Myeloid molecular characteristics of human γδ T cells support their acquisition of tumor antigen-presenting capacity

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Abstract

Human T cells expressing γδ 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 γδ T cells remain largely unclear. In this study, we have investigated the molecular characteristics of human γδ T cells related to their acquisition of antigen presenting capacity in comparing with activated αβ T cells. We found that activated γδ but not αβ 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-κB pathways. Confocal microscopical analysis revealed that activated γδ T cells can phagocytose protein antigens. Activated γδ 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/EBPα, a critical transcription factor for the development of myeloid-lineage cells, is expressed much higher in γδ T cells than in αβ T cells. These results unveiled the molecular mechanisms for the elicitation of antigen presenting functions in γδ T cells and would also help designing new approaches for γδ T cell-mediated human cancer immunotherapy.

Key words: γδ T cells, zoledronate, antigen presenting capacity, CD36, C/EBPα, myeloid

Precis: Activated human γδ T cells express myeloid cell characteristics and can function as antigen presenting cells.

Abbreviations

APC: antigen-presenting cell

C/EBPα: 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 γδ TCR comprise approximately 1-10% of peripheral blood mononuclear cells (PBMCs). The majority of γδ T cells in peripheral blood express the Vδ2 chain in combination with Vγ9 [1]. $V\gamma9V\delta2^{+}$ 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 $V\gamma9V\delta2^{+}$ 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 γδ T cells [8-10]. We have previously established an efficient large-scale ex vivo expansion method of γδ 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 γδ T cells show professional antigen-presentation function upon activation [12]. IPP- or Zoledronate-activated γδ T cells possess functional

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

In addition to γδ T cells, αβ 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 γδ T cells have several particular molecular features to obtain APC phenotype and functions. For example, we found that activated human γδ T cells express CD36, a scavenger receptor, whose expression was upregulated via MAPK and/or NF-κB signal pathways. Furthermore, we also found that CCAAT/enhancer-binding protein α (C/EBPα), a critical transcription factor for the development of myeloid-lineage cells, is expressed much higher in γδ T cells than in αβ T cells. These results may unveil the molecular mechanisms for the elicitation of APC functions in γδ 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 Coporation (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 μM Zoledronate and 1000 IU/ml IL-2 for γδ T cells and 10 μg/ml plate-immobilized anti-CD3 antibody (BD Biosciences: San Jose, CA, USA) and 175 IU/ml IL-2 for αβ T cells, respectively. Fresh media including IL-2 were added to the culture to keep the cell concentration (0.5-2 x 10^6 cells/ml). In some experiments, γδ T cells were stimulated with 50 μM IPP in the presence of 50 IU/ml of IL-2, and betulinic acid (50 μM), an inhibitor of C/EBPα [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-TCRVγ9-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 γδ or αβ T cells was detected by flow cytometry using the FC500 (Beckman

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

Antigen uptake

Activated γδ or αβ T cells (day 5-8 of culture) were pulsed for 120 minutes or overnight with 50 μg/ml of Alexa Fluor 555-labeled ovalbumin (OVA) (Molecular Probes). After incubation, cells were stained with anti-TCRVγ9 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 αβ or γδ T cells were used as APCs. Apoptosis was induced in JCOCB by irradiation (50 Gy). Apoptotic or untreated live tumor cells and αβ or γδ T cells (day 14 of culture) were co-cultured for 48 or 96 hours, and then Pan-T cells and γδ 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 αβ or γδ 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 rations for 4 hours at 37 ºC. 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/EBPα, Pu.1, HES1, MFG-E8, Tim-4, and GATA3 in cDNA samples taken from sorted αβ and γδ 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 γδ T cells (day 8 of culture) were washed and rested on ice for 1 hour, and then stimulated with 50 μM IPP at 37 ºC. 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 Na_3VO_4, and 10 μg/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 reprobed 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 γδ T cell expansion and molecular expressions

We propagated γδ T cells using Zoledronate and IL-2 from PBMCs of human healthy volunteers as previously reported [11]. The number of $V\gamma9^+$ 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 γδ and αβ T cells. Those molecules were not detected in both γδ and αβ 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 γδ T cells than αβ T cells. In contrast, the expression level of CD80 was similar or higher in αβ T cells after activation. Additionally, the expression levels of adhesion molecules such as CD54, CD11a, CD11b and CD11c were comparable in resting γδ and αβ T cells (Fig. 1B). Following activation, the expression levels of these molecules were detected at higher levels in γδ T cells, except for CD11c which was higher in αβ T cells (Fig. 1B). Together, these results suggest that both γδ T and αβ T cells acquire characteristics of antigen presenting cells following activation.

