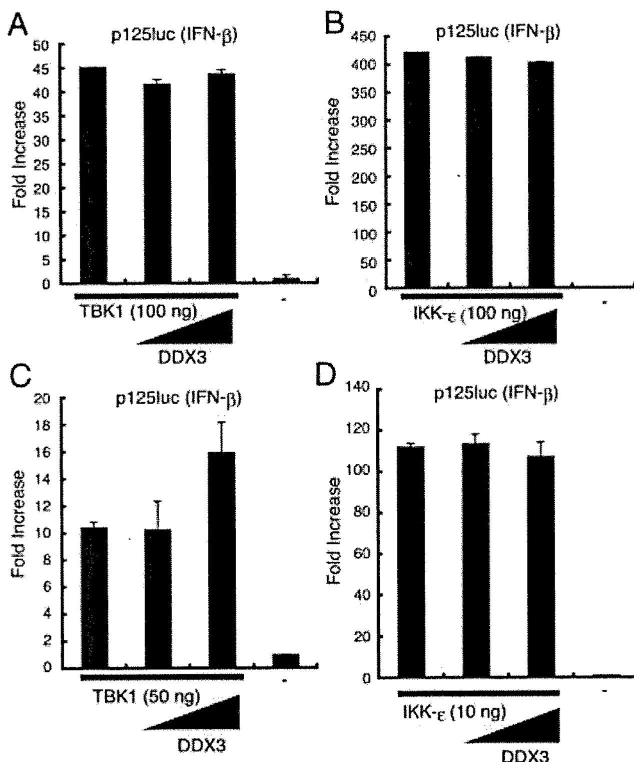

Figure 6. TBK1 and IKKε are not main targets for DDX3-mediated IFN-β up-regulation. (A–D) The activation of IFN-β promoter was examined by reporter gene assay. HEK293 cells were transfected in 24-well plates with DDX3 (0, 100 or 300 ng)-, TBK1 (0, 50 or 100 ng)- or IKKε (0, 10 or 100 ng)-encoding plasmid together with reporter (p125luc) and control plasmid. After 24 h, the cell lysate was prepared and the luciferase activities were measured. Data show mean+SD of three independent experiments.

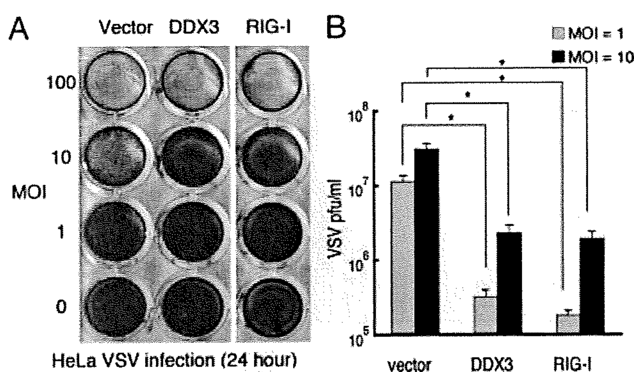

Figure 7. VSV infection is suppressed by overexpressed DDX3. (A) HeLa cells were transfected with DDX3, RIG-I or empty vector. After 24 h, the transfected cells were infected with VSV at indicated MOI. 24 h after VSV infection, the cells were fixed with formaldehyde and stained with crystal violet. (B) The VSV titers of culture supernatant of HeLa cells infected with VSV at MOI = 1 or 10 were measured by plaque assay. Data show mean+SD of three independent experiments. *$p<0.05$, Student's t-test.

Proteins involved in type I IFN induction are found ubiquitinated for their functional regulation. It has been reported that TRIM25 [19] and Riplet/RNF135 [20] act as ubiquitin ligases to activate RIG-I for IFN-β induction in their different sites of RIG-I ubiquitination. Another ubiquitin ligase RNF125 polyubiquitinates RIG-I through Lys48, leading to degradation of RIG-I [21]. The RIG-I level is highly susceptible to not only IFN but also ubiquitination in host cells. In addition, many viral factors may suppress the RIG-I function. It remains unknown what factor maintains a minimal level of RIG-I/MDA5 in resting cells. We favor the interpretation that DDX3 can be an alternative factor for compensating the low RLR contents in a certain infectious situation such that RIG-I is degraded or poorly up-regulated by other viral factors.

