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Failure of mycoplasma lipoprotein MALP-2 to induce NK cell activation through dendritic cell TLR2

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Running title: NK activation by MALP-2

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Abstract

Macrophage-activating lipopeptide 2 (MALP-2), a mycoplasmal diacylated lipopeptide with palmitic acid moiety (Pam2), activates Toll-like receptor (TLR) 2 to induce inflammatory cytokines. TLR2 is known to mature myeloid dendritic cells (mDC) to drive mDC contact-mediated natural killer (NK) cell activation. Here we tested if MALP-2 activates NK cells through stimulation of TLR2 on mDC. Although synthetic MALP-2 with 6 or 14 amino acids (a.a.) stretch (designated as s and f) matured mDC to induce IL-6, IL-12p40 and TNF-α to a similar extent, they far less activated NK cells than Pam2CSK4, a positive control of 6 a.a.-containing diacyl lipopeptide. MALP-2s and f were TLR2/6 agonists and activate the MyD88 pathway similar to Pam2CSK4, but MALP-2s having the CGNNDE sequence acted on mDC TLR2 to barely induce external NK activation. Even the s form, with slightly high induction of IL-6 compared to the f form, barely induced *in vivo* growth retardation of NK-sensitive implant tumor. Pam2CSK4 and MALP-2 have the common lipid moiety but different peptides, which are crucial for NK cell activation. The results infer that MALP-2 is applicable to a cytokine inducer but not to an adjuvant for antitumor NK immunotherapy.

Keywords: Toll-like receptor 2, MyD88, macrophage-activating lipopeptide 2, dendritic cells, NK activation.

1. *Introduction*

Macrophage-activating lipopeptide 2 (MALP-2) is a mycoplasmal diacylated lipopeptide with agonistic activity for Toll-like receptor (TLR) 2 (1). Myeloid dendritic cells (mDC) and macrophages produce inflammatory cytokines in response to MALP-2 (1,2). MALP-2 is proteolytically liberated from the parent lipoprotein of M161Ag (2,3) or MALP-404 (4), which is anchored on the outer membrane of *Mycoplasma fermentans*. Although the protease that specifically cleaves M161Ag into MALP-2 has not been identified, the peptide sequence of MALP-2 is determined by Mass spectrometry as S-[2,3-bis(palmitoyl)propyl]cysteine (Pam2Cys) followed by 14 amino acids (5) (Table 1). In fact, the TLR2 agonistic functions are conserved in a synthetic compound (herein referred to MALP-2f) (5). This synthetic MALP-2 has been applied to clinical phase studies to develop a new adjuvant (6).

Several reports suggested that microbial pattern molecules have the ability to activate natural killer (NK) cells in vitro (7-10). TLR and cytoplasmic pattern sensors are representative pattern-recognition receptors (PRR) which may be associated with mDC-mediated NK activation (7-9). TLR3 and the adaptor TICAM-1 (TRIF) in mDC typically participate in driving NK activation in response to dsRNA (10). Recent studies on TLR2 agonists including lipopeptides also revealed that stimulation of TLR2 on mDC results in activation of the MyD88 pathway in mDC to drive external NK activation (11,12). NK cells play a role in early defense against various pathogens.

We have looked into the immuno-modulatory function of M161Ag and MALP-2, to develop a new adjuvant for cancer immunotherapy (1). MALP-2 possessed high activity to induce IL-6, TNF-α, IL-10 and IL-12p40 from myeloid cells, but its functional potential for NK activation has not been examined yet. We test in the present studies whether MALP-2 has sufficient activity to induce NK cell activation *in vitro* and NK-mediated tumor regression *in vivo* using a mouse tumor implant model.

2. *Materials and Methods*

2.1. *Reagents and antibodies*

The following materials were obtained as indicated: Fetal calf serum (FCS) from Bio Whittaker (Walkersville, MD), mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) from PeproTech EC, Ltd (London, UK), the enzyme-linked immunosorbent assay (ELISA) kits for mouse (m)IFN-γ from Biolegend (San Diego, CA), IL-12p40 and IL-6 from eBioscience (San Diego, CA). Two forms of synthetic macrophage-activating lipopeptide 2 (MALP-2) were ordered to Biologica Co., Nagoya,

Japan. MALP-2s: Pam2CGNNDE (MW; 1201.5) and MALP-2f: Pam2CGNNDESNISFKEK (MW; 2135.6). The synthesis of lipopeptides was achieved with a combination of solution- and solid-phase methods as described (13). Pam2Cys12 (Pam_2CSTSEVIGEKI), Pam2CSK4 (Pam_2CSKKKK) and control Pam2CSK were prepared as referenced (12). Poly I:C, a TLR3 agonist for induction of antitumor immunity, was used for this study as a positive control.

The following antibodies were used: fluorescein isothiocyanate (FITC)-labeled anti-mouse CD69, I-Ab, IFN-γ mAb, phycoerythrin (PE)-labeled anti-mouse CD86, CD25, and allophycocyanin (APC)-labeled anti-mouse NK1.1 were purchased from Biolegend (San Diego, CA).

2.2. *Mouse and cell lines*

TLR2 -/- and MyD88 -/- mice were gifts from Dr. S. Akira (Osaka Univ., Osaka) as previously reported (14). Female C57BL/6 mice were purchased from Clea Japan (Tokyo). Mice were maintained in our institute under specific pathogen-free conditions. All animal work was performed under guidelines established by the Hokkaido University Animal Care and Use Committee. Mice (12 weeks female C57BL/6) were housed four per cage and allowed food and water ad libitum. Animal studies were carefully performed without ethical problems.

HEK293 cells were obtained from ATCC and maintained in RPMI 1640/10% FCS. B16D8 cells were established in our laboratory as a subline of the B16 melanoma cell line (15) and cultured in RPMI 1640/10% FCS. This subline was characterized by its low MHC levels with no metastatic properties when injected s.c. into syngeneic C57BL/6 mice. The B16D8 cell line is a typical NK target (10).