Uptake of protein antigen

We then examined the potential of Zoledronate-activated γδ T cells to uptake protein antigen by using ovalbumin (OVA) as a model. The activated γδ T cells and OVA-Alexa555 were co-incubated on ice or at 37 ºC for 2 hours, and uptake of protein antigen was examined by flow cytometer. Comparing to γδ T cells incubated on ice, γδ T cells incubated at 37 ºC showed increased fluorescence which reflects increased uptake of protein antigen (Fig. 2A). Similar uptake of protein antigen was not observed in αβ 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 γδ but not αβ T cells. Thus, these results suggest that activated γδ but not αβ T cells have the capacity to uptake protein antigens.

Antigen presenting function of γδ and αβ T cells using A27L peptide

Next, we examined antigen presenting function of γδ and αβ T cells. To do so, we evaluated the ability of γδ or αβ 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 γδ or αβ T cells were stimulated with apoptotic tumor cells (Fig. 3B). Interestingly, when live tumor cells were used as the source of antigen, only activated γδ T cells could support the induction of antigen-specific T cell proliferation (Fig. 3B). Furthermore, γδ T cells showed higher cytotoxic activities against tumor cells compared to αβ T cells (Fig. 3C). Together, these results suggest that activated γδ T cells have the ability to present tumor cell-derived antigens.

Comparison of scavenger/phagocytosis-related molecules expression between γδ and αβ T cells

We next investigated expression of scavenger/phagocytosis-related molecules in both γδ and αβ T cells. γδ 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 αβ 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 γδ and αβ 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 γδ 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 γδ T cells. So far, it has been reported that MAPK or NF-κB 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 γδ T cells. In this experiment, we activated γδ 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 ± 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-κB (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 γδ T cells seems to be regulated by signaling pathway through MAPK and NF-κB. Particularly, CD36 expression seems to be highly regulated by these pathways.

Comparison of myeloid-related transcription factors between γδ and αβ T cells

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

However, it barely altered the expression level of CD54 and MHC class I in both γδ and αβ T cells (Fig. 6B). Therefore, these results suggested that the high expression of C/EBPα in γδ 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 αβ and γδ 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 αβ and γδ 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 γδ and αβ T cells. Regarding expression of antigen-presenting molecules, MHC class II expression was higher in γδ T cells than in αβ T cells (Fig. 1). Furthermore, we found that human γδ T cells expressed the scavenger receptor CD36 (Fig. 4), as previously indicated that bovine γδ 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 γδ 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 γδ 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 γδ T cells activation, and found that CD36 upregulation was mediated by MAPK and NF-κB pathways (Fig. 5). A previous study has suggested that the phosphorylation of C/EBPα is regulated by p38 or ERK1/2 signaling pathway [26]. Consistently, C/EBPα 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/EBPα is important for the induction of APC function in activated γδ T cells.

In this study, we showed that γδ T cells possess a capacity to take up not only peptide but also protein antigen (Fig. 2). Furthermore, γδ T cells possess strong cytotoxicity and ability to uptake cell debris, followed by antigen presentation, as described previously [28]. Therefore, it seems that γδ T cells are able to process wide range of antigens and mediate the antigen presentation. In αβ 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 γδ T cells use similar mechanisms to exert the antigen presenting capacity.

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

through Vγ9Vδ2^{+} TCR, which activates MAPK cascade including p38 and ERK1/2 resulting in the phosphorylation of C/EBPα. Following phosphorylation, C/EBPα upregulates CD36 expression which helps the uptake of cell-derived antigens. Activated γδ 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 γδ T cells would help to understand the anti-cancer immune responses and design new strategies to improve the therapeutic effects of γδ T cells-based cancer immunotherapy.

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Disclosure of Conflicts of Interest

The authors declare no competing financial interest.

References

1. Kabelitz D, Wesch D, Pitters E, Zöller M (2004) Potential of human gammadelta T lymphocytes for immunotherapy of cancer. Int J Cancer 112(5): 727-732
2. Tanaka Y, Morita CT, Tanaka Y (1995) Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. Nature 375: 155-158
3. Bennouna J, Bompas E, Neidhardt EM, Rolland F, Philip I, Galéa C, Salot S, Saiagh S, Audrain M, Rimbert M, Lafaye-de Micheaux S, Tiollier J, Négrier S (2008) Phase-I study of Innacell gammadelta, an autologous cell-therapy product highly enriched in gamma9delta2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma. Cancer Immunol Immunother 57(11): 1599-1609
4. Kobayashi H, Tanaka Y, Yagi J, Minato N, Tanabe K (2011) Phase I/II study of adoptive transfer of γδ T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma. Cancer Immunol Immunother 60(8): 1075-1084
5. A J Nicol, H Tokuyama, S R Mattarollo, T Hagi, K Suzuki, K Yokokawa and M Nieda. (2011) Clinical evaluation of autologous gamma delta T cell-based immunotherapy for metastatic solid tumours. British Journal of Cancer 105: 778–786