DDX3 is functionally complicated since its protective role against viruses may be modulated after the synthesis of viral proteins. DDX3 couples with the HCV core protein in HCV-infected cells and promotes viral replication [22]. This alternative function of DDX3 is accelerated by the HCV core protein, since the core protein withdraws DDX3 from the IFN-β-inducing facility, leading to suppression of IFN-β induction and positive regulation of HCV propagation in infected cells. DDX3 is also involved in HIV RNA translocation [14]. The DDX3 gene is conserved among eukaryotes, and Ded1 is a budding yeast homolog [23]. Ded1 helicase is essential for initiation of host mRNA translation, and human DDX3 can complement the lethality of Ded1-null yeast cells [24, 25]. Hence, another function of DDX3 is to bind viral RNA to modulate RNA replication and translocation. It is not surprising that DDX3 is implicated in various steps of RNA metabolism in cells with both host and viral RNA.

Materials and methods

Cell culture and reagents

HEK293 cells and HEK293FT cells were maintained in Dulbecco's Modified Eagle's low or high glucose medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS (Invitrogen) and antibiotics. HeLa cells were maintained in MEM (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FBS. Anti-FLAG M2 mAb, anti-HA polyclonal Ab, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor®-conjugated secondary Ab were from Invitrogen.

Plasmids

DDX3 cDNA encoding the entire ORF was cloned into pCR-blunt vector using primers, DDX3N F-Xh (CTC GAG CCA CCA TGA GTC ATG TGG CAG TGG AA) and DDX3C R-Ba (GGA TCC GTT ACC CCA CCA GTC AAC CCC) from human lung cDNA library. To make an expression plasmid, HA tag was fused at the C-terminal end of the full length DDX3 (pEF-BOS DDX3-HA). pEF-BOS DDX3 (1–224 aa) vector was made by using primers DDX3 N-F-Xh and DDX3D1 (GGA TCC GGC ACA AGC CAT CAA GTC TCT TTT C).

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pEF-BOS DDX3-HA (225–662) was made by using primers DDX3D2-3 (CTC GAG CCA CCA TGC AAA CAG GGT CTG GAA AAA C) and DDX3C R-Ba. To make pEF-BOS DDX3-HA (225–484) and pEF-BOS DDX3-HA (485–663), the primers DDX3D2 R-Ba (GGA TCC AAG GGC CTC TTC TCT ATC CCT C) and DDX3D3 F-Xh (CTC GAG CCA CCA TGC ACC AGT TCC GCT CAG GAA AAA G) were used, respectively. Reporter and internal control plasmids for reporter gene assay are previously described [26].

RNAi

Knockdown of DDX3 was carried out using siRNA, DDX3 siRNA-1: 5′-GAU UCG UAG AAU AGU CGA ACA-3′, siRNA-2: 5′-GGA GUG AUU ACG AUG GCA UUG-3′, siRNA-3: 5′-GCC UCA GAU UCG UAG AAU AGU-3′ and control siRNA: 5′-GGG AAG AUC GGG UUA GAC UUC-3′. Twenty picomoles of each siRNA was transfected into HEK293 cells in 24-well plates with Lipofectamin 2000 according to manufacture's protocol. Knockdown of DDX3 was confirmed 48 h after siRNA transfection. Experiments were repeated twice for confirmation of the results.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed as described previously [27]. The yeast AH109 strain (Clontech, Palo Alto, CA, USA) was transformed using bait (pGBKT7) and prey (pGADT7) plasmids. The transformants were streaked onto plates and incubated for 3–5 days. The IPS-1 CARD vector was constructed by inserting IPS-1 partial fragment encoding from 6 to 136 aa region into pGBKT7 multicloning site. Yeast two-hybrid screening was performed using human lung cDNA libraries. We obtained four independent clones, and one encoded DDX3 partial cDNA. SD-WLH is a yeast synthetic dextrose medium that lacks Trp, Leu and His aa. SD-WLHA lacks adenine in addition to Trp, Leu and His. SD-WL lacks Trp and Leu and thus non-selective plate.

