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Adjuvant engineering for cancer immunotherapy: development of a synthetic TLR2 ligand with increased cell adhesion

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Running title: **A synthetic TLR2 ligand containing an RGD motif**

Footnote

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Abbreviations: **BCG**, Mycobacterium bovis bacillus Calmette-Guérin; **BMDCs**, bone marrow–derived dendritic cells; **BRM**, biological response modifiers; **CWS**, cell-wall skeleton; **DCs**, dendritic cells; **IFN**, interferon; **IL**, interleukin; **MALP-2**, macrophage-activating lipopeptide-2; **MyD88**, myeloid differentiation protein 88; **Pam2Cys** or **P2C**, S-(2,3-bispalmitoyloxypropyl)-cysteine; **TICAM**, Toll/IL-1 receptor homology-containing adaptor molecule; **TLR**, Toll-like receptor

Abstract

It is essential to develop effective immunoadjuvants for tumor immunotherapy. Various applications of mycobacterium bovis bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) to tumor immunotherapy have been examined. Because the BCG-CWS is a macromolecule that cannot be synthesized, development of an alternative synthetic adjuvant is required for a stable supply. Here, we designed a new adjuvant based on the structure of macrophage-activating lipopeptide-2 (MALP-2), which is a TLR2 ligand same as BCG-CWS. MALP-2 (S-(2,3-bisphosphatidyl)Cys-Gly-Asn-Asn-Asp-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-Lys, P2C-GNNDESNISFKEK) is a lipopeptide identified from a Mycoplasma species that can be chemically synthesized. We substituted a functional motif peptide, RGDS, for bacterial origin in the structure of MALP-2, creating P2C-RDGS, a novel molecule. We selected RGDS because the RGDS sequence is an integrin-binding motif, and various integrins are expressed on immune cells, including dendritic cells (DCs). Thus, this motif adds functionality to the ligand. P2C-RGDS activated DCs *in vitro* as much as MALP-2, and the RGDS motif partially contributed to the DC activation. P2C-RGDS largely depended on the RGDS motif to induce IFN- γ from splenocytes. This suggests that the upregulation of lipopeptide adhesion correlates with the activation of immune cells. P2C-RGDS showed higher

activity than MALP-2 in inducing IFN- γ production from splenocytes *in vitro* and cytotoxicity, and in inhibiting tumor growth *in vivo*. This process of designing and developing synthetic adjuvants has been named “adjuvant engineering”, and the evaluation and improvement of P2C-RGDS is the first step in developing a stronger synthetic adjuvant in the future.

Introduction

Recently, bacterial adjuvants used for cancer immunotherapy in the 1970s as biological response modifiers (BRM) have been re-evaluated.¹⁻³ Cancer antigens that had been identified in many laboratories were tested as peptide vaccines for clinical applications, but the peptides alone did not activate the immune system enough.⁴ These results suggested that the activation of the innate immune system, including dendritic cells (DCs), by a supporting adjuvant was important.⁵⁻⁸ In peptide vaccine therapy, T cells of the acquired immune system play an important role in attacking the tumor.⁹ DCs have a key role in an initial start of immune systems to an antigen and control the acquired immune system. The identification of the Toll-like receptor (TLR) family advanced the understanding of DC function and of adjuvants, because almost all microbial adjuvants work as TLR ligands and activate DCs.¹⁰⁻¹² These findings provide a mechanism for adjuvant therapy.

Dr. Azuma developed BCG-CWS, a cell-wall skeleton preparation of *Mycobacterium bovis* bacillus Calmette-Guérin,^{13,14} as an anticancer immunotherapeutic adjuvant. Although many studies of BRM have been discontinued, basic and clinical research on BCG-CWS have continued at Osaka Medical Center for Cancer. We reported that BCG-CWS is a ligand of TLR2/4 (refs. 15,16,17) and acts as an effective adjuvant to induce CTLs when treated with irradiated tumors in a mouse experimental model. These

activities are mediated via myeloid differentiation protein 88 (MyD88),¹⁸ which is a TLR adaptor molecule. The effectiveness of BCG-CWS in improving the prognosis for cancer patients after surgery was confirmed in our clinical research.¹⁹

Interleukin (IL)-23 and interferon (IFN)- γ are the main cytokines induced by BCG-CWS *in vivo*^{14,19,20} and are important for antitumor immunity.^{21,22} IL-12 is well known as an anticancer cytokine,²³ and IL-23 shares the IL12p40 subunit with IL-12.²² Unexpectedly, IL-23 advanced tumor growth in experiments with IL-23R^{-/-} mice or neutralizing antibodies by interacting with Th17 cells.²⁴⁻²⁶ However, it was also reported that systemic administration of IL-23 produces antitumor effects similar to those of IL-12,²⁷ and TLR2 ligands exhibit antitumor activity²⁸⁻³⁰ that may be mediated by the induction of IL-23.