6. Nakajima J, Murakawa T, Fukami T, Goto S, Kaneko T, Yoshida Y, Takamoto S, Kakimi K (2010) A phase I study of adoptive immunotherapy for recurrent non-small-lung cancer patients with autologous gammadelta T cells. Eur J Cardiothorac Surg 37: 1191-1197

7. Wada I, Matsushita H, Noji S, Mori K, Yamashita H, Nomura S, Shimizu N, Seto Y, Kakimi K (2014) Intraperitoneal injection of in vitro expanded Vγ9Vδ2 T cells together with zoledronate for the treatment of malignant ascites due to gastric cancer. Cancer Med 3(2): 362-375.

8. Kunzmann V, Bauer E, Wilhelm M (1999) γ/δ T-cell stimulation by pamidronate. N Engl J Med 340:737

9. Kunzmann V, Bauer E, Feurle J, Weißinger F, Tony H-P, Wilhelm M (2000) Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood 96: 384-392

10. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, Tony HP (2003) Gammadelta T cells for immune therapy of patients with lymphoid malignancies. Blood 102(1): 200-206

11. Abe Y, Muto M, Nieda (2009) Clinical and immunological evaluation of zoledronate-activated Vgamma9gammadelta T-cell-based immunotherapy for patients with multiple myeloma. Exp Hematol 37: 956-968

12. Brandes M, Willimann K, Moser B (2005) Professional antigen-presentation function by human gammadelta T Cells. Science 309(5732): 264-268

13. Wu Y, Wu W, Wong WM, Ward E, Thrasher AJ, Goldblatt D, Osman M, Digard P, Canaday DH, Gustafsson K (2009) Human gamma delta T cells: a lymphoid lineage cell capable of professional phagocytosis. J Immunol 183(9): 5622-5629

14. Landmeier S, Altvater B, Pscherer S, et al (2009) Activated human gammadelta T cells as stimulators of specific CD8+ T-cell responses to subdominant Epstein Barr virus epitopes: potential for immunotherapy of cancer. J Immunother 32: 310–321

15. Altvater, B., Pscherer, S., Landmeier, S., Kailayangiri, S., Savoldo, B., Juergens, H., and Rossig, C (2012) Activated human γδ T cells induce peptide-specific CD8+ T-cell responses to tumor-associated self-antigens. Cancer Immunol. Immunother. 61: 385–396

16. Brandes M, Willimann K, Bioley G, Lévy N, Eberl M, Luo M, Tampé R, Lévy F, Romero P, Moser B (2009) Cross-presenting human γδ T cells induce robust $CD8^+$ αβ T cell responses. Proc Natl Acad Sci U S A 106(7): 2307-2312

17. Himoudi N, Morgenstern DA, Yan M, Vernay B, Saraiva L, Wu Y, Cohen CJ, Gustafsson K, Anderson J (2012) Human γδ T lymphocytes are licensed for professional antigen presentation by interaction with opsonized target cells. J Immunol 188(4): 1708-1716

18. Umeshappa CS, Huang H, Xie Y, Wei Y, Mulligan SJ, Deng Y, Xiang J (2009) CD4+ Th-APC with acquired peptide/MHC class I and II complexes stimulate type 1 helper CD4+ and central memory CD8+ T cell responses. J Immunol 182(1): 193-206

19. Hao S, Yuan J, Xiang J (2007) Nonspecific CD4+ T cells with uptake of antigen-specific dendritic cell-released exosomes stimulate antigen-specific CD8+ CTL responses and long-term T cell memory. J Leukoc Biol 82(4): 829-838

20. Hollis A, Speri B, Graber M, Berg T (2012) The natural product betulinic acid inhibits C/EBP family transcription factors. ChemBioChem 13(2): 302-307

21. Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, Bhardwaj N (1998) Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J Exp Med 188(7): 1359-1368

22. Greenberg ME1, Sun M, Zhang R, Febbraio M, Silverstein R, Hazen SL (2006) Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells. J Exp Med 203(12): 2613-2625

23. Ardeshna KM1, Pizzey AR, Devereux S, Khwaja A (2000) The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. Blood 96(3): 1039-1046

24. Yoshimura S, Bondeson J, Foxwell BM, Brennan FM, Feldmann M (2001) Effective antigen presentation by dendritic cells is NF-kappaB dependent: coordinate regulation of MHC, co-stimulatory molecules and cytokines. Int Immunol 13(5): 675-683

25. Lubick K, Jutila MA (2006) LTA recognition by bovine gammadelta T cells involves CD36. J Leukoc Biol 79(6):1268-70

26. Ross SE, Radomska HS, Wu B, Zhang P, Winnay JN, Bajnok L, Wright WS, Schaufele F, Tenen DG, MacDougald OA (2004) Phosphorylation of C/EBPalpha inhibits granulopoiesis. Mol Cell Biol 24(2): 675-686