2.3. *Preparation of BMDC and spleen NK cells of mice*

Mouse bone marrow-derived DC (BMDC) were prepared as described previously (16). Spleen NK cells were positively isolated from spleens with DX5 Micro Beads kit (Miltenyi Biotech) (10). The purity of NK cells ($DX5^+$ cells) was routinely about 80%. $DX5^+$ NK cells were used within 24 h.

2.4. *Reporter assay*

Plasmids (pEFBos) for expression of human TLR1, TLR2, TLR6 and TLR10 were prepared in our laboratory as described previously (16). HEK293 cells were seeded onto 24-well plates and transfected with various amounts of expression vectors, the ELAM

reporter gene, and the phRL-TK control plasmid using FuGene HD (Roche) according to the manufacturer's instructions. After 24 h, the cells were harvested in 50 μl lysis buffer. The luciferase activity was measured using Dual-Luciferase Reporter assay systems (Promega) and was shown as the means ± S.D. of three experiments.

2.5. *ELISA, Flow cytometric (FACS) analysis of cell surface antigens*

The levels of cytokines (IL-6, IL-12p40, IFN-γ etc.) were determined by sandwich ELISA (Amersham Pharmacia Biotech, Buckinghamshire, UK) or the message levels assessed by quantitative PCR (27). Surface CD86 and I-Ab were determined by FACS using specific mAbs. The practical methods for FACS were described previously (16).

2.6. *Assessment of in vitro cytolytic activity*

The cytolytic activity of spleen NK cells was determined by ^{51}Cr assay as described previously (10). NK cells were prepared from the spleen of C57BL/6 mice. NK cells were co-cultured with BMDCs at a ratio of 2:1 and 24 hrs later the mixtures were subdivided to assess NK-mediated cytotoxicity (10). A B16 subline (D8) was used as a target cell. Target cells ($2x10^3$ cells/well) were coincubated with NK cells at the indicated lymphocyte to target (E/T) cell ratio (typically 15 and 30) in U-bottom 96-well plates in a total volume of 200 μl of RPMI 1640/10% FCS medium at 37 °C. Four hours later, the liberated ^{51}Cr in the medium was measured using the scintillation counter. Specific cytolytic activity was obtained by the formula: Specific cytotoxic activity (%) =[(experimental ^{51}Cr activity - spontaneous ^{51}Cr activity)/(total ^{51}Cr activity – spontaneous ^{51}Cr activity)] x 100. Each experiment was done in triplicate to confirm reproducibility of the results, and representative results are shown.

2.7. *Tumor challenge and the treatment with Pam2Cys-containing peptides*

B16D8 cells ($6x10^5$ cells) were subcutaneously (s.c.) transplanted into the back of mice at day 0. Pam2Cys-containing peptides (10 μg/head) or PBS (vehicle) only were injected around tumor at day 0, 3, 7, 9, 13, and 17. Tumor surfaces were measured twice a week by using a caliper.

3. *Results*

3.1. *BMDC maturation and cytokine liberation in response to MALP-2*

Pam2Cys-containing lipopeptides, Pam2Cys12, Pam2CSK, Pam2CSK4, and two forms of MALP-2 (s and f) were synthesized with reference to a previous report (Fig. S1)

(13,17). Pam2CSK was used as a negative control (17), which has virtually no cytokine-inducing activity (Fig. 1). By ELAM reporter assay, we assessed NF-κB activation potential of these lipopeptides (10~500 nM), and confirmed that all except Pam2CSK possess similar luciferin-activating potentials (data not shown).

IL-6 and IL-12p40 levels were determined by ELISA with the supernatant of the media where BMDC and each of the lipopeptides were co-cultured for 24 hrs. These cytokines were detected with high levels in the wells with Pam2Cys12, Pam2CSK4, MALP-2s and MALP-2f but not in Pam2CSK (Fig. 1A,B). These lipopeptides neither induced the mRNA of type I interferon (IFN), IL-15 and I-18 (data not shown) nor produced less than the detection limit (<5 pg/ml) of IL-12p70 protein (Fig 1C).

The degrees of CD86 upregulation were examined with the these lipopeptides, and similar DC maturation was evaluated by flow cytometry (Fig. 1D,E). Although the levels of CD86 were increased in response to Pam2 peptides (100 nM), no significant difference was observed among the Pam2 peptides tested (Fig. 1E). At the dose of Pam2 where cytokine induction sufficiently occurs, CD86 expression inadequately takes place in BMDC, as reported previously (12). Thus, NF-κB activation, cytokine liberation and DC maturation are partially co-related in these lipopeptides.

3.2. *NK activation by Pam2CSK4 and MALP-2*

Previous reports suggested that TLR2 agonists have ability to induce NK activation (11,12). To investigate whether the *mycoplasma* lipopeptides harbor activity of NK cell activation, we added the Pam2Cys peptides (100 nM) to BMDC, NK cells or BMDC/NK co-culture as in a previous method assessing polyI:C activity for BMDC-mediated NK activation (10). Although BMDC *per se* can induce IFN-γ production in response to some TLR stimuli (17,18), we could detect only minure amounts of IFN-γ in our setting using Pam2 lipopeptides (Fig. 2A). In this context, three markers for NK activation (19) were assessed with this system, IFN-γ production, up-regulation of NK activation markers and target cell (B16D8) cytotoxicity by NK cells (Fig. 2). IFN-γ was generated in the supernatants (sup) of NK cells (Fig. 2B) or BMDC/NK coculture (Fig. 2C, Fig. 3A left hand panel) in response to the control lipopeptides, Pam2CSK4 and Pam2Cys12. However, MALP-2s and f showed significantly low potentials for IFN-γ induction comparable to the negative control Pam2CSK.