Reporter assay

HEK293 cells (4×10^4 cells/well) cultured in 24-well plates were transfected with the expression vectors for IPS-1, DDX3 or empty vector together with the reporter plasmid (100 ng/well) and an internal control vector, phRL-TK (Promega) (2.5 ng/well) using FuGENE (Roche) as described previously [28]. The p-125 luc reporter containing the human IFN-β promoter region (−125 to +19) was provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). The total amount of DNA (500 ng/well) was kept constant by adding empty vector. After 24 h, cells were lysed in lysis buffer (Promega), and the *Firefly* and *Renella* luciferase activities were determined using a dual-luciferase reporter assay kit (Promega). The *Firefly* luciferase activity was normalized by *Renella* luciferase activity and is expressed as the fold stimulation relative to the activity in vector-transfected cells. Experiments were performed three times in duplicate (unless otherwise indicated in the legends).

PolyI:C stimulation

PolyI:C was purchased from GE Healthcare company, and solved in milliQ water. For polyI:C treatment, polyI:C (50 μg/mL) was mixed with DEAE-dextran (0.5 mg/mL) (Sigma) in the culture medium, and the cell culture supernatant was replaced with the medium containing polyI:C and DEAE-dextran. Using DEAE-dextran, polyI:C is incorporated into the cytoplasm to activate RIG-I/MDA5.

Virus preparation and infection

VSV Indiana strain or poliovirus type 1 Mahoney strain were used for virus assay. Vero derived cell (Vero-SLAM) was used for propagation and plaque assay for VSV indiana strain or poliovirus type 1 Mahoney strain. HEK293 cells were infected with viruses at MOI = 0.001 in a 24-well plate. The virus titers of culture media at indicated hours post infection in the figures were determined by plaque assay using Vero-SLAM cells. In some experiments that require rapid virus propagation, high MOI ($0.1\sim1$) was used for infection.

Immunoprecipitation

HEK293FT cells were transfected in a 6-well plate with plasmids encoding DDX3, IPS-1, RIG-I or MDA5 as indicated in the figures. Twenty-four hours after tranfection, the total cell lysate was prepared by lysis buffer (20 mM Tris-HCl (pH 7.5) containing 125 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 30 mM NaF, 5 mM Na3Vo4, 20 mM IAA and 2 mM PMSF), and the protein was immunoprecipitated with anti-HA polyclonal (Sigma) or anti-FLAG M2 mAb (Sigma). The precipitated samples were resolved on SDS-PAGE, blotted onto a nitrocellulose sheet and stained with anti-HA (HA1.1) monoclonal (Sigma), anti-HA polyclonal or anti-FLAG M2 mAb.

Confocal analysis

HeLa cells were plated onto cover glass in a 24-well plate. In the following day, cells were transfected with indicated plasmids using Fugene HD (Roch). The amount of DNA was kept constant by adding empty vector. After 24 h, cells were fixed with 3% of paraformaldehyde in PBS for 30 min, and then permeabilized with PBS containing 0.2% of Triton

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X-100 for 15 min. For the polyI:C stimulation, 100 ng of polyI:C were transfected into HeLa cell in 24-well plates together with IPS-1 or DDX3 expressing vectors, and 24 h after the transfection, the cells were fixed and stained for confocal microscopic analysis. Permeabilized cells were blocked with PBS containing 1% BSA and were labeled with anti-Flag M2 mAb (Sigma), anti-HA polyclonal Ab (Sigma) or Mitotracker in 1% BSA/PBS for 1 h at room temperature. The cells were then washed with 1% BSA/PBS and treated for 30 min at room temperature with Alexa-conjugated Ab (Molecular Probes). Thereafter, micro-cover glass was mounted onto slide glass using PBS containing 2.3% DABCO and 50% of glycerol. The stained cells were visualized at $\times 60$ magnification under a FLUOVIEW (Olympus, Tokyo, Japan).

