘍効果を検討した．はじめにNK細胞とEGFR抗体であるセツキシマブとの併用療法を考案し肺癌細胞株に対する抗腫瘍効果を検討した．LAK細胞（リンフォカイン活性化キラー細胞)とセツキシマブの併用ではセツキシマブによる抗腫瘍効果の増強は認められなかったが，NK細胞との併用によって10～25%の抗腫瘍効果の増強が認められた．また，EGFR非発現細胞株に対する抗腫瘍効果は認められなかった．次にγδT細胞と高カルシウム血症治療薬の併用による抗腫瘍効果について検討した．まず，第2世代の高カルシウム血症治療薬であるパミドロネートによる肺癌細胞株に対しての検討を行った．パミドロネートによる腫瘍細胞株の前処置後，γδT細胞による細胞傷害活性を検討した．パミドロネートによる前処置は時間，濃度依存性であり前処置時間は16時間また，濃度は50 μM以上必要であった．しかし，前処置された細胞はγδT細胞による抗腫瘍効果が15%以上上乗せされ，効果的であった．次に，第3世代高カルシウム血症治療薬であるゾレドロネートによる効果を検討した．ゾレドロネートも時間依存性であったが，前処置濃度は5 μM以上で効果は一定であった．臨床的にはパミドロネートによりゾレドロネートによる前処置が現実的であり，将来的にはゾレドロネートによるγδT細胞療法が理想的であると考えられた．養子培養による活性化NK細胞療法およびγδT細胞による免疫細胞療法は抗腫瘍効果が期待でき，化学療法などとの併用も効果が期待できることが示唆された．