The NK cell activation markers CD25 and CD69 were analyzed with BMDC co-cultured NK cells with or without the lipopeptide treatment by flow cytometry (Fig.

2D). Up-regulation of surface CD25 and CD69 was observed in NK cells incubated with BMDC stimulated with Pam2CSK4 and Pam2Cys12 but far less with MALP-2s and f.

Activated NK cells are a major source of IFN-γ which causes a variety of responses of the immune system. To further examine whether BMDC matured with Pam2CSK4 or MALP-2 drive NK-dependent IFN-γ secretion, we stimulated BMDC with these lipopeptide reagents for 4 hrs and then mixed with NK cells (ratio 1:2) for 20 hrs. Breferdin was added to the mixture in order to accumulate IFN-γ in the NK cells for the last 4 hrs of incubation. As shown in Fig. 2E, the TLR2 ligands Pam2CSK4 and Pam2Cys12 significantly increased the frequency of IFN-γ-secreting NK cells, while MALP-2f and s showed far less activity to produce IFN-γ in the NK cells.

Cytotoxic activity was evaluated using B16D8 cells as a target (10). BMDC-activated NK cells (see above) were incubated with B16D8 cells at a ratio of 30:1. Again, MALP-2s and f showed less effective killing against the target (Fig. 3B left hand panel). The other lipopeptides had sufficient killing activity compared to the control polyI:C: one of two examples assayed with different BMDC lots are shown in the figure.

3.3. *Participation of TLR2/MyD88 in MALP-2-mediated NK activation*

We next examined whether the lipopeptide-mediated IFN-γ secretion was dependent on MyD88 of BMDC. IFN-γ secretion was almost completely abrogated in the co-culture with MyD88-/- BMDC and wild-type NK cells in the presence of Pam2Cys12 and Pam2CSK4 (Fig. 3A), Similar tendencies were observed with MALP-2 peptides, which essentially evoked a minimal IFN-γ production, and the IFN-γ-induction was largely abrogated with MyD88-/- BMDC (Fig. 3A). The results were less prominently reproduced with TLR2-/- BMDC and wild-type NK cells (data not shown). Further confirmation was performed using the mixtures with wild-type NK cells and various lipopeptides. No direct activation of NK cells was observed in the absence of BMDC (data not shown).

The results were further confirmed with NK cytotoxic assay using NK cells cocultured with Pam2Cys lipopeptide-stimulated BMDC (Fig. 3B). When wild-type BMDC was used as a NK cell cytotoxicity inducer, full NK activation was induced by Pam2Cys12 or Pam2CSK4. MALP-2f and s were found to be inefficient NK activators (Fig. 3B). If wild-type BMDC were replaced with MyD88-/- BMDC, BMDC-enhanced NK cytotoxicity was abrogated (Fig. 3B). The MyD88 pathway in BMDC is crucial for BMDC-mediated NK cell activation.

3.4. *Combinational recognition of MALP-2s and f by TLR2 and TLR6*

TLR2 recognizes diacyl lipopeptide in combination with TLR6 (20) while TLR2 recognizes triacyl lipopeptide together with TLR1 (21). We found TLR2/6 cooperate to recognize *S. aureus* lipopeptides using HEK293 cells with TLR2/6 expression. Data testing MALP-2f for the usage of TLR2/6 are shown in Fig. 4. Single receptors of TLR1, TLR6 and TLR10 barely activate NF-κB by reporter assay and only TLR2 exhibited <60 fold ELAM promoter activation (data not shown). No enhanced activation was observed in combination with TLR2 and TLR1 or TLR2 and TLR10. Similar results on TLR2/6-mediated augmentation of reporter activation were observed with MALP-2s and Pam2CSK4 (data not shown). Hence, TLR6 helps to amplify the TLR2 signal by MALP-2 lipopeptide as in other Pam2Cys lipopeptides, but IFN-γ was minimally induced in the NK cells.

3.5. *Antitumor adjuvant activity against NK-sensitive tumor in vivo*

Recent studies revealed that intratumoral or i.p. injection of MALP-2 supresses pancreatic carcinoma in a mouse model (22). Tumor suppression is also observed with Pam2Cys type lipopeptides in B16D8 (NK-sensitive) implant mice (23). The antitumor function by MALP-2 is abrogated in MyD88-/- mice, suggesting that TLR2/MyD88 and following cell-mediated immunity play a major part of tumor suppression (23). We tested whether MALP-2 injected s.c. induces growth retardation of the tumor (NK-target B16D8 cells) via NK activation (Fig. 5). Pam2CSK4 s.c. injected around tumor exhibited tumor growth retardation (Fig. 5A). This Pam2CSK4 activity was abrogated by injection of asialoGM-1 Ab (data not shown). In contrast, no tumor growth retardation was observed in this NK-sensitive tumor by s.c. injected MALP-2 (Fig. 5B). The results infer that MALP-2 exerts only minimal potential if any, to activate NK cells through BMDC *in vitro* and *vivo*. Unlike BCG-CWS (19) or polyI:C (8-10), MALP-2 barely suppresses tumor growth in this mouse system.

4. Discussion

Recent studies demonstrated that mDC induce NK activation by stimulation with TLR2 in mDC (12). This NK activation occurs in a distinct mode of those reported in the TICAM-1/IPS-1 pathways for type I IFN induction because this mode of NK activation is derived through the MyD88 pathway in the mouse system. Indeed, MyD88 has been reported to participate in NK cell activation induced by *Plasmodium falciparum*-infected erythrocytes, but TLR2 response was not mandatory in the reported human case (24). In

another report, direct TLR2 stimulation in NK cells but not mDC was critical for NK cell activation in a vaccinia infection system (25). We then tested whether a TLR2 agonist MALP-2 harbors adjuvant potential of mDC-mediated NK activation.