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Abbreviations: **CARD:** caspase recruitment domain · **DEAD:** Asp-Glu-Ala-Asp · **DDX3:** DEAD/H BOX 3 · **IKKε:** I-kappa-B kinase ε · **IRF-3:** IFN regulatory factor-3 · **IP:** immunoprecipitation · **IPS-1:** IFN-β promoter stimulator-1 · **MDA5:** melanoma differentiation-associated gene 5 · **RIG-I:** retinoic acid inducible gene-I · **RLR:** RIG-I-like receptor · **TBK1:** TANK-binding kinase 1 · **VSV:** vesicular stomatitis virus

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

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

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For Review

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Summary

The development of effective immunoadjuvants for tumor immunotherapy is of fundamental importance. The use of mycobacterium bovis bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) in tumor immunotherapy has been examined in various clinical applications. Because BCG-CWS is a macromolecule that cannot be chemically synthesized, the development of an alternative synthetic molecule is necessary to ensure a constant supply of adjuvant. In the present study, a new adjuvant was designed based on the structure of macrophage-activating lipopeptide-2 (MALP-2), which is a TLR2 ligand similar to BCG-CWS. MALP-2 [S-(2,3-bispalmitoyloxypropyl)Cys (P2C) –GNNDESNISFKEK], originally identified in a Mycoplasma species, is a lipopeptide that can be chemically synthesized. A MALP-2 peptide was substituted with a functional motif, RGDS, creating a novel molecule named P2C-RGDS. RGDS was selected because its sequence constitutes an integrin-binding motif and various integrins are expressed in immune cells including dendritic cells (DCs). Thus, this motif adds functionality to the ligand. P2C-RGDS activated DCs and splenocytes more efficiently than MALP-2 over short incubation times *in vitro*, and the RGDS motif contributed to their activation. Furthermore, P2C-RGDS showed higher activity than MALP-2 in inducing migration of DCs to draining lymph node, 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 constitutes a first step in the development stronger synthetic adjuvants in the future.

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

Introduction

Bacterial adjuvants that were used as biological response modifiers (BRM) for cancer immunotherapy in the 1970s have recently 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 were not sufficient to fully activate the immune system.[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, the T cells of the acquired immune system play an important role in recognizing and attacking tumor cells.[9] DCs play a key role in the regulation of the acquired immune system by presenting antigen and inducing a primary immune response. The identification of the Toll-like receptor (TLR) family advanced the understanding of DC function and of the role of adjuvants, because almost all microbial adjuvants work as TLR ligands and activate DCs.[10-12] These findings provide the basis for the understanding of the mechanism of adjuvant therapy.

Dr. Azuma developed BCG-CWS, a cell-wall skeleton preparation of Mycobacterium bovis bacillus Calmette-Guérin,[13,14] as an antitumor immunotherapeutic adjuvant. Although many BRM studies 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

For Review

TLR2/4 (refs. 15,16,17) and acts as an effective adjuvant to induce CTLs in irradiated tumors in a mouse experimental model. These activities are mediated by the 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 through 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 antitumor 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, systemic administration of IL-23 was also reported to have antitumor effects similar to those of IL-12,[27] and TLR2 ligands exhibit antitumor activity[28-30] that may be mediated by the induction of IL-23.

Although BCG-CWS is effective as an adjuvant, its clinical use is limited in purity, stability and a stable supply because it cannot be chemically synthesized and is therefore prepared from bacterial cells. These factors indicate that there is a need to develop new synthetic adjuvants as effective as BCG-CWS. The present report describes the design of 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

For Review

can be chemically synthesized. No functional consensus peptide 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, FSL[32], P2C-SKKKK[33]) 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 $\alpha V/\beta 3$ and $\alpha 5/\beta 1$, and the RGDS motif specifically binds to these integrins.[36,37] Virus particles expressing proteins containing the RGD motif efficiently infect DCs.[37] Therefore, a new TLR2 ligand was developed by replacing the peptide of bacterial origin with a hydrophilic functional motif (adjuvant engineering). P2C and the RGDS peptide were linked to increase the efficiency of ligand adherence to DCs or other immune cells, and the effect of the new adjuvant on antitumor activities *in vitro* and *in vivo* was examined.