27. Qiao L, Zou C, Shao P, Schaack J, Johnson PF, Shao J (2008) Transcriptional regulation of fatty acid translocase/CD36 expression by CCAAT/enhancer-binding protein alpha. J Biol Chem 283(14): 8788-8795

28. Meulter S, Eberl M, Moser B (2010) Prolonged antigen survival and cytosolic export in cross-presenting human gammadelta T cells. Proc Natl Acad Sci U S A 107(19): 8730-8735

Figure legends

Figure 1. Expansion of γδ T cells and their expression of antigen presentation-related molecules

PBMCs were cultured with Zoledronate for expanding γδ T cells or with plate-immobilized anti-CD3 antibody for αβ T cells, respectively. (**A**) Left, representative histogram data indicating expression of MHC Class II, CD80, and CD86 in αβ and γδ 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 γδ T cells (closed circle) and αβ T cells (open circle) were shown (mean ± SD; n = 5-9). (**B**) Time courses of the expression of adhesion molecules (CD54, CD11a, CD11b, and CD11c) on γδ T cells (closed circle) and αβ T cells (open circle) were shown (mean ± SD; n = 5-9).

Figure 2. Activated γδ T cells, but not αβ T cells, took the protein antigen up into the cells

Activated γδ or αβ T cells were co-incubated with ovalbumin (OVA) as a protein antigen. (**A**) Activated γδ T cells (day 5 of culture) and OVA-Alexa555 were co-incubated on ice (solid

lines) or at 37 ºC (gray shade) for 2 hours, and the cells were examined with flow cytometer. (B) Activated γδ or αβ T cells (day 8 of culture) were co-incubated for overnight with OVA-Alexa555 (red). After incubation, γδ or αβ T cells were stained with anti-TCRVγ9 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 γδ T cells, but not αβ T cells, induce antigen-specific T cells when co-cultured with live tumor cells as a source of antigen

Antigen pulsed activated αβ or γδ 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 αβ and γδ T cells against MART-1 positive tumor cells. Data are presented as percentage of activity (mean ± SD; n = 3).

Figure 4. CD36, a scavenger receptor is expressed in naïve or activated γδ T cells, but not αβ T cells

PBMCs were cultured with Zoledronate for γδ T cells or with plate-immobilized anti-CD3 antibody for αβ cells, respectively. (**A**) Left, representative flow cytometry data indicating expression of CD36 in αβ and γδ T cells at the indicated time points. Right, time courses of the expression of CD36 on γδ T cells (closed circle) and αβ T cells (open circle) (mean ± SD; n = 5-9). **P* < 0.05, ***P* < 0.01, ***P < 0.005. (**B**) Left, time courses of the expression of CD206 on γδ T cells (closed circle) and αβ T cells (open circle) (mean ± SD; n = 5-9). Right, expression levels of MFG-E8 and Tim4 in cDNA samples taken from sorted αβ and γδ 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 γδ T cells

γδ 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-κB (PS-1145), respectively. (**B**) MFI of antigen presentation-related molecules in the presence or absence of inhibitors (mean ± 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 γδ T cells were measured by Western blotting. Grb2 was a protein loading control.

Figure 6. Resting γδ T cells expressed CCAAT/enhancer-binding protein α (C/EBPα) more than αβ T cells

(**A**) Expression levels of C/EBPα, Pu.1, HES-1 and GATA3 in cDNA samples taken from sorted αβ and γδ 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 ± SD of 3 samples are shown. (**B**) γδ or αβ T cells were activated in the presence of C/EBP inhibitor, betulinic acid (50 μM). Seventy-two hours later, cell surface expression of the indicated molecules was estimated with flow cytometer. The inhibition ratio (%) was calculated as follows; {(% positive without the inhibitor - % positive with the inhibitor) / % positive without the inhibitor} x 100. Representative 2 results out of 5 are shown.

