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Myeloid molecular characteristics of human $\gamma\delta$ T cells support their acquisition of tumor antigen-presenting capacity

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Abstract

Human T cells expressing $\gamma\delta$ T cell receptor have a potential to show antigen presenting cell-like phenotype and function upon their activation. However, the mechanisms that underlie the alterations in human $\gamma\delta$ T cells remain largely unclear. In this study, we have investigated the molecular characteristics of human $\gamma\delta$ T cells related to their acquisition of antigen presenting capacity in comparing with activated $\alpha\beta$ T cells. We found that activated $\gamma\delta$ but not $\alpha\beta$ T cells upregulated cell surface expression of a scavenger receptor, CD36, which seemed to be mediated by signaling through mitogen activated protein kinase (MAPK) and/or NF- κ B pathways. Confocal microscopical analysis revealed that activated $\gamma\delta$ T cells can phagocytose protein antigens. Activated $\gamma\delta$ T cells could induce tumor antigen-specific CD8⁺ T cells using both apoptotic and live tumor cells as antigen resources. Furthermore, we detected that C/EBP α , a critical transcription factor for the development of myeloid-lineage cells, is expressed much higher in $\gamma\delta$ T cells than in $\alpha\beta$ T cells. These results unveiled the molecular mechanisms for the elicitation of antigen presenting functions in $\gamma\delta$ T cells and would also help designing new approaches for $\gamma\delta$ T cell-mediated human cancer immunotherapy.

Key words: $\gamma\delta$ T cells, zoledronate, antigen presenting capacity, CD36, C/EBP α , myeloid

Precis: Activated human $\gamma\delta$ T cells express myeloid cell characteristics and can function as antigen presenting cells.

Abbreviations

APC: antigen-presenting cell

C/EBP α : CCAAT/enhancer-binding protein α

IPP: isopentenyl pyrophosphate

MAPK: mitogen activated protein kinase

OVA: ovalbumin

PBMCs: peripheral blood mononuclear cells

Introduction

In human, T cells expressing $\gamma\delta$ TCR comprise approximately 1-10% of peripheral blood mononuclear cells (PBMCs). The majority of $\gamma\delta$ T cells in peripheral blood express the V δ 2 chain in combination with V γ 9 [1]. V γ 9V δ 2⁺ T cells have a unique reactivity towards phosphoantigens (such as isopentenyl pyrophosphate: IPP), which are non-peptide antigens most commonly associated with metabolites of bacterial isoprenoid biosynthesis or the mevalonate pathway [2]. Activated V γ 9V δ 2⁺ T cells show strong cytotoxicity against stressed cells such as cancer cells, and thus serve as potent candidates for cancer immunotherapy [3-7]. It has been also known that one of bisphosphonates, Zoledronate, can stimulate and activate $\gamma\delta$ T cells [8-10]. We have previously established an efficient large-scale ex vivo expansion method of $\gamma\delta$ T cells using Zoledronate [11]. Zoledronate is used to prevent skeletal fractures in patients with malignancies such as multiple myeloma or prostate cancer. It can be also used to treat hypercalcemia associated with malignant diseases and can be helpful for decreasing pain from bone metastases.

A recent report has indicated that $\gamma\delta$ T cells show professional antigen-presentation function upon activation [12]. IPP- or Zoledronate-activated $\gamma\delta$ T cells possess functional

properties of phagocytosis [13-15] and cross-presentation of an antigen [16, 17]. However, antigen-presenting function by Zoledronate-expanded $\gamma\delta$ T cells remain unclear. Moreover, molecular mechanisms for the induction of antigen-presenting function in activated $\gamma\delta$ T cells are not completely understood.

In addition to $\gamma\delta$ T cells, $\alpha\beta$ T cells also show some antigen-presenting cell (APC) phenotype and function upon activation [18, 19]. However, the differences among their acquisition of APC phenotype and function have not been clearly shown. In this study, we have investigated the differences, and found that $\gamma\delta$ T cells have several particular molecular features to obtain APC phenotype and functions. For example, we found that activated human $\gamma\delta$ T cells express CD36, a scavenger receptor, whose expression was upregulated via MAPK and/or NF- κ B signal pathways. Furthermore, we also found that CCAAT/enhancer-binding protein α (C/EBP α), a critical transcription factor for the development of myeloid-lineage cells, is expressed much higher in $\gamma\delta$ T cells than in $\alpha\beta$ T cells. These results may unveil the molecular mechanisms for the elicitation of APC functions in $\gamma\delta$ T cells, and would also help designing new approaches for Zoledronate-based human cancer immunotherapy.

Materials and Methods

Reagents

Zoledronate acid was purchased from Novartis Pharmaceuticals (Basel, Switzerland). HLA-A*0201-restricted, modified MRAT-1 (A27L) 10 mer synthetic peptides (ELAGIGILTV) and A27L tetramer were obtained from Operon (Tokyo, Japan) and MBL (Nagoya, Japan), respectively. MART-1 recombinant protein was obtained from Abnova Corporation (Taipei, Taiwan). MART-1-positive tumor cell line (JCOCB) was kindly provided as a gift by Dr. Chris Schmidt at the Queensland Institute of Medical Research (Brisbane, Australia). This cell line was originally established from fresh surgical specimens. IPP and betulinic acid were purchased from Sigma-Aldrich (St. Louis, MO).