For Review

Materials and Methods

Mice, cells, and reagents

TICAM-1$^{-/-}$ mice were generated in our laboratory.[2] TLR2$^{-/-}$ and 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 approved by the committee in Osaka Medical Center for Cancer. EG7 cells are ovalbumin-transfected EL4 and were obtained from ATCC (Manassas, VA, USA).[39] B16D8 was established in our laboratory as a subline of the B16 melanoma cell line[18]. Cell lysates were prepared by the freeze-thaw method.

Preparation of mouse BMDCs, splenocytes, and lymph node cells

Bone marrow-derived DCs (BMDCs) were prepared as previously described [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), 50 μM 2-mercaptoethanol (Invitrogen, Carlsbad, CA), 10 mM HEPES and 10% FCS (Bio Whittaker, Walkersville, MD). The inguinal lymph

For Review

node cells and splenocytes were prepared by using Lympholyte-M (Cedarlane, Ontario, Canada). CD11c-positive and negative cells, and CD90-positive and negative cells were separated from splenocytes by using CD11c or CD90 microbeads (Miltenyi Biotec, Auburn, CA).

In vitro assay

For the simple stimulation assay *in vitro*, BMDCs or splenocytes were cultured with 10 μg/ml of BCG-CWS[18], 100 nM of MALP-2 and the designed lipopeptide (purity >90%; Biologica Co., Aichi, Japan) for 24 h (BMDC, FACS), 48 h (BMDC, ELISA) or 72 h (splenocytes, ELISA). For the inhibition assay, BMDCs or splenocytes were pre-incubated with the indicated concentrations of RGDS peptide or anti-CD29 antibody (eBioscience, San Diego, CA) at 4°C for 30 min before stimulating them with TLR2 ligands at 4°C for 60 min. The cells were then washed and re-cultured for 48 h (BMDC, ELISA) or 72 h (splenocytes, ELISA). The Mixed Lymphocyte Reaction (MLR) assay was performed as previously described,[40] and the results were analyzed as uptake of [^{3}H]thymidine (1 μCi/well; Amersham Biosciences, Piscataway, NJ). BMDCs stimulated with TLR2 ligands for 24 h (C57BL/6, 5 × 10^4 cells) were co-cultured 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, Intracellular Cytokine Staining 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 antibody, or with phycoerythrin-conjugated anti-mouse CD4 or isotype control antibody (eBioscience). The cells were washed, and their fluorescence intensities were measured by FACS analysis. For Intracellular Cytokine Staining, splenocytes were stimulated with P2C-RGDS for 72 h and Brefeldin A (GolgiPlug, BD Biosciences, San Diego, CA) for the last 6 h. Cells were stained with phycoerythrin-conjugated anti-mouse CD3ε, anti-mouse CD4, anti-mouse CD8α antibodies, allophycocyanin-conjugated anti-mouse CD11c or anti-mouse NK1.1 antibodies (eBioscience), followed by fixation and permeabilization with Cytofix/Cytoperm plus Kit (BD Biosciences). Cells were stained intracellularly with FITC-labeled anti-IFN-γ antibody (XMG1.2, eBioscience). 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 ʃl of 1-2 × 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. A volume of 50 μl of a mixture consisting of 10 nmol of lipopeptide and the cell lysate of 2 x 10^5 EG7 cells with or without 10 nmol of RGDS peptide was injected intradermally around the transplanted tumor. Tumor volume was calculated using the formula: Tumor volume (cm^3) = (long diameter) × (short diameter) × (short diameter) × 0.4. Statistical analysis was performed with the Student's 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, and then the splenocytes and lymph node cells were prepared and cultured for 4 d to be primed by DCs and macrophages. The cytolytic activities of lymph node cells were then analyzed with a ^{51}Cr release assay.[18] The percentage of specific lysis was calculated using the formula: %Specific lysis = [(experimental release – spontaneous release)/(total release – spontaneous release)] × 100. The proportions of CD8- and CD11c-positive cells in lymph nodes or spleen were analyzed by FACS.