Fig.1

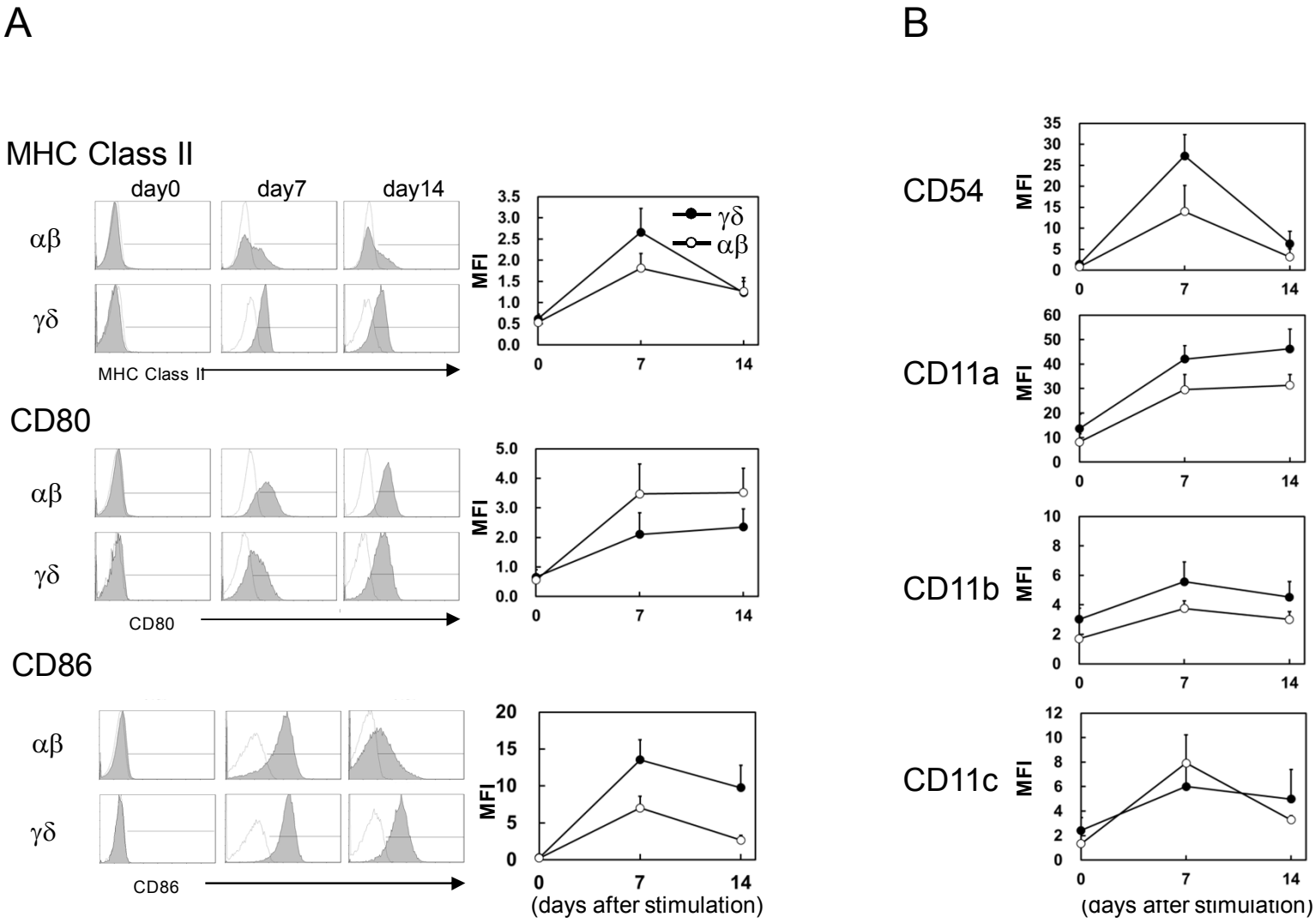
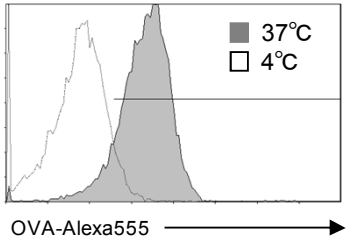

A
MHC Class II
day0
day7
day14
αβ
γδ
MHC Class II
MFI
γδ
αβ
CD80
CD80
CD86
CD86
(days after stimulation)
B
CD54
CD11a
CD11b
CD11c
MFI
(days after stimulation)