Cell preparation

To separate PBMCs, whole bloods from healthy volunteers were centrifuged on Lymphocyte Separation Medium (MP Biomedicals) at 400g for 30 minutes. Intermediate mononuclear fraction was collected and washed with PBS. After separation, PBMCs were cultured in ALyS203 (Cell Science & Technology Institute, Inc., Sendai, Japan) supplemented with 10%

AB serum or 10% heat-inactivate autologous plasma, and human recombinant IL-2 (Chiron Benelux B.V., The Netherlands). Cell stimulation was done with 5 μ M Zoledronate and 1000 IU/ml IL-2 for $\gamma\delta$ T cells and 10 μ g/ml plate-immobilized anti-CD3 antibody (BD Biosciences: San Jose, CA, USA) and 175 IU/ml IL-2 for $\alpha\beta$ T cells, respectively. Fresh media including IL-2 were added to the culture to keep the cell concentration ($0.5-2 \times 10^6$ cells/ml). In some experiments, $\gamma\delta$ T cells were stimulated with 50 μ M IPP in the presence of 50 IU/ml of IL-2, and betulinic acid (50 μ M), an inhibitor of C/EBP α [20], was added to the culture. Written informed consent was obtained from the volunteers and the study was approved by the Ethical Committee of our institution.

Flow cytometry

The following monoclonal antibodies (mAbs) were used for cell surface staining and purchased from Beckman Coulter (Indianapolis, IN), BD Biosciences (San Jose, CA), or BioLegend (San Diego, CA): anti-TCRV γ 9-FITC, anti-CD3-PC5, anti-CD8-FITC, anti-CD11a-PE, anti-CD11b-PE, anti-CD11c-PE, anti-CD80-PE, anti-CD86-PE, anti-HLA-DR-PE, anti-CD54-PE, anti-CD206-PE, and anti-CD36-PE. The cell surface phenotype of $\gamma\delta$ or $\alpha\beta$ T cells was detected by flow cytometry using the FC500 (Beckman

Coulter), and data were analyzed using CXP software (Beckman Coulter).

Antigen uptake

Activated $\gamma\delta$ or $\alpha\beta$ T cells (day 5-8 of culture) were pulsed for 120 minutes or overnight with 50 $\mu\text{g/ml}$ of Alexa Fluor 555-labeled ovalbumin (OVA) (Molecular Probes). After incubation, cells were stained with anti-TCRV γ 9 or anti-HLA ABC mAbs followed by Alexa Fluor 488-labeled secondary antibody (Invitrogen). Thereafter, the fluorescence intensity of the cells was analyzed by flow cytometry and confocal microscopy for uptake of labeled antigen.

Antigen Presentation Assay

MART-1 protein- or MART-1-positive tumor cell line (JCOCB)-pulsed $\alpha\beta$ or $\gamma\delta$ T cells were used as APCs. Apoptosis was induced in JCOCB by irradiation (50 Gy). Apoptotic or untreated live tumor cells and $\alpha\beta$ or $\gamma\delta$ T cells (day 14 of culture) were co-cultured for 48 or 96 hours, and then Pan-T cells and $\gamma\delta$ T cells were negatively selected, respectively. The APCs were irradiated (20 Gy) before starting co-culture with CD3⁺ T cells (responder cells) from peripheral blood of HLA-A*0201-positive healthy volunteer. IL-2 (50 IU/mL) was added to the cultures every 2-3 days. After 1 or 2 weeks of culture, cells were stained with

A27L tetramer.

Killing assay

The Annexin V/PI flow cytometric assay (BD Biosciences) was used to determine the extent of activated $\alpha\beta$ or $\gamma\delta$ T cell (day 14 of culture) cytotoxicity towards MART-1-positive tumor cell line. Following PKH26 dye (Sigma-Aldrich) staining of target cells to allow distinction on the flow cytometer, effector cells and target cells were co-cultured at various E/T ratios for 4 hours at 37 °C. All cells were then harvested and stained with Annexin V and PI according to the manufacturer's instructions. Early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+), and necrotic (Annexin V-/PI+) cells were distinguished from viable cells (Annexin V-/PI-) in PKH26 positive target cells. Cytotoxicity was determined as the percentage of Annexin V and/or PI positive events in PKH26 positive target cells after subtracting values from appropriate control wells containing targets only.

Real-time PCR analysis

Expression levels of C/EBP α , Pu.1, HES1, MFG-E8, Tim-4, and GATA3 in cDNA samples taken from sorted $\alpha\beta$ and $\gamma\delta$ T cells were measured by real-time PCR. Primer pairs for

real-time PCR were purchased from QIAGEN (Germantown, MD). The data were normalized by hypoxanthine-guanine phosphoribosyltransferase (HPRT) and relative expression levels were calculated using Comparative-Ct Method.

Immunoblotting

Antibodies to Phospho-Erk1/2, Erk1/2, Phospho-p38, p38 were purchased from Cell Signaling Technology (Tokyo, Japan). Anti-Grb2 antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). Expanded $\gamma\delta$ T cells (day 8 of culture) were washed and rested on ice for 1 hour, and then stimulated with 50 μ M IPP at 37 °C. After stimulation, cells were lysed with 1 x Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 2 mM Na₃VO₄, and 10 μ g/ml each of PMSF and Leupeptin). Samples were subjected 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membrane (Millipore). Membranes were probed with the indicated primary antibodies, followed by HRP-conjugated secondary antibodies. Membranes were then washed and visualized with the enhanced chemiluminescence detection system (Amersham) and LAS-3000 imaging system. When necessary, membranes were stripped by incubation in stripping buffer (Thermo Scientific) for 15 min with constant agitation, washed,

and then reprobbed with various other antibodies.