NK activation fails to be induced by MALP-2-stimulated mDC judged by IFN-γ production, up-regulation of NK-activation marker CD25 and CD69, and cytotoxicity against the NK target B16D8 cells. Cytokines with NK activation properties such as IFN-α/β, IL-15 and IL-12p70 are not up-regulated in mDC in response to MALP-2, although a regulatory cytokine IL-10 is produced by stimulation with MALP-2 (3,5). Finally, s.c. administration of MALP-2 did not result in retardation of tumor growth in mice with B16D8 tumor burden. Although Pam2CSK4 having 6 a.a.-stretch following the diacyl residue acts as an NK-activating reagent (10,18), two forms of MALP-2 with short (6 a.a.) or long (14 a.a.) peptide barely exhibits antitumor activity. Hence, NK activation is a phenotype induced by a limited group of Pam2Cys lipopeptides, and the peptide sequence is critical for inducing mDC-mediated NK activation. Our results infer that TLR2-dependent mDC response to drive NK activation largely relies on the peptide sequences of lipoproteins. TLR2-stimulating lipopeptides are not unifunctional: some are active on NK cells but not others.

TLR2 in conjunction with TLR6 serves as an adjuvant receptor with potent cytokine inducing ability, accompanied with up-regulation of IL-1β, IL-6, IL-12p40 and TNF-α. Apoptosis and NO production are also evoked through TLR2 stimulation (2). The cytokine profile induced by MALP-2 indicates that macrophages and mDC differentiated from monocytes are targets for MALP-2 via their TLR2. TLR2 agonists facilitate induction of CTL and CD4 T cells against specific antigens (23,26). In fact, M161Ag potently induces complement-associated inflammation (27) and maturation of immature mDC (16,28). Although cytoplasmic sensors for bacterial lipoproteins may in part participate in the functional properties of MALP-2 (29), TLR2 agonistic activity in MALP-2 would involve cytokine-inducing properties but not antitumor function by intensifying *in vivo* NK activation.

Adjuvants are important for induction of vaccine immunity. Cancer immunotherapy has been developed using a variety of adjuvants. Intratumoral or intraperitoneal injection of MALP-2 has been attempted to induce suppression of pancreatic carcinoma in a mouse model (22). In clinical trials, MALP-2 alone or in combination with gemcitabine was used for the treatment of unresectable pancreas carcinoma (6). The rationale of this approach is based on the ability of MALP-2 to [1] act as a cytokine inducer (30), [2] activate murine as well as human DC to express co-stimulatory molecules (31), [3]

induce a T-helper (Th) 1/2 response (32) and, most importantly, prolong survival in a mouse model of an orthotopic, syngeneic pancreas tumour (22). Although Pam2 lipoproteins often induce an inhibitory cytokine IL-10 and regulatory T cells (Yamazaki S, unpublished data), no report mentioned the effect of these factors on MALP-2 adjuvant potential. We favored interpretation that the beneficial effects were due to immune activation, as we observed an increase in the expression of co-stimulatory molecules on lymphocytes, and cytotoxic T and NK cells infiltrating the tumor. However, our experiments with tumor-loaded mice showed that s.c. administration of MALP-2 confers no NK cell-mediated tumor regression on B16D8-implant mice. This unexpected result may be due to specific TLR2-agonistic properties of MALP-2 compared to peptidoglycan (that induces IL-12p70 in human mDC) (33) or instability of the lipid moiety of MALP-2. MALP-2 is degraded by two different mechanism in inflamed tissue: de-esterification and oxidation of the thioester bridge (6,34), thereby disappearing from the skin with a half time of ~ 20 hrs. Further modification will be required for *in vivo* use of this reagent.

5. ***Acknowledgements***

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Figure legends

Figure 1. BMDC cytokine production and maturation in response to TLR2 agonists. (A, B, C) Cytokine production by BMDC stimulated with Pam2Cys-containing peptides. BMDC prepared from wild-type mice were treated with indicated Pam2Cys-containing peptides (100 nM) for 24 hrs. IL-6 (A), IL-12p40 (B), and IL-12p70 (C) concentrations in the supernatant were measured by ELISA. (D, E) Flow cytometric analysis of CD86 and I-Ab expression of BMDC stimulated with Pam2Cys-containing peptides. Typical examples of flow cytometric analysis (D). Summary of CD86 and I-Ab expression on the BMDC (E).

Figure 2. Pam2Cys-containing peptides activates NK cells through TLR2 in BMDC. (A, B, C) BMDC and NK cells prepared from wild-type mice were stimulated with Pam2Cys-containing peptides for 24 hrs (A, B). Alternatively, BMDC were stimulated with Pam2Cys-containing peptides for 4 hrs. Then, NK cells prepared from wild-type mouse spleen were cocultured with the BMDC for 24 hrs. IFN-γ levels in the culture supernatant are shown. (D) CD25 and CD69 expression on NK cells co-cultured with BMDC in the presence of Pam2Cys-containing peptides. The NK cell populations (marked with NK1.1) were gated on the display of FACS and levels of CD25 or CD69 (inset values) were examined as shown in the graphs. (E) Intracellular IFN-γ staining of NK cells cocultured with BMDC in the presence of Pam2Cys-containing peptides as above. Cells were treated with breferdin and then permeabilized. Intracellular IFN-γ was detected by specific mAb. IFN-γ positive cells are marked with square and their frequencies are indicated by inset values.

Figure 3. IFN-γ production and cytotoxic activity of NK cells co-cultured with BMDC in the presence of Pam2Cys-containing peptides. Wild-type and MyD88 -/- BMDC were stimulated with Pam2Cys-containing peptides for 4 hrs. Then, the BMDC were co-cultured with wild-type NK cells for 24 hrs. (A) IFN-γ levels in culture supernatant were determined by ELISA. (B) Cytotoxic activities of NK cells were measured by ^{51}Cr release assay. B16D8 cells were used as a target. E/T ratio = 20.