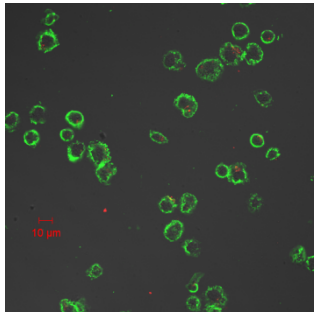
Fig.2

A



B

αβ



γδ

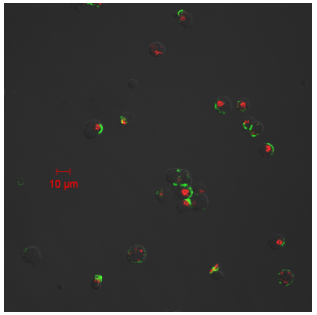
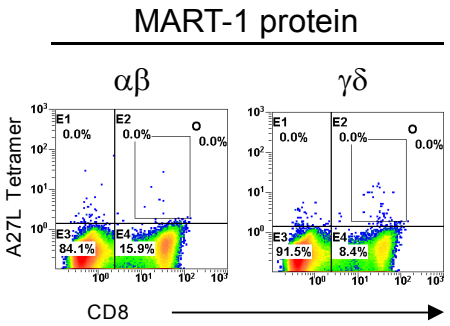
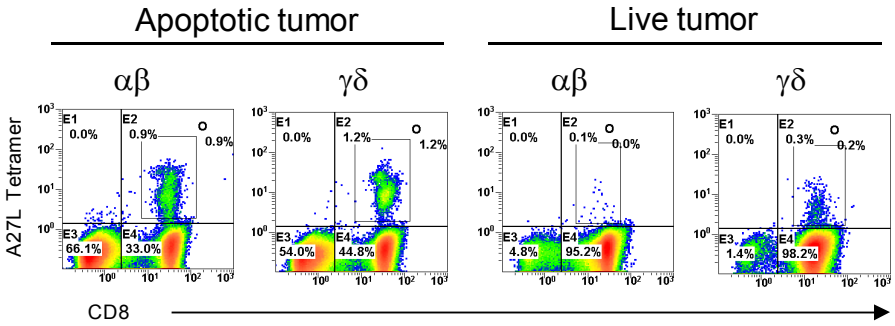


Fig.3

A


MART-1 protein
αβ
γδ
A27L Tetramer
CD8
E1 0.0%
E2 0.0%
0.0%
E3 84.1%
E4 15.9%
E1 0.0%
E2 0.0%
0.0%
E3 91.5%
E4 8.4%

B


Apoptotic tumor
Live tumor
αβ
γδ
αβ
γδ
A27L Tetramer
CD8
E1 0.0%
E2 0.9%
0.9%
E3 66.1%
E4 33.0%
E1 0.0%
E2 1.2%
1.2%
E3 54.0%
E4 44.8%
E1 0.0%
E2 0.1%
0.0%
E3 4.8%
E4 95.2%
E1 0.0%
E2 0.3%
0.2%
E3 1.4%
E4 98.2%

C

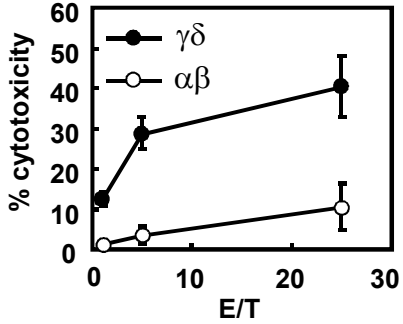
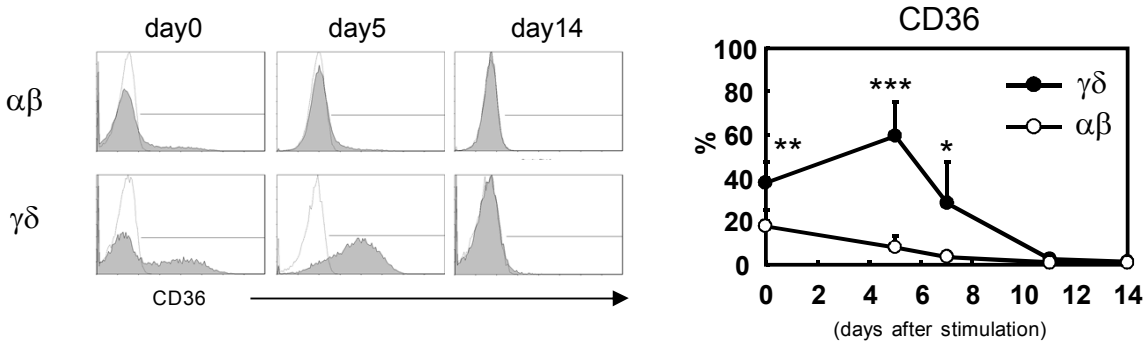

% cytotoxicity
E/T
γδ
αβ
0
10
20
30
40
50
60

Fig.4

A


day0
day5
day14
αβ
γδ
CD36
CD36
100
80
60
40
20
0
%
**
***
*
γδ
αβ
0 2 4 6 8 10 12 14
(days after stimulation)