Statistical Analyses

P values were calculated using the Student's t-test and considered significant at a P value < 0.05.

Results

Zoledronate-mediated $\gamma\delta$ T cell expansion and molecular expressions

We propagated $\gamma\delta$ T cells using Zoledronate and IL-2 from PBMCs of human healthy volunteers as previously reported [11]. The number of $V\gamma 9^+$ T cells became 237 ± 68 -fold at day 7 and 4317 ± 2565 -fold at day 14 compared to day 0 (data not shown). We examined the cell surface expression of MHC class II and CD80/86 in activated $\gamma\delta$ and $\alpha\beta$ T cells. Those molecules were not detected in both $\gamma\delta$ and $\alpha\beta$ T cells before the culture, whereas significantly upregulated after the activation (Fig. 1A). The expression levels of MHC class II and CD86 were higher in $\gamma\delta$ T cells than $\alpha\beta$ T cells. In contrast, the expression level of CD80 was similar or higher in $\alpha\beta$ T cells after activation. Additionally, the expression levels of adhesion molecules such as CD54, CD11a, CD11b and CD11c were comparable in resting $\gamma\delta$ and $\alpha\beta$ T cells (Fig. 1B). Following activation, the expression levels of these molecules were detected at higher levels in $\gamma\delta$ T cells, except for CD11c which was higher in $\alpha\beta$ T cells (Fig. 1B). Together, these results suggest that both $\gamma\delta$ T and $\alpha\beta$ T cells acquire characteristics of antigen presenting cells following activation.

Uptake of protein antigen

We then examined the potential of Zoledronate-activated $\gamma\delta$ T cells to uptake protein antigen by using ovalbumin (OVA) as a model. The activated $\gamma\delta$ T cells and OVA-Alexa555 were co-incubated on ice or at 37 °C for 2 hours, and uptake of protein antigen was examined by flow cytometer. Comparing to $\gamma\delta$ T cells incubated on ice, $\gamma\delta$ T cells incubated at 37 °C showed increased fluorescence which reflects increased uptake of protein antigen (Fig. 2A). Similar uptake of protein antigen was not observed in $\alpha\beta$ T cells (not shown). To confirm whether the increased fluorescence was actual intracellular uptake but not cell surface attachment, we examined the cells with confocal microscopy. As shown in Fig. 2B, protein antigens were located in the cytoplasm of activated $\gamma\delta$ but not $\alpha\beta$ T cells. Thus, these results suggest that activated $\gamma\delta$ but not $\alpha\beta$ T cells have the capacity to uptake protein antigens.

Antigen presenting function of $\gamma\delta$ and $\alpha\beta$ T cells using A27L peptide

Next, we examined antigen presenting function of $\gamma\delta$ and $\alpha\beta$ T cells. To do so, we evaluated the ability of $\gamma\delta$ or $\alpha\beta$ T cells to induce MART-1-specific CD8⁺ T cells when stimulated with MART-1 protein (Fig. 3A), or apoptotic or live MART-1-positive tumor cells (Fig. 3B). When MART-1 protein was used as an antigen resource, the induction of antigen-specific T cells

could not be observed (Fig. 3A). On the other hand, the fraction of MART-1-specific CD8⁺ T cells was significantly increased when $\gamma\delta$ or $\alpha\beta$ T cells were stimulated with apoptotic tumor cells (Fig. 3B). Interestingly, when live tumor cells were used as the source of antigen, only activated $\gamma\delta$ T cells could support the induction of antigen-specific T cell proliferation (Fig. 3B). Furthermore, $\gamma\delta$ T cells showed higher cytotoxic activities against tumor cells compared to $\alpha\beta$ T cells (Fig. 3C). Together, these results suggest that activated $\gamma\delta$ T cells have the ability to present tumor cell-derived antigens.

Comparison of scavenger/phagocytosis-related molecules expression between $\gamma\delta$ and $\alpha\beta$ T cells

We next investigated expression of scavenger/phagocytosis-related molecules in both $\gamma\delta$ and $\alpha\beta$ T cells. $\gamma\delta$ T cells showed expression of the scavenger receptor CD36, even when they are in a naïve state, and CD36 expression was upregulated following activation (Fig. 4A). On the other hand, CD36 was barely expressed in $\alpha\beta$ T cells, and was not upregulated after activation. We also examined the expression of the mannose receptor CD206, and apoptosis-related MFG-E8 or Tim4, but did not detect any significant difference between $\gamma\delta$ and $\alpha\beta$ T cells (Fig. 4B). CD36 has been reported to be involved in the uptake of apoptotic

cells in immature dendritic cells (DCs) and macrophages [21, 22]. Therefore, these results suggest that $\gamma\delta$ T cells possess a potential to take apoptotic cells up into the cells.