Although BCG-CWS is an effective adjuvant, it cannot be chemically synthesized and is prepared from bacterial cells, there is a problem in purity, stability and stable supply. Therefore, there is a need to develop new synthetic adjuvants as effective as BCG-CWS that can be stably produced. We have designed such adjuvants based on the structure of the TLR2 ligand and in consideration of the need for IL-23 induction. MALP-2, a lipopeptide of mycoplasmic origin, is a TLR2 ligand that can be chemically synthesized. No functional consensus sequences were identified in MALP-2. The N-terminal

cysteine of the 13-amino-acid peptide of bacterial origin was modified with 2 palmitates (Pam2Cys or P2C),³¹ but P2C alone does not work as a TLR2 ligand.³² Bacterial and synthetic TLR2 ligands (MALP-2, FSL³², P2C-SKKKK³³) contain mostly hydrophilic peptides, and the presence of solubilizers critically affects their TLR2 agonistic ability,³⁴ suggesting that the activity of compounds as TLR2 agonists correlates with their solubility.

CD11c is a member of the integrin superfamily and is known to be a marker of DCs.³⁵ DCs also express other integrin molecules such as α V/ β 3 and α 5/ β 1, and the RGDS motif specifically binds to these integrins.³⁶ Virus particles expressing proteins containing the RGD motif efficiently infect DCs.³⁷ Therefore, we developed a new TLR2 ligand by replacing the peptide of bacterial origin with a hydrophilic functional motif (adjuvant engineering). We linked P2C and the RGDS peptide to increase the efficiency of ligand adherence to DCs or other immune cells, and examined the effect of the new adjuvant on antitumor activities *in vitro* and *in vivo*.

Materials and Methods

Mice, cells, and reagents

TICAM-1^{-/-} mice were generated in our laboratory.² MyD88^{-/-} mice were provided by Shizuo Akira (Osaka Univ.).³⁸ The mice were maintained under specific pathogen-free conditions in the animal facility of the Osaka Medical Center. They were backcrossed with C57BL/6 mice >8 times before use. Wild-type C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). All animal experiments were consented by the committee in our institute. EG7 cells are ovalbumin-transfected EL4 and were obtained from ATCC (Manassas, VA, USA).³⁹ Cell lysates were prepared by the freeze-thawing method.

Preparation of mouse BMDCs, splenocytes, and lymph node cells

Bone marrow-derived DCs (BMDCs) were prepared as reported^{35, 40} with minor modifications. BMDCs were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) containing 10 ng/ml mouse granulocyte-macrophage colony-stimulating factor (PeproTech EC Ltd., London, UK), and 50 μ M 2-mercaptoethanol (Invitrogen, Carlsbad, CA), 10 mM HEPES and 10% FCS (Bio Whittaker, Walkersville, MD). The lymph node cells and splenocytes were prepared from inguinal lymph nodes and spleens of mice by using

Lympholyte-M (Cedarlane, Ontario, Canada).

In vitro assay

Simple stimulation assay, BMDCs or splenocytes were cultured with 10µg/ml of BCG-CWS¹⁸, 100nM of MALP-2 and the designed lipopeptide (purity >90%; Biologica Co., Aichi, Japan) for 24 h (BMDC, FACS), 48 h (BMDC, ELISA) or 72hr (splenocytes, ELISA). In the inhibition assay, BMDCs or splenocytes were pre-incubated with 5 or 50 µM of RGDS peptide at 4°C for 30 min before stimulation of TLR2 ligands at 4°C for 60 min. The cells were then washed and recultured for 24 h (BMDC, FACS) or 72 h (splenocytes, ELISA). The Mixed Lymphocyte Reaction (MLR) assay was previously described,⁴⁰ and the results were analyzed as uptake of [³H]thymidine (1 µCi/well; Amersham Biosciences, Piscataway, NJ). BMDCs stimulated with TLR2 ligands for 24 h (C57BL/6, 5×10^4 cells) were cocultured with CD90-positive T cells (BALB/c, 10^5 cells) for 72 h. To exclude the possible effect of contaminating lipopolysaccharide, lipopeptides were pretreated with polymyxin B (SIGMA-Aldrich Chemical Company, St. Louis, MO) at 37°C for 60 min.