Figure 4. TLR6 facilitates the recognition of MALP-2 by TLR2. HEK293 cells were transfected with the plasmids encoding TLR2 and/or TLR6, and ELAM-luciferase reporter. After 24 hrs, the cells were treated with MALP-2f for 6 hrs. Then, luciferase activity of the cell lysates was measured. Similar results were obtained with MALP-2s

(not shown).

Figure 5. MALP-2 fails to inhibit tumor growth *in vivo*. B16D8 cells were transplanted subcutaneously into mice at day 0. Mice (16 week-old, female) were treated with Pam2CSK4 (A) or MALP-2s (B) at day 0, 3, 7, 9, 13, 17 as described in *Materials and Methods*. No tumor growth retardation was observed when MALP-2s was replaced with MALP-2f (not shown). Each group consists of n=4. Surface diameters of the implanted tumors were measured. Mean ± SD are shown.

Table 1. Lipopeptides used in this study

Name	Peptide structure	Mr (Dalton)	Ref.
Pam2CSK	CSK	887.3	(19)
Pam2CSK4	CSKKKK	1271.8	(13)
Pam2Cys12	CSTSEVIGEKI	1716.2	(19)
MALP-2s	CGNNDE	1201.5	(*)
MALP-2f	CGNNDESNISFKEK	2135.6	(13)

* This paper.

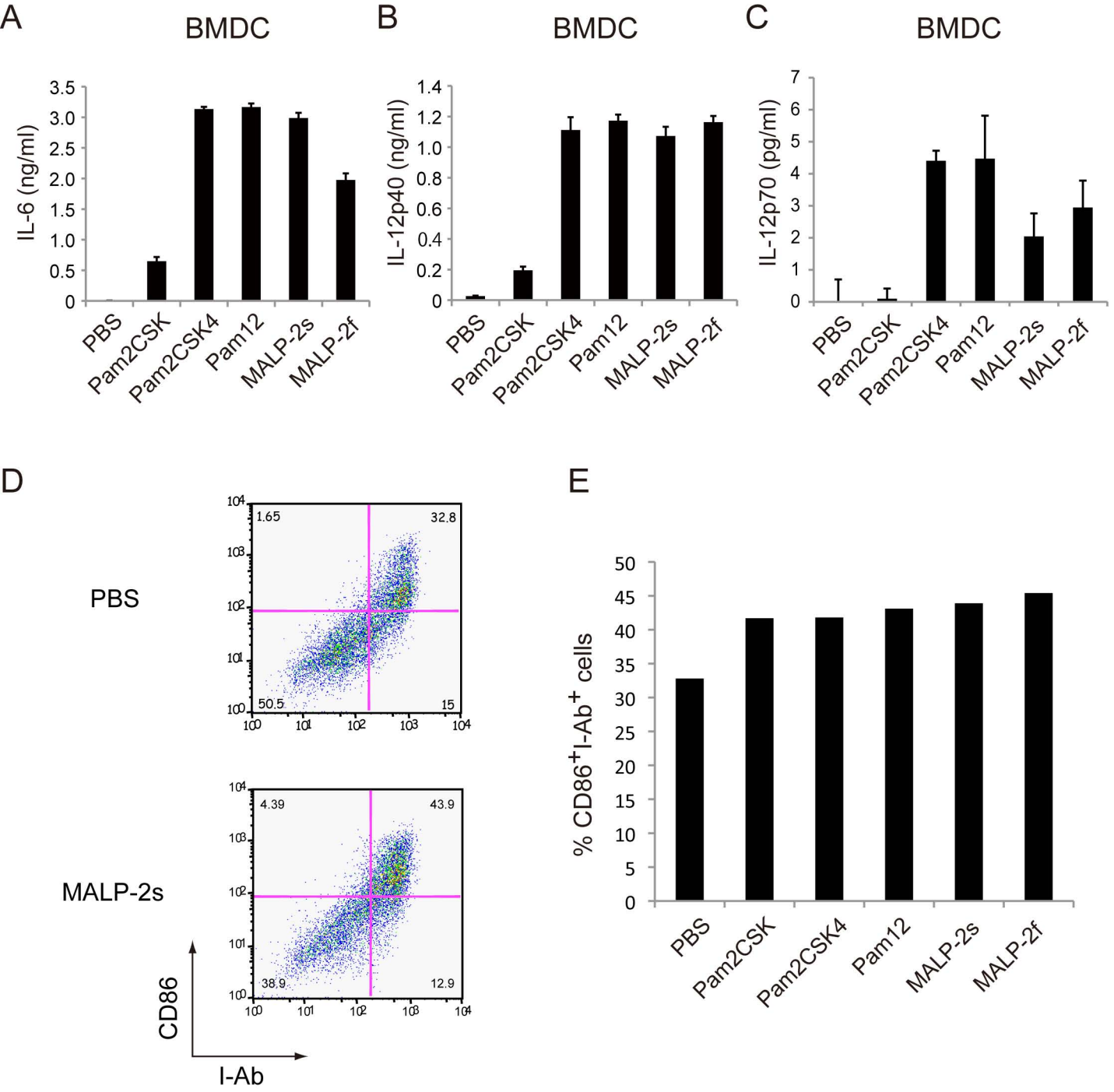

A
BMDC
IL-6 (ng/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
B
BMDC
IL-12p40 (ng/ml)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
C
BMDC
IL-12p70 (pg/ml)
7
6
5
4
3
2
1
0
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
D
PBS
1.65
32.8
50.5
15
MALP-2s
4.39
43.9
38.9
12.9
CD86
I-Ab
E
% CD86+I-Ab+ cells
50
45
40
35
30
25
20
15
10
5
0
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f