B

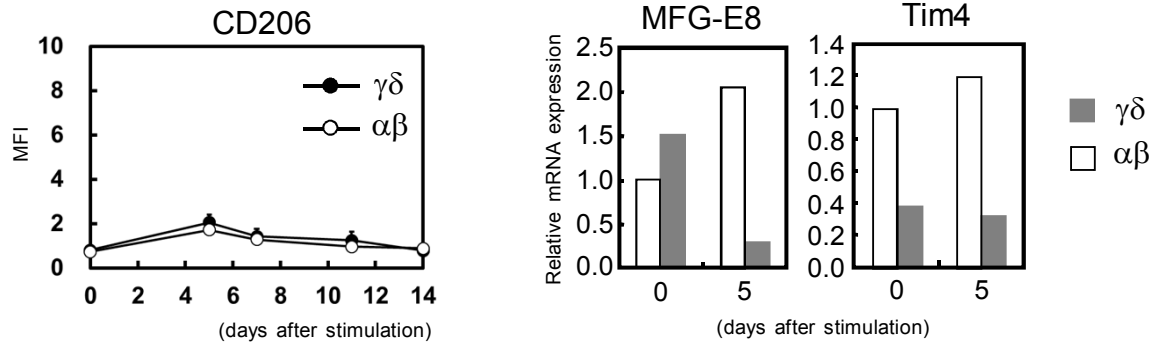

CD206
MFI
10
8
6
4
2
0
γδ
αβ
0 2 4 6 8 10 12 14
(days after stimulation)
MFG-E8
Tim4
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
0
5
(days after stimulation)
γδ
αβ