FACS analysis and ELISA

For FACS analysis, cells were suspended in PBS containing 0.1%

sodium azide and 1% FCS, and then incubated for 30 min at 4°C with FITC-conjugated anti-mouse CD80, anti-mouse CD86, anti-mouse CD8, or isotype control antibodies, phycoerythrin-conjugated anti-mouse CD4 or isotype control antibodies (eBioscience, San Diego, CA). The cells were washed, and their fluorescence intensities were measured by FACS analysis. For ELISA, samples were stored at -80°C and analyzed with ELISA kits for IFN- γ , TNF- α , and IL-12p40 (Biosource, Camarillo, CA).

In vivo therapy model

C57BL/6 mice were shaved on the back and injected subcutaneously with 200 μ l of $1-2 \times 10^6$ syngeneic EG7 cells in PBS on day 0. Thereafter, the treatment was performed 3 times, on days 16, 20, and 23, and tumor volumes were measured using a caliper every 2 d. 50 μ l of a mixture, 10 nmol of lipopeptide and the cell lysate of 2×10^5 EG7 cells, was injected intradermally around the transplanted tumor. Tumor volume was calculated using the formula: Tumor volume (cm^3) = (long diameter) $\times$ (short diameter) $\times$ (short diameter) $\times$ 0.4. Statistical analysis was performed with the student t-test.

Ex vivo assay

C57BL/6 mice were treated intradermally with a mixture of 10 nmol of lipopeptide and the cell lysate of 2×10^5 EG7 cells every 3 d for >4 treatments.

At 24 h after the last treatment, the mice were sacrificed by etherization, splenocytes and lymph node cells were prepared and cultured for 4 d to be primed by migrating DCs and macrophages. Then, the cytolytic activities of lymph node cells were analyzed with a ^{51}Cr release assay.¹⁸ The percentage of specific lysis was calculated using the formula: %Specific lysis = $[(\text{experimental release} - \text{spontaneous release})/(\text{total release} - \text{spontaneous release})] \times 100$. The ratios of CD8-positive in lymph node cells, and of CD4 or CD8-positive in splenocytes were analyzed by FACS.

Results

To design a new TLR2 ligand with activity equivalent to BCG-CWS, we connected the minimum lipopeptide unit, P2C, to the RGDS integrin-binding motif to increase adherence to DCs (Fig. 1a). The hydrophobicity and pI of P2C-RGDS were similar to those of MALP-2, and the molecular weight of P2C-RGDS was half that of MALP-2 (Fig. 1b).

First, we evaluated whether these synthetic adjuvants activated BMDCs *in vitro* when these compounds were simply added to the culture medium. The P2C-RGDS enhanced the expression of CD80 and CD86 on BMDCs to a level equal to that of MALP-2, the positive control (Fig. 2a), whereas P2C did not. P2C-RGDS also enhanced the production of IL12p40 and TNF- α (Fig. 2b) and the proliferation of allogeneic T cells cocultured with the BMDCs. Thus, P2C-RGDS and MALP-2 stimulate DCs equally *in vitro*.

MALP-2 is a ligand of TLR2/6 that activates DCs through MyD88. To examine the TLR signaling pathway of P2C-RGDS, BMDCs were prepared from MyD88^{-/-} or TICAM-1^{-/-} mice and stimulated with these synthetic lipopeptides. Both MALP-2 and P2C-RGDS enhanced the expression of CD80 and CD86 on BMDCs derived from WT or TICAM-1^{-/-} mice, but not MyD88^{-/-} mice (Fig. 3), suggesting that P2C-RGDS is a TLR2 ligand with activity similar to that of MALP-2 *in vitro*.