Signaling pathway responsible for the expression of antigen presenting molecules

Next we aimed to identify which signaling pathway is responsible for the expression of antigen presentation-related molecules in activated $\gamma\delta$ T cells. So far, it has been reported that MAPK or NF- κ B pathway is involved in MHC class II or costimulatory molecule expression in DCs [23, 24]. Therefore, we employed several inhibitors for signaling pathways to identify their contribution to the antigen presentation in activated $\gamma\delta$ T cells. In this experiment, we activated $\gamma\delta$ T cells with IPP, as Zoledronate stimulation requires APCs, and in this situation the inhibitors might influence the APCs as well. We show representative histogram of antigen presentation-related molecules 72 hours after activation (Fig. 5A) and means \pm SD (n = 3-4) of MFI (Fig. 5B). The expression of MHC class II, CD54, and CD80 were not significantly changed with the addition of inhibitors. On the other hand, expressions of MHC class I, CD86 and CD36 were significantly downregulated with addition of inhibitors of MAPK and NF- κ B (Fig. 5A and B). We next examined whether extracellular signal-regulated kinase (ERK) 1/2 and p38 were phosphorylated by activation with IPP. Activation with not only

PMA/ionomycin but also IPP induced phosphorylation of both ERK1/2 and p38 (Fig. 5C).

Taken together, upregulation of antigen presentation-related molecules in activated $\gamma\delta$ T cells seems to be regulated by signaling pathway through MAPK and NF- κ B. Particularly, CD36 expression seems to be highly regulated by these pathways.

Comparison of myeloid-related transcription factors between $\gamma\delta$ and $\alpha\beta$ T cells

Lastly, we investigated the expression levels of myeloid-related transcription factors such as PU.1, C/EBP α , HES1 and GATA3 in $\gamma\delta$ and $\alpha\beta$ T cells. As a result, we found that resting $\gamma\delta$ T cells expressed CCAAT/enhancer-binding protein α (C/EBP α) more than $\alpha\beta$ T cells, while the expressions of other transcription factors were comparable between $\gamma\delta$ and $\alpha\beta$ T cells (Fig. 6A). After activation, the expression of C/EBP α was downregulated to the level observed in $\alpha\beta$ T cells (data not shown). To examine the impact of C/EBP α expression in $\gamma\delta$ T cell function, we employed an inhibitor for C/EBP α , betulinic acid [20]. In the presence of this inhibitor, we activated both $\gamma\delta$ and $\alpha\beta$ T cells, and observed expression change of antigen presentation-related molecules. We found that the inhibition of C/EBP α blocked the upregulation of CD36 and MHC class II more preferentially in $\gamma\delta$ T cells than in $\alpha\beta$ T cells (Fig. 6B). In Exp. 2, superior suppression of CD86 expression in $\gamma\delta$ T cells was also observed.

However, it barely altered the expression level of CD54 and MHC class I in both $\gamma\delta$ and $\alpha\beta$ T cells (Fig. 6B). Therefore, these results suggested that the high expression of C/EBP α in $\gamma\delta$ T cells might support their acquisition of APC function at least through upregulation of CD36 and MHC class II.

Discussion

It has been known that T cells, including both $\alpha\beta$ and $\gamma\delta$ T cells, have a potential of antigen-presentation to a certain extent [12-19]. However, it has not been clarified whether there are differences in antigen-presentation between $\alpha\beta$ and $\gamma\delta$ T cells and the related molecular mechanisms. In this study, we compared the expression levels of antigen presentation-related molecules, antigen uptake and cross presentation, in addition to the molecular mechanisms that underlie the differences between activated $\gamma\delta$ and $\alpha\beta$ T cells. Regarding expression of antigen-presenting molecules, MHC class II expression was higher in $\gamma\delta$ T cells than in $\alpha\beta$ T cells (Fig. 1). Furthermore, we found that human $\gamma\delta$ T cells expressed the scavenger receptor CD36 (Fig. 4), as previously indicated that bovine $\gamma\delta$ T cells express CD36 [25]. CD36 is known to be involved in the uptake of apoptotic cells in immature DCs and macrophages [21, 22]. Activated $\gamma\delta$ T cells showed cytotoxic activities against tumor cells and potentials to induce antigen-specific T cells when co-cultured with live tumor cells (Fig. 3). Therefore, it is conceivable that $\gamma\delta$ T cells kill live tumor cells, followed by uptake of their debris through CD36, process tumor cells-derived antigens, and then exert the APC functions to induce tumor antigen- specific CD8⁺ T cell response.

We also examined which signaling pathways are involved in upregulating CD36 expression of upon $\gamma\delta$ T cells activation, and found that CD36 upregulation was mediated by MAPK and NF- κ B pathways (Fig. 5). A previous study has suggested that the phosphorylation of C/EBP α is regulated by p38 or ERK1/2 signaling pathway [26]. Consistently, C/EBP α is involved in the regulation of CCAAT box, an enhancer region that locates within the regulatory element of CD36 gene [27]. Taken together, it is highly suggested that the high expression of C/EBP α is important for the induction of APC function in activated $\gamma\delta$ T cells.

In this study, we showed that $\gamma\delta$ T cells possess a capacity to take up not only peptide but also protein antigen (Fig. 2). Furthermore, $\gamma\delta$ T cells possess strong cytotoxicity and ability to uptake cell debris, followed by antigen presentation, as described previously [28]. Therefore, it seems that $\gamma\delta$ T cells are able to process wide range of antigens and mediate the antigen presentation. In $\alpha\beta$ T cells, it has been reported that their antigen-presenting capacity was mediated via trogocytosis or exosomes [18, 19]. Thus, it is of great interest to examine whether $\gamma\delta$ T cells use similar mechanisms to exert the antigen presenting capacity.