Figure 1

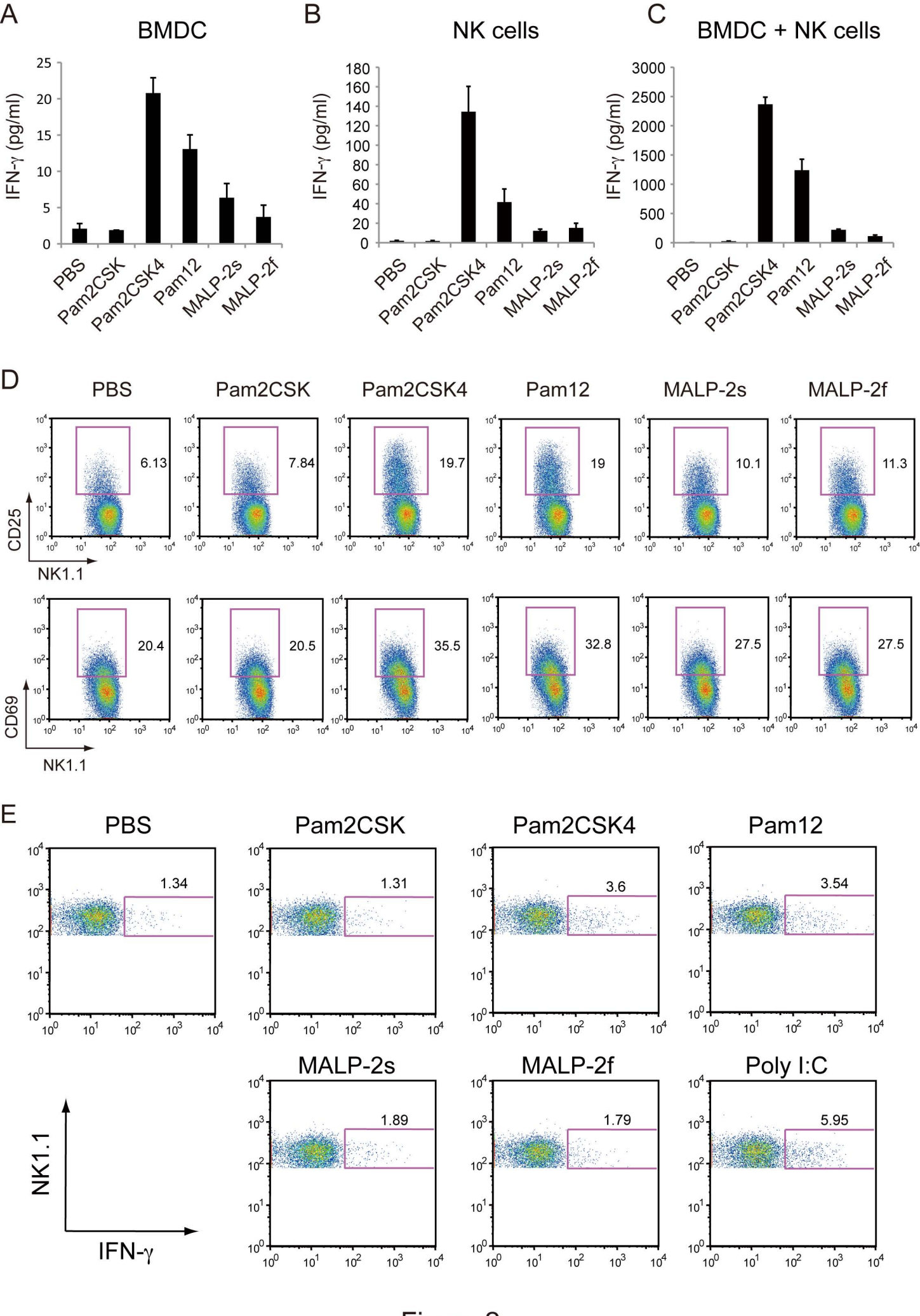
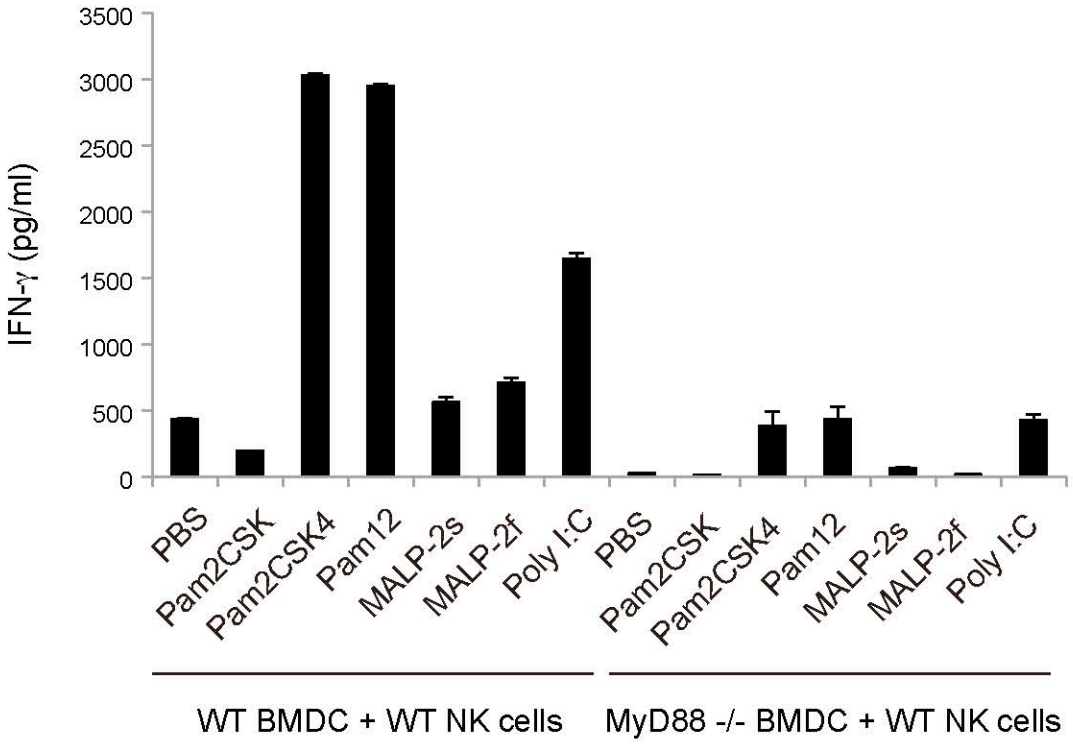