To test whether the function of P2C-RGDS as a TLR2 ligand depends

not only on its hydrophilicity, but also on the motif-specificity of the peptide sequences, we performed peptide-dependent inhibition assays. At first, the inhibition peptide did not work in any experiments when DCs were treated with each compound for 24 h (data not shown); therefore, we changed the DC stimulation conditions to affect the adherence of the compounds. DCs were treated with each compound at 4°C for 1 h, then washed and recultured for 24 h. The addition of RGDS peptide partially attenuated the expression of CD86 on BMDCs induced by P2C-RGDS, but not that induced by MALP-2 (Fig. 4a). This result indicates that the hydrophilicity of RGDS is a major contributor to the *in vitro* stimulation of DCs, but the function of the RGDS motif also weakly contributes to its activity.

Next, we evaluated the activities of P2C-RGDS using *in vitro* whole splenocyte stimulation and an *in vivo* tumor-implantation model. Although the activities of P2C-RGDS and MALP-2, as measured by IFN- γ production, were almost the same and were weaker than BCG-CWS when splenocytes were simply stimulated with each compound for 72 h (Fig. 4b). Then we evaluated the inhibitory effect of IFN γ production of splenocytes under 4°C for 1 h. P2C-RGDS induced IFN γ more strongly than MALP-2 and its induction was most attenuated by the RGDS peptide. Furthermore, the splenocytes stimulated with P2C-RGDS produced as much IFN- γ as those stimulated with BCG-CWS under 4°C for 1 h. Finally, we investigated the

antitumor activity of P2C-RGDS *in vivo* using a tumor-implantation model. The mice were transplanted with EG7 on day 0, and treated with synthetic lipopeptide (10 nmol) and the cell lysate of EG7 cells (2×10^5) on day 16, 20, 23. Although the tumor volume was slightly smaller in the MALP-2–treated mice than in the control mice treated with P2C, the difference was not significant. However, P2C-RGDS showed significant anti-tumor effect (student t-test, $P < 0.05$ vs. control). We next isolated lymph node cells from the mice immunized with EG7 lysate and P2C-RGDS or MALP-2, and measured the cytotoxicity against EG7 using a ^{51}Cr release assay. P2C-RGDS induced stronger cytotoxic activity against EG7 than MALP-2 (Fig. 5b). Then we continuously cultured these lymph node cells with live EG7 cells for 96 h and analyzed the proportion of CD8-positive cells by FACS. CD8-positive cells in lymph nodes derived from P2C-RGDS–treated mice remained at about 16% of total cells, but most CD8-positive cells from MALP-2–treated mice were lost after culture with EG7 (Fig. 5c). We also used FACS to analyze the ratios of CD4- or CD8-positive cells of total splenocytes derived from immunized mice. CD8-positive cells increased more among splenocytes derived from mice treated with P2C-RGDS than among those from mice treated with MALP-2 (Fig. 5d). Although the necrosis appeared on surface of tumor by P2C-RGDS but not MALP-2 treatment, and CD8-positive cells were detected around necrosis area by immunostaining (data not shown). These

data suggest that P2C-RGDS induces and activates CTL more efficiently than MALP-2.

Discussion

It has been reported that natural TLR2 ligands of bacterial origin, such as FSL-1 and MALP-2, effectively activate CD8-positive T cells and induce antitumor activity. Because these lipopeptides are hydrophilic, it was predicted that the hydrophilicity of the peptide might be important for its activity.³¹⁻³⁴ Many researchers have used a chemobiologic strategy of making amino acid replacements in nature bacterial lipopeptide to explore which lipopeptide peptide sequences provide effective adjuvant activity.⁴¹ Using this strategy, Takeda Co Ltd. developed Tan-1511 analogues, which has high activity for the induction of granulopoiesis.⁴¹ Another strategy has to randomly search for effective sequences or peptides that enhance TLR2 ligand activity.⁴² The purpose of our current project was to develop as a new synthetic adjuvant a TLR2 ligand with an additional function through the addition of a hydrophilic, functional peptide (Fig. 1). Our adjuvant-engineering project is a new strategy to incorporate biological findings into drug design.