As suggested by our results, C/EBP α expression in naïve $\gamma\delta$ T cells is important for the acquisition of antigen presenting capacity (Fig. 6). First, a signal is induced by an antigen

through $V\gamma 9V\delta 2^+$ TCR, which activates MAPK cascade including p38 and ERK1/2 resulting in the phosphorylation of C/EBP α . Following phosphorylation, C/EBP α upregulates CD36 expression which helps the uptake of cell-derived antigens. Activated $\gamma\delta$ T cells process the cell debris to generate antigen, and then cross-present the antigen to CD8 $^+$ T cells. The clarification of these mechanisms of antigen presentation by $\gamma\delta$ T cells would help to understand the anti-cancer immune responses and design new strategies to improve the therapeutic effects of $\gamma\delta$ T cells-based cancer immunotherapy.

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Disclosure of Conflicts of Interest

The authors declare no competing financial interest.

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Figure legends

Figure 1. Expansion of $\gamma\delta$ T cells and their expression of antigen presentation-related molecules

PBMCs were cultured with Zoledronate for expanding $\gamma\delta$ T cells or with plate-immobilized anti-CD3 antibody for $\alpha\beta$ T cells, respectively. (A) Left, representative histogram data indicating expression of MHC Class II, CD80, and CD86 in $\alpha\beta$ and $\gamma\delta$ T cells at the indicated time points (shaded histograms). Open histograms, isotype control. Right, time course of the expression of MHC Class II, CD80, and CD86 on $\gamma\delta$ T cells (closed circle) and $\alpha\beta$ T cells (open circle) were shown (mean \pm SD; n = 5-9). (B) Time courses of the expression of adhesion molecules (CD54, CD11a, CD11b, and CD11c) on $\gamma\delta$ T cells (closed circle) and $\alpha\beta$ T cells (open circle) were shown (mean \pm SD; n = 5-9).

Figure 2. Activated $\gamma\delta$ T cells, but not $\alpha\beta$ T cells, took the protein antigen up into the cells

Activated $\gamma\delta$ or $\alpha\beta$ T cells were co-incubated with ovalbumin (OVA) as a protein antigen.

(A) Activated $\gamma\delta$ T cells (day 5 of culture) and OVA-Alexa555 were co-incubated on ice (solid

lines) or at 37 °C (gray shade) for 2 hours, and the cells were examined with flow cytometer.

(B) Activated $\gamma\delta$ or $\alpha\beta$ T cells (day 8 of culture) were co-incubated for overnight with OVA-Alexa555 (red). After incubation, $\gamma\delta$ or $\alpha\beta$ T cells were stained with anti-TCRV γ 9 or anti-HLA ABC mAbs, respectively, followed by Alexa Fluor 488-labeled secondary antibody (green), and analyzed by confocal microscopy. Representative data from three independent experiments.

Figure 3. Activated $\gamma\delta$ T cells, but not $\alpha\beta$ T cells, induce antigen-specific T cells when co-cultured with live tumor cells as a source of antigen

Antigen pulsed activated $\alpha\beta$ or $\gamma\delta$ T cells were used as APCs. APCs were irradiated before starting co-culture with CD3⁺ T cells (responder cells). (A) MART-1 protein, (B) apoptotic or live MART-1 positive tumor cells were used as antigen resources. Apoptosis was induced in tumor cells by irradiation. After 1 or 2 weeks of co-culture, cells were stained with A27L tetramer. Representative data from three independent experiments. (C) Cytotoxic activities of $\alpha\beta$ and $\gamma\delta$ T cells against MART-1 positive tumor cells. Data are presented as percentage of activity (mean \pm SD; n = 3).

Figure 4. CD36, a scavenger receptor is expressed in naïve or activated $\gamma\delta$ T cells, but not $\alpha\beta$ T cells

PBMCs were cultured with Zoledronate for $\gamma\delta$ T cells or with plate-immobilized anti-CD3 antibody for $\alpha\beta$ cells, respectively. (A) Left, representative flow cytometry data indicating expression of CD36 in $\alpha\beta$ and $\gamma\delta$ T cells at the indicated time points. Right, time courses of the expression of CD36 on $\gamma\delta$ T cells (closed circle) and $\alpha\beta$ T cells (open circle) (mean \pm SD; n = 5-9). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. (B) Left, time courses of the expression of CD206 on $\gamma\delta$ T cells (closed circle) and $\alpha\beta$ T cells (open circle) (mean \pm SD; n = 5-9). Right, expression levels of MFG-E8 and Tim4 in cDNA samples taken from sorted $\alpha\beta$ and $\gamma\delta$ T cells were measured by real-time PCR. The data were normalized by HPRT and relative expression levels were calculated using Comparative-Ct Method. Means of two samples are shown.

Figure 5. Signaling pathway responsible for the expression of antigen presentation-related molecules in activated $\gamma\delta$ T cells

$\gamma\delta$ T cells were activated with IPP. (A) Representative histogram of antigen presentation-related molecules 72 hours after activation with IPP alone (control) or the