A
BMDC
IFN-γ (pg/ml)
25
20
15
10
5
0
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
B
NK cells
IFN-γ (pg/ml)
180
160
140
120
100
80
60
40
20
0
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
C
BMDC + NK cells
IFN-γ (pg/ml)
3000
2500
2000
1500
1000
500
0
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
D
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
6.13
7.84
19.7
19
10.1
11.3
CD25
NK1.1
20.4
20.5
35.5
32.8
27.5
27.5
CD69
NK1.1
E
PBS
Pam2CSK
Pam2CSK4
Pam12
1.34
1.31
3.6
3.54
MALP-2s
MALP-2f
Poly I:C
1.89
1.79
5.95
NK1.1
IFN-γ

Figure 2

A


IFN-γ (pg/ml)
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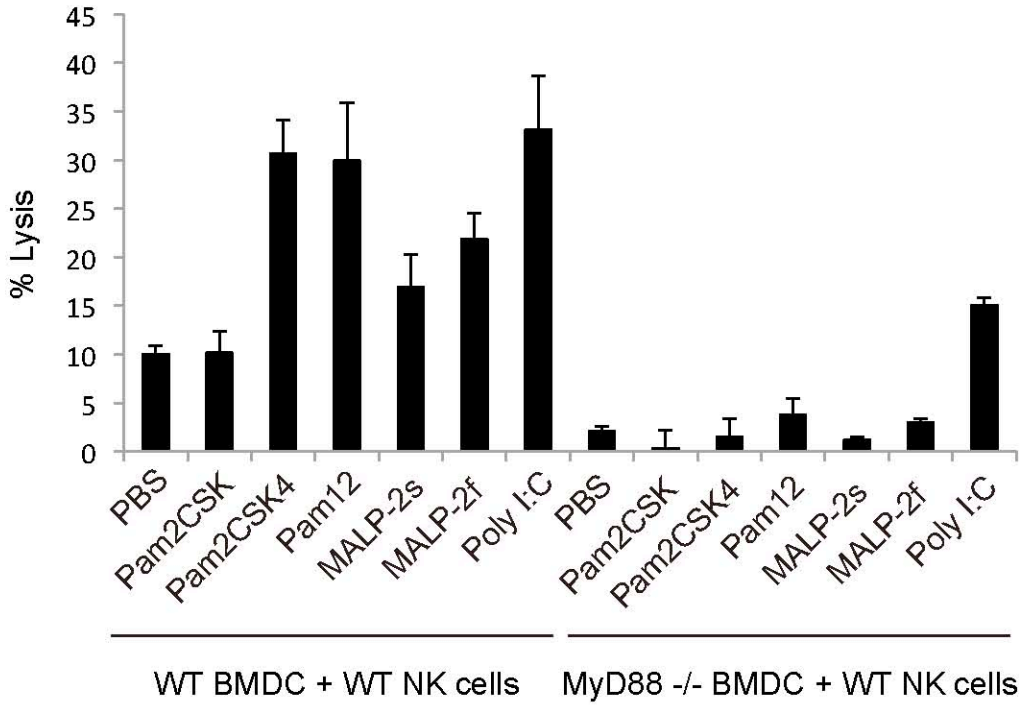
3000
2500
2000
1500
1000
500
0
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
Poly I:C
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
Poly I:C
WT BMDC + WT NK cells
MyD88 -/- BMDC + WT NK cells

B


% Lysis
45
40
35
30
25
20
15
10
5
0
PBS
Pam2CSK
Pam2CSK4
Pam12
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MALP-2f
Poly I:C
PBS
Pam2CSK
Pam2CSK4
Pam12
MALP-2s
MALP-2f
Poly I:C
WT BMDC + WT NK cells
MyD88 -/- BMDC + WT NK cells