We developed the compound P2C-RGDS, which was as effective as MALP-2 in generating BMDCs responses *in vitro*, such as the enhancement of a maturation marker (CD80 and CD86) and cytokine induction when DCs were cultured with each compound for 24-48 h (Fig. 2). P2C-RGDS and MALP-2 activated DCs through MyD88 (Fig. 3), which is a major TLR2

signal adaptor molecule.³⁸ In this study, although P2C-RGDS has an additional sequence (RGDS) that was required, in part, to activate DCs, P2C-RGDS and MALP-2 showed similar activities *in vitro* (Fig. 4). We therefore predicted that the peptide sequence of MALP-2 may also adhere to DCs, because MALP-2 also activated BMDCs when incubated even at 4°C for 60 min. We also hypothesized that if the adhesive abilities of RGDS were not reflected in this simple experimental system, even though diffused lipopeptide stimulated DCs in culture medium, the differences might appear *in vivo* or in the responses of whole splenocytes stimulated with compounds for a short time. In fact, whole splenocytes stimulated with P2C-RGDS produced more IFN- γ than MALP-2 (Fig. 4), and P2C-RGDS more effectively retarded tumor growth and induced CD8-positive cells than MALP-2 (Fig. 5). The stronger IFN- γ induction by P2C-RGDS might be due to its retention in the culture system by adherence to various cells among the splenocytes. These data suggest that the adhesion efficiency of compounds also contributes to adjuvanticity and antitumor activity *in vivo*. In another experiments, the tumor volume of BCG-CWS treatment group was about 60% of control on day 22 (data not shown), the therapeutic effects of P2C-RGDS were almost equivalent to BCG-CWS. However BCG-CWS must be emulsified with drakeol, there is an advantage for P2CR to use easily.

The induction of IFN- γ production by BCG-CWS treatment is one of

the indexes for continuing treatment in our clinical application^{14, 19}, and the response can be confirmed in the mouse. IFN- γ stimulation upregulates the expression of MHC class I on tumor cells,⁴³ and therefore presumably improves recognition of tumors by immune cells, leading to increased suppression of tumor growth. Although we did not design this compound specifically to induce IFN γ , we thought it was possible to support IFN- γ production by stimulation of CD8-positive cells with antigen peptides. In fact, splenocytes stimulated with only P2C-RGDS in the absence of antigen peptide produced IFN- γ more efficiently than MALP-2. It will be necessary to analyze this mechanism in the future.

Integrins are expressed on various tumor cells.⁴⁴ We confirmed that EG7 cells also express integrin α V, β 1, and β 3 (data not shown). However, there was no direct cytotoxicity to EG7 cells treated with P2C-RGDS at concentrations up to 100 nM (data not shown). On this basis, we predict that these compounds exhibit their anticancer activity via activation of the immune system. Here, we examined whether including an integrin-binding motif in a TLR2 ligand would increase the effectiveness of the compound as an antitumor adjuvant by enhancing adhesion of the ligand to DCs. It seemed that DCs efficiently recognized P2C-RGDS, which adhered to and were maintained around cells, and P2C-RGDS showed stronger antitumor activity than MALP-2 *in vivo*. Targeting peptides are used to elicit a strictly selective

response among immune cells. A targeted strategy can effectively activate immune cells at a low concentration while not affecting other cells whose activation might lead to side effects. The lipopeptides we obtain from our strategy do not exist in nature and may therefore have new activities. We have already made more than 20 TLR2 ligands with 10 alternative functions by adjuvant engineering and are working to develop the strongest adjuvant through continued evaluation and improvements.

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Figure Legends

Fig. 1 The structure of our adjuvant, a synthetic Toll-like receptor 2 ligand containing the RGDS motif.

(a) The structure of MALP-2. The N-terminal cysteine of a peptide derived from a mycobacterium is modified with 2 palmitates (Pam2Cys, P2C). We conjugated RGDS to P2C to form P2C-RGDS because of its hydrophilicity and its additional function as an integrin-binding motif for cell adhesion. (b) The structure, molecular weight, isoelectric point (pI), and hydrophobicity of synthetic (P2C-RGDS, P2C-SKKKK) and natural (MALP-2, FSL-1) lipopeptides used in this study. The pI and hydrophobicity were calculated by ProtParam tools (<http://br.expasy.org/tools/protparam.html>). The pI and hydrophobicity of P2C-RGDS were almost equivalent to those of MALP-2. The molecular weight of P2C-RGDS was about half that of MALP-2. N: natural TLR2 ligand, S: synthetic TLR2 ligand. The asterisks (*) indicate acidic peptides. The gray boxes indicate hydrophobic amino acids.