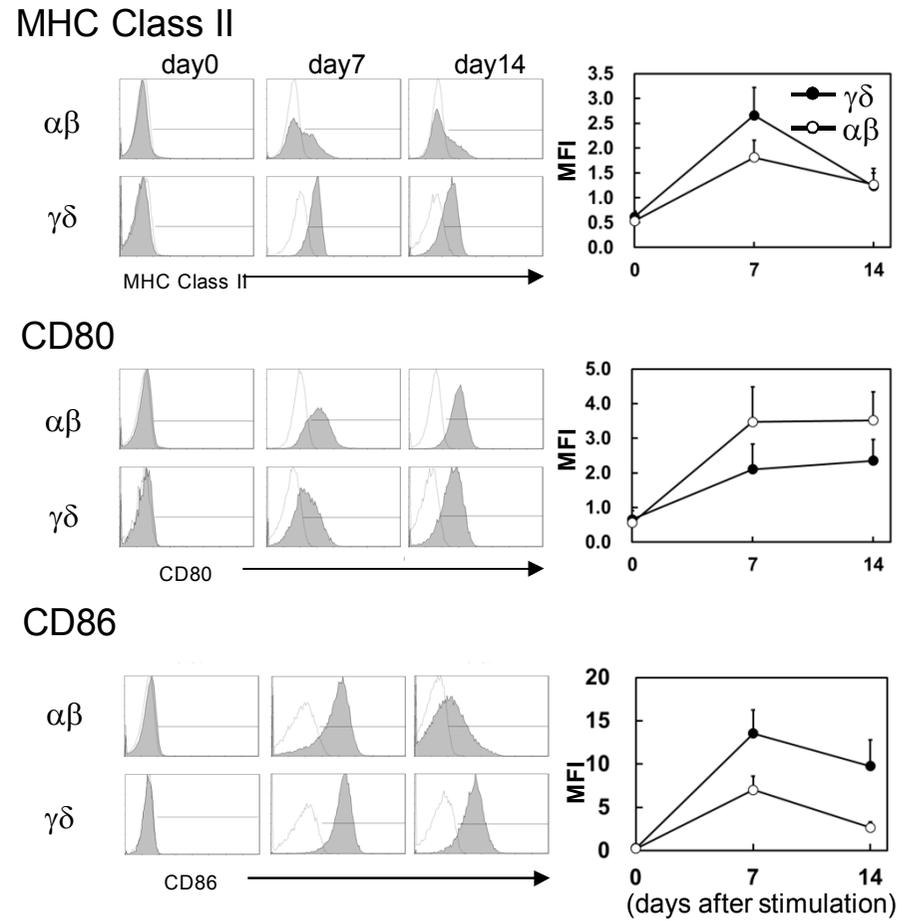
addition of inhibitors for ERK 1/2 (PD98059), p38 MAP kinase (SB203580), or NF- κ B (PS-1145), respectively. **(B)** MFI of antigen presentation-related molecules in the presence or absence of inhibitors (mean \pm SD; n = 3-4). * P < 0.05, ** P < 0.01. **(C)** Time course of phosphorylation of ERK1/2 and p38 after stimulation with IPP or PMA/ionomycin on $\gamma\delta$ T cells were measured by Western blotting. Grb2 was a protein loading control.

Figure 6. Resting $\gamma\delta$ T cells expressed CCAAT/enhancer-binding protein α (C/EBP α) more than $\alpha\beta$ T cells

(A) Expression levels of C/EBP α , Pu.1, HES-1 and GATA3 in cDNA samples taken from sorted $\alpha\beta$ and $\gamma\delta$ T cells were measured by real-time PCR. The data were normalized by HPRT and relative expression levels were calculated using Comparative-Ct Method. Mean \pm SD of 3 samples are shown. **(B)** $\gamma\delta$ or $\alpha\beta$ T cells were activated in the presence of C/EBP inhibitor, betulinic acid (50 μ M). Seventy-two hours later, cell surface expression of the indicated molecules was estimated with flow cytometer. The inhibition ratio (%) was calculated as follows; $\{(\% \text{ positive without the inhibitor} - \% \text{ positive with the inhibitor}) / \% \text{ positive without the inhibitor}\} \times 100$. Representative 2 results out of 5 are shown.

Fig.1

A



B

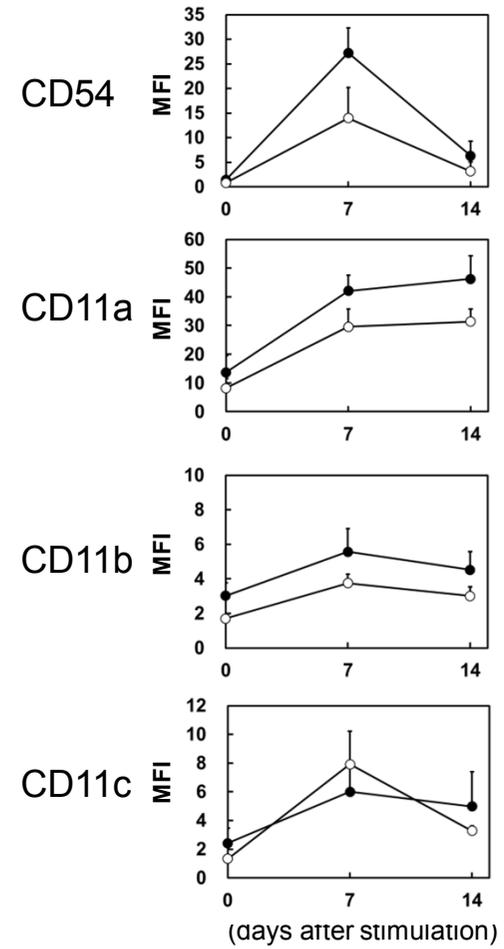
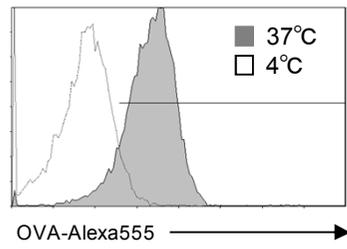