Figure 3

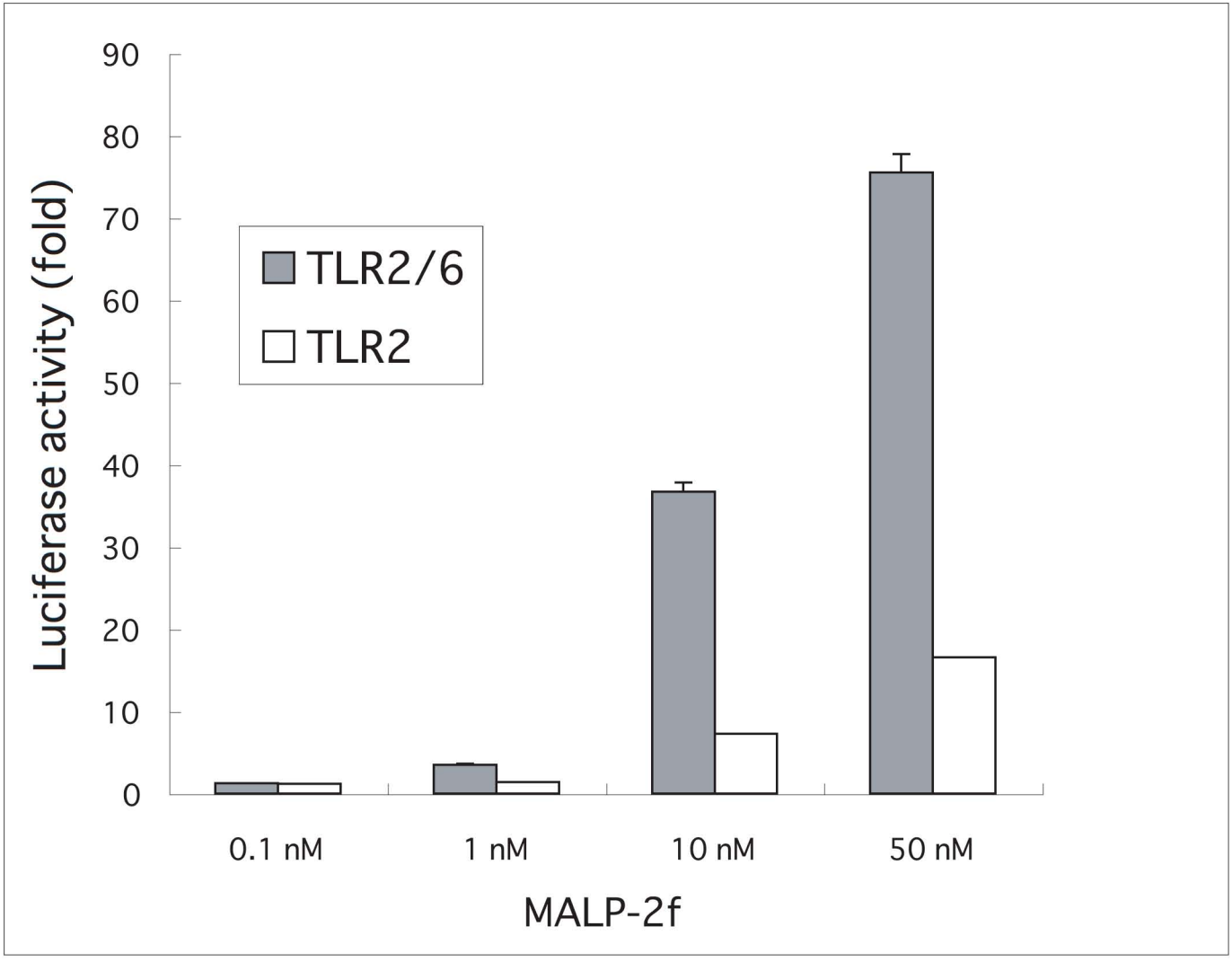
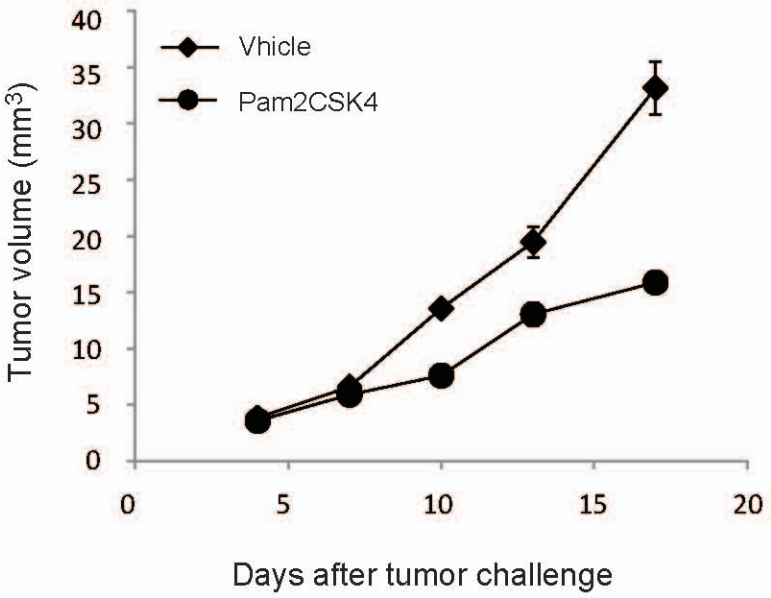

90
80
70
60
50
40
30
20
10
0
Luciferase activity (fold)
TLR2/6
TLR2
0.1 nM
1 nM
10 nM
50 nM
MALP-2f

Figure 4

A


Vhicle
Pam2CSK4
Tumor volume (mm3)
Days after tumor challenge
0
5
10
15
20
25
30
35
40

B

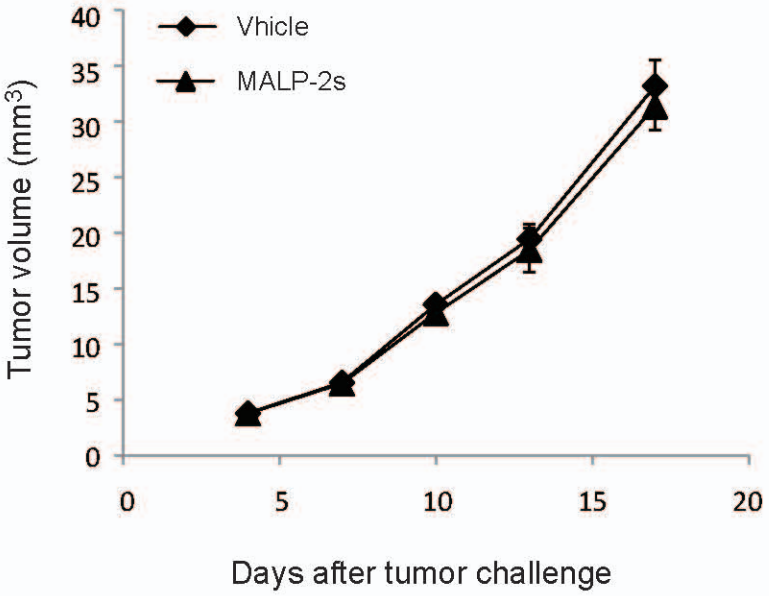

Vhicle
MALP-2s
Tumor volume (mm3)
Days after tumor challenge
0
5
10
15
20
25
30
35
40

Figure 5