Fig. 2 P2C-RGDS activates bone marrow–derived dendritic cells (BMDCs) as much as MALP-2 *in vitro*.

(a) The enhancement of CD80/CD86 expression of BMDCs stimulated with the indicated compounds (100 nM) for 24 h was observed with FACS analysis.

P2CR, P2C-RGDS. (b) IL12p40 and TNF- α production of BMDCs stimulated with each compound for 48 h was determined by ELISA. (c) The proliferation of allogeneic T cells co-cultured with activated-BMDCs for 72 h were measured with the [3 H]thymidine uptake method. BMDCs were treated with each compound for 24hr before co-cultured with T cells. CPM, count per minute.

Fig. 3 P2C-RGDS and MALP-2 equivalently activate BMDCs in a MyD88-dependent manner *in vitro*.

BMDCs were prepared from mice lacking TLR adaptor molecules (MyD88^{-/-} or TICAM-1^{-/-}) and analyzed with FACS. CD80 and CD86 expression were observed by FACS analysis 24 h after BMDCs were stimulated with MALP-2 or P2C-RGDS. The numbers in the panels represent mean fluorescence intensity. P2C-RGDS and MALP-2 activated BMDCs via the MyD88 but not the TICAM-1 pathway. P2CR, P2C-RGDS.

Fig. 4 P2C-RGDS activates BMDCs and splenocytes in an RGDS motif-dependent manner *in vitro*.

(a) BMDCs were pretreated with indicated concentrations of competitor RGDS peptide (P2C-modification free) at 4°C for 30 min. Then, the BMDCs were stimulated with TLR2 ligands at 4°C for 1 h, washed, and recultured for

24 h. CD86 expression on BMDCs stimulated by P2C-RGDS, but not by MALP-2, was partially attenuated by RGDS peptide in a dose-dependent manner. The numbers in the panels represent mean fluorescence intensity. (b) IFN- γ production from splenocytes-stimulated with each compound for 72 h. (c) The pretreatment of splenocytes with competitor RGDS peptide attenuated the induction of IFN- γ from splenocytes-stimulated with P2C-RGDS at 4°C for 1 h. Splenocytes were recultured for 72 h after stimulation.

Fig. 5 P2C-RGDS retards tumor growth and induces CD8-positive cells more strongly than MALP-2 *in vivo* and *ex vivo*.

(a) Antitumor effect of P2C-RGDS and MALP-2 in the EG7-implanted mouse model (C57BL6-EG7 model). Mice were treated with TLR2 ligand and EG7 lysate on days 16, 20, and 23 (arrows). The data are shown as means $\pm$ s.e. (n=9). * $P < 0.05$ vs. control (student t-test). (b) Lymph node cells derived from P2C-RGDS-immunized mice showed stronger cytotoxicity than cells from MALP-2-immunized mice (^{51}Cr release assay). (c) CD8-positive T cells were more effectively induced in lymph nodes derived from mice immunized with P2C-RGDS and EG7 lysate than immunized with MALP-2. The lymph node cells in Fig. 5b were cultured with live EG7 for 96 h, then the ratio of CD8-positive T cells to total was analyzed. (d) Immunization with P2C-RGDS and EG7 lysate induced CD8-positive T cells from splenocytes

more efficiently than immunization with MALP-2. The ratios of CD8- or CD4-positive in whole splenocytes were determined by FACS analysis at 96 h after the splenocytes were cultured.