Fig.2

A



B

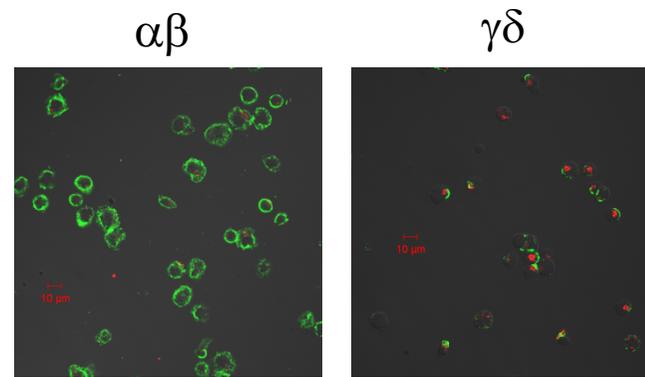
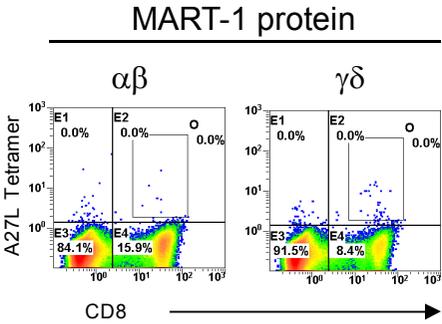
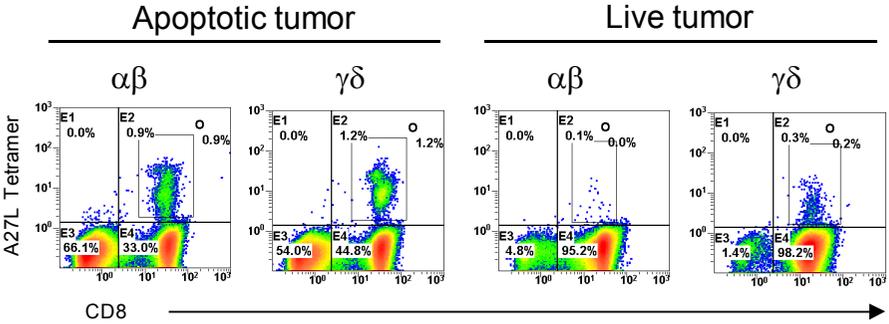


Fig.3

A



B



C

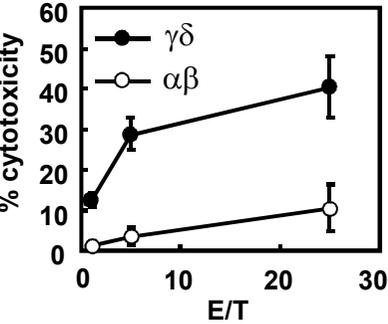
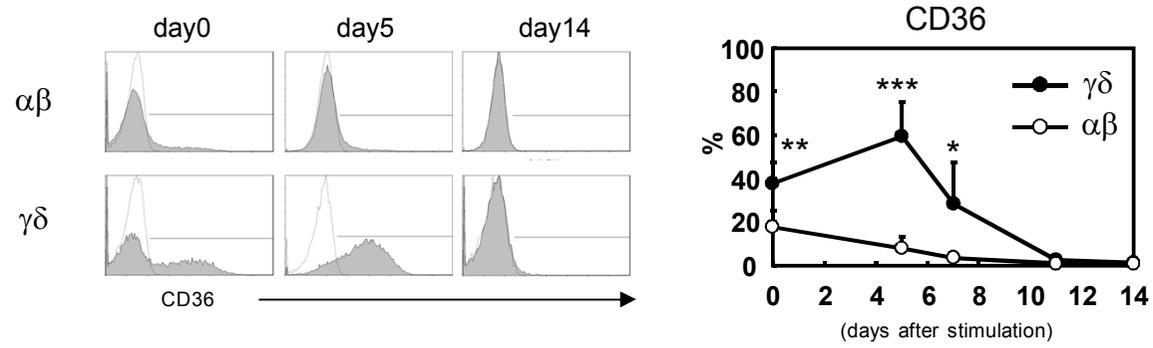