Fig.5

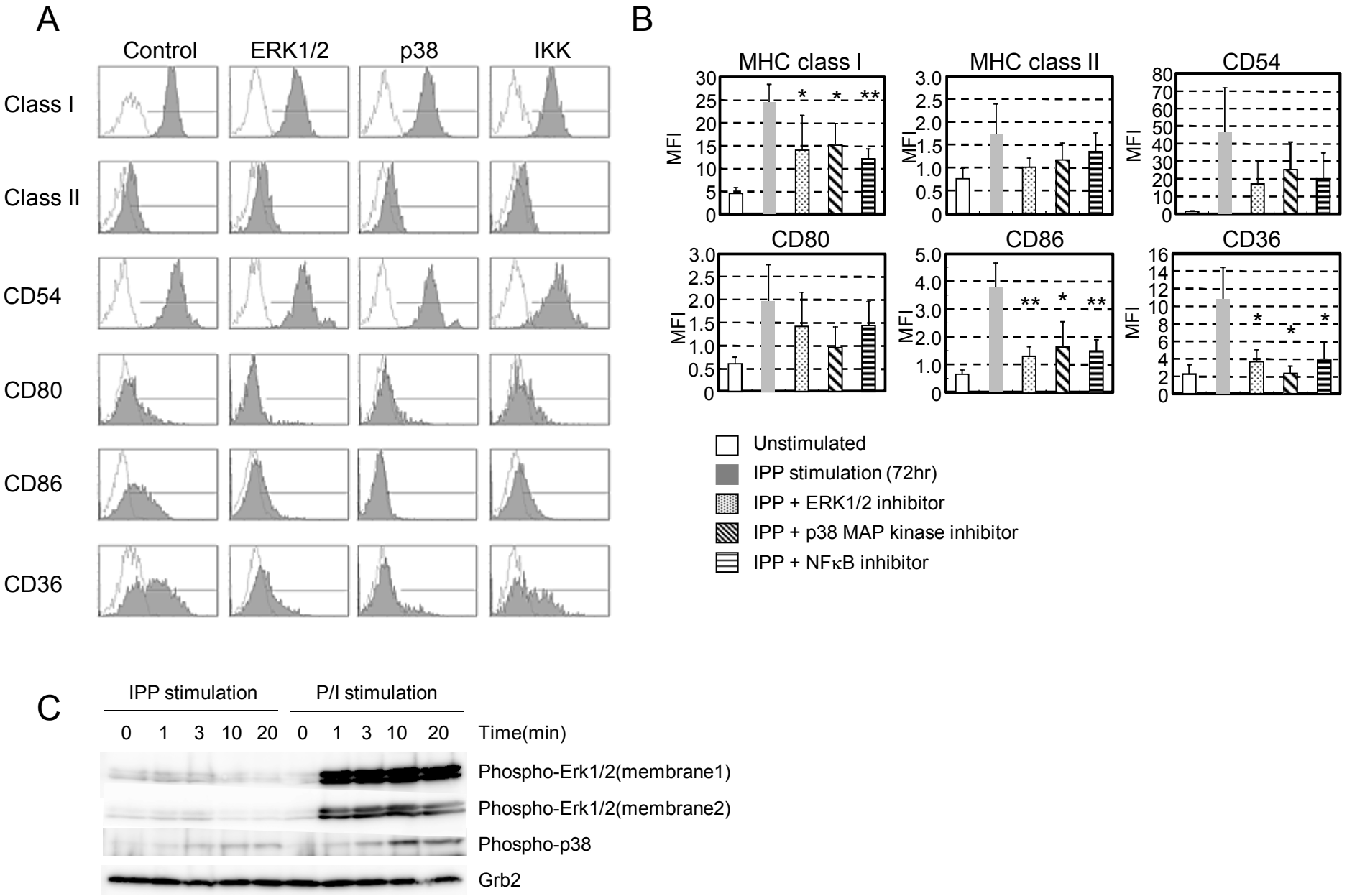
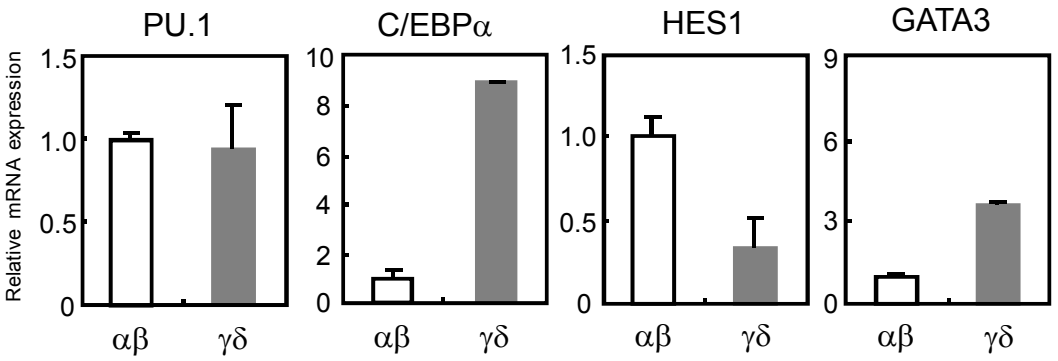
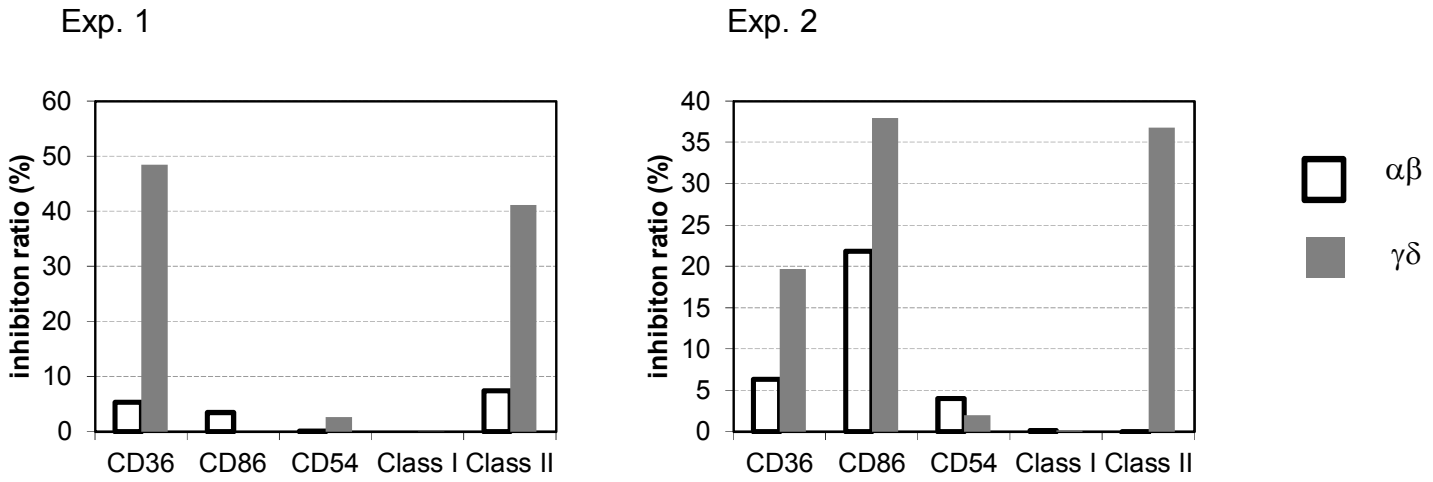

A
Control
ERK1/2
p38
IKK
Class I
Class II
CD54
CD80
CD86
CD36
B
MHC class I
MHC class II
CD54
CD80
CD86
CD36
MFI
Unstimulated
IPP stimulation (72hr)
IPP + ERK1/2 inhibitor
IPP + p38 MAP kinase inhibitor
IPP + NFκB inhibitor
C
IPP stimulation
P/I stimulation
0 1 3 10 20 0 1 3 10 20
Time(min)
Phospho-Erk1/2(membrane1)
Phospho-Erk1/2(membrane2)
Phospho-p38
Grb2

Fig.6

A


PU.1
C/EBPα
HES1
GATA3
Relative mRNA expression
1.5
1.0
0.5
0
10
8
6
4
2
9
3
αβ
γδ

B


Exp. 1
Exp. 2
inhibiton ratio (%)
60
50
40
30
20
10
0
35
25
15
5
CD36
CD86
CD54
Class I
Class II
αβ
γδ