Fig1

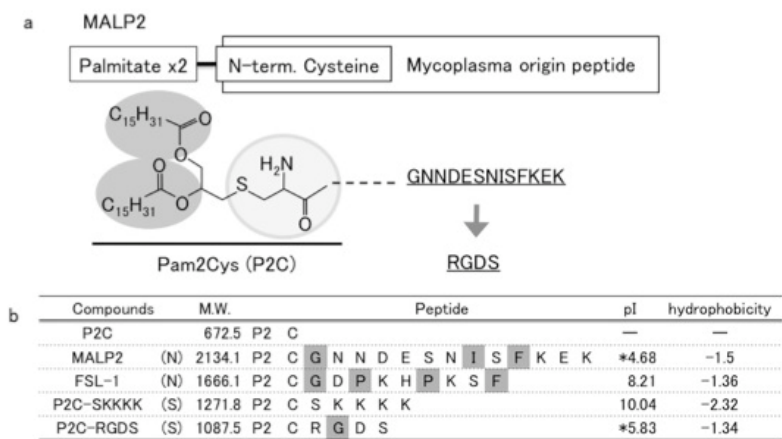


Fig2

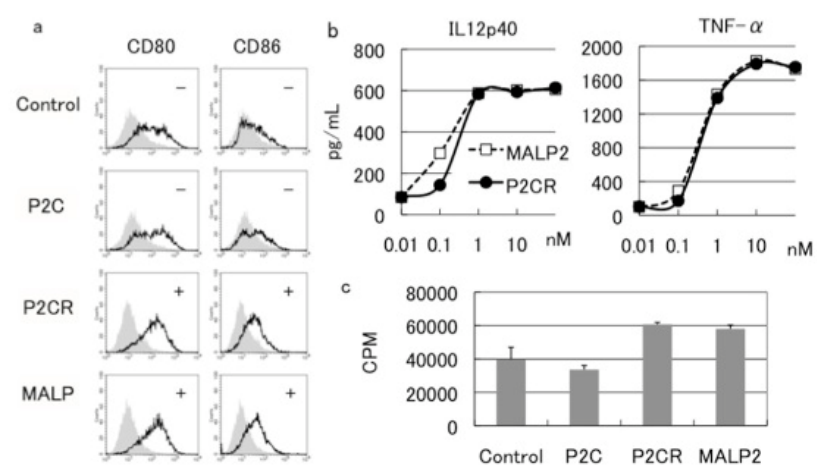


Fig3

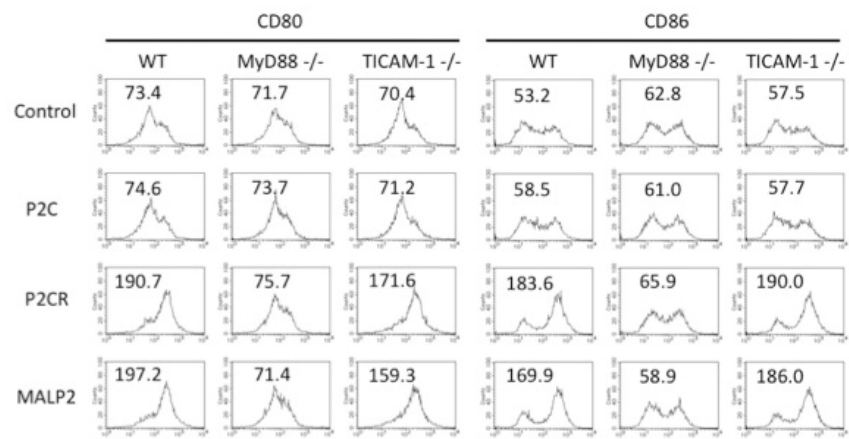


Fig4

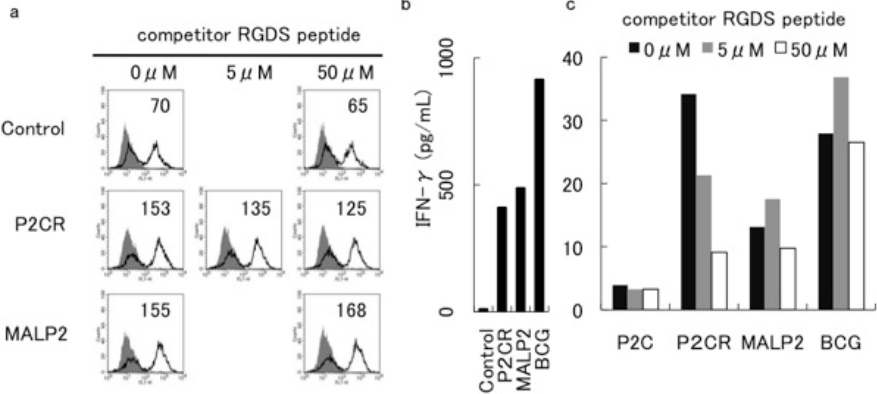


Fig5