Fig.4

A



B

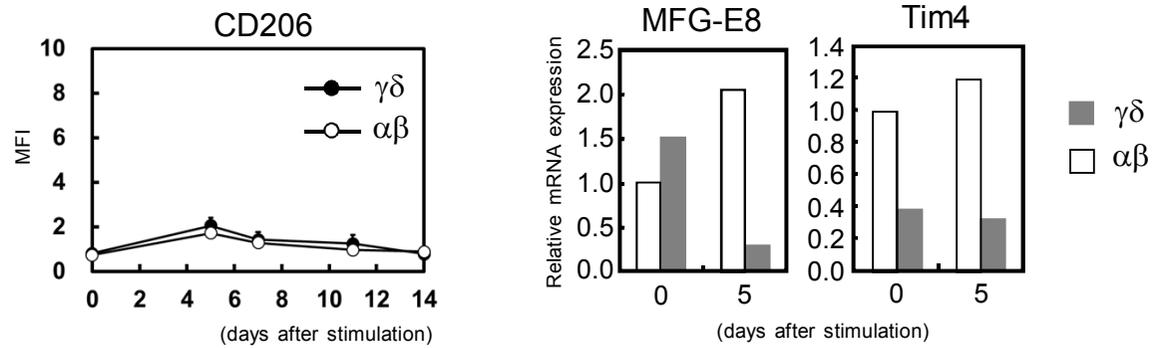


Fig.5

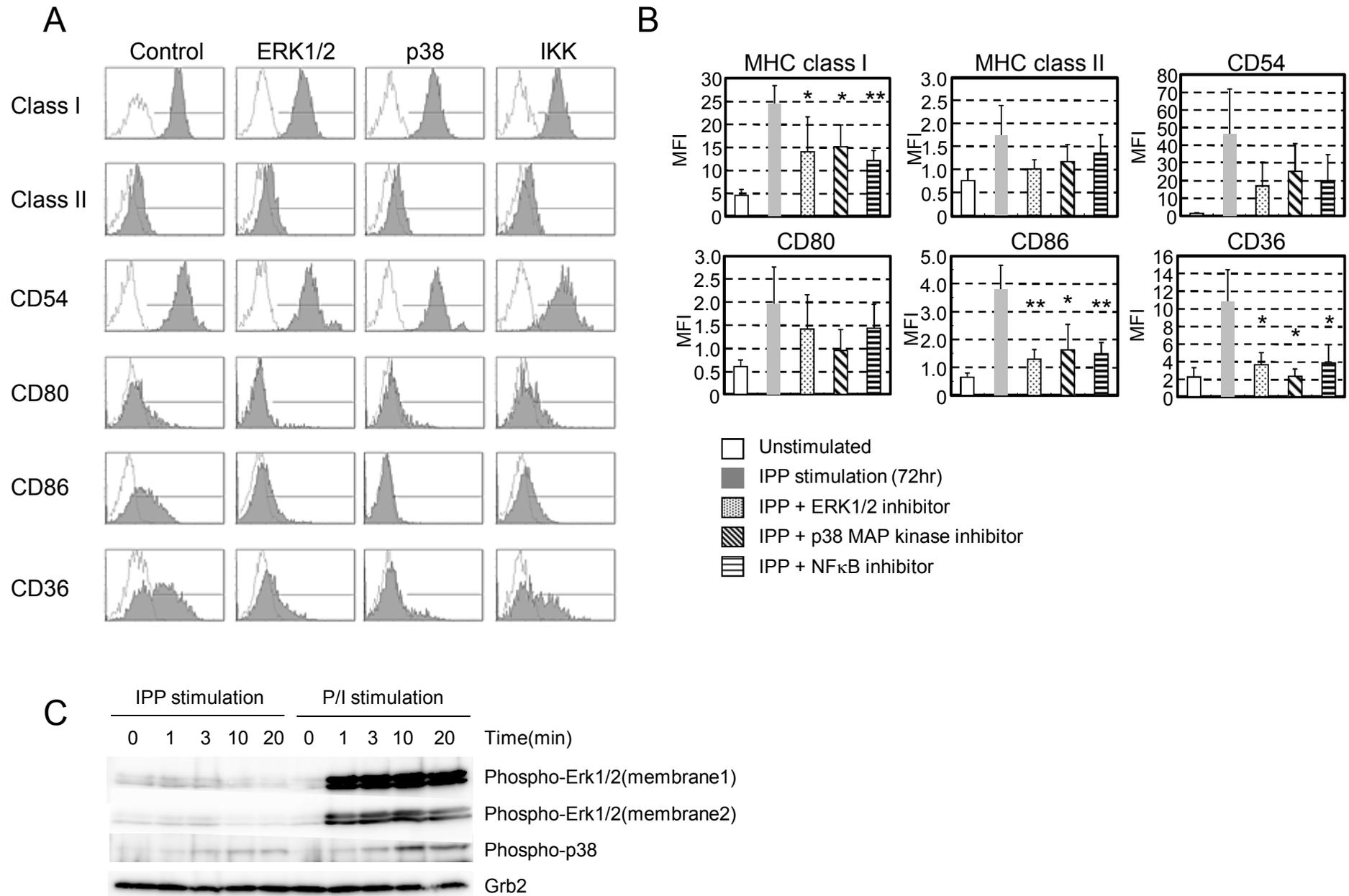
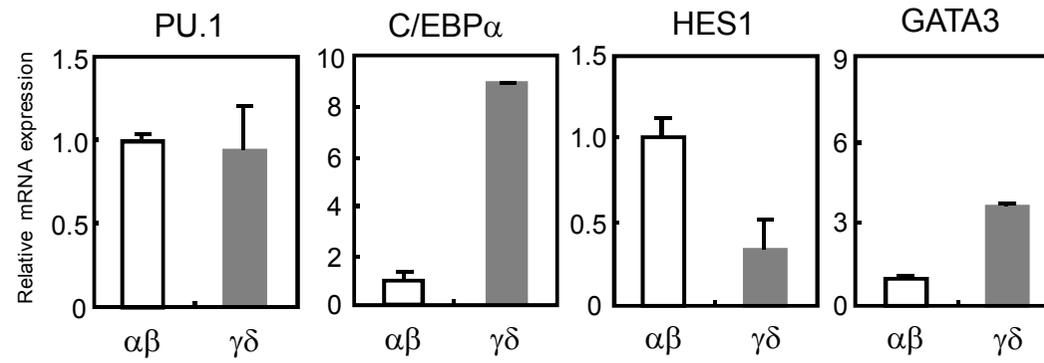


Fig.6

A



B