Results

To design a new TLR2 ligand with activity equivalent to that of BCG-CWS, the minimum lipopeptide unit, P2C, was connected to the RGDS integrin-binding motif to increase adherence to DCs, forming P2C-RGDS (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, the synthetic adjuvants were tested for their capacity to activate BMDCs *in vitro* when these compounds were added to the culture medium. P2C-RGDS enhanced the expression of CD80 and CD86 in BMDCs at a level equal to that of MALP-2, the positive control (Fig. 2a). P2C-RGDS also enhanced the production of IL12p40 and TNF-α (Fig. 2b) and the proliferation of allogeneic T cells co-cultured with the BMDCs. Thus, P2C-RGDS and MALP-2 stimulate DCs equally *in vitro*, whereas P2C dose not show the same activity.

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

For Review

P2C-RGDS is a TLR2 ligand with activity similar to that of MALP-2 *in vitro*.

Next, the functional dependence of P2C-RGDS as a TLR2 ligand on not only its hydrophilicity, but also on the motif-specificity of the peptide sequences, was tested. Since MALP2 and P2C-RGDS activated DCs to the same extent at 37°C for 48 h, DCs were instead stimulated at 4°C for 1 h, then washed and re-cultured at 37°C for 48 h. Under these conditions, P2C-RGDS induced IL12p40 more efficiently than MALP2 (Fig. 4a). To analyze the specificity of the RGDS peptide, IL12p40 production was inhibited by the addition of an RGDS competitor peptide or anti-integrin β1 antibody to the assay. The addition of the RGDS competitor peptide and the integrin blocking antibodies partially attenuated the production of IL12p40 induced by P2C-RGDS, but not that induced by MALP-2 (Fig. 4b and c). These results indicate that not only hydrophilicity but also the functional RGDS motif contributes to the activation of DCs *in vitro* at short incubation times.

The P2C-RGDS–induced production of IFN-γ was evaluated next, using *in vitro* whole splenocyte stimulation. The activities of P2C-RGDS and MALP-2, as measured by IFN-γ production, were comparable and weaker than that of BCG-CWS when splenocytes were simply stimulated with each compound for 72 h (Fig. 5a). However, when splenocytes were stimulated with each compound at 4°C for 1 h and re-cultured at 37°C for 72 h, the P2C-RGDS-induced production of IFN-γ was stronger than that induced by

MALP-2 and it was attenuated mostly by the RGDS peptide. Furthermore, the splenocytes stimulated with P2C-RGDS produced as much IFN-γ as those stimulated with BCG-CWS at 4°C for 1 h (Fig. 5b). IFN-γ production was not detected in splenocytes depleted of CD11c-positive dendritic cells, and IFN-γ production could be restored under these conditions by adding back CD11c-positive cells. These data indicate that IFN-γ production by splenocytes following stimulation with P2C-RGDS was mediated by DC activation (Fig. 5c). IFNγ production was also impaired by depletion of CD90 (Thy1) positive T cells (Fig. 5c). Further assessment of IFN-γ producing cells by intracellular cytokine staining, revealed that IFN-γ was mainly detected in CD3- or CD8-positive cells, but in CD4-, NK1.1-, CD11c-negative cells stimulated with P2C-RGDS (Fig. 5d).

Finally, the antitumor activity of P2C-RGDS *in vivo* was investigated 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 days 16, 20, 23. The minimum lipopeptide unit P2C showed no *in vivo* antitumor activity similar to the activation of DCs *in vitro*. Although MALP-2–treated mice showed a slightly smaller tumor volume than control mice, this difference was not significant. However, P2C-RGDS showed a significant antitumor effect (student t-test, $P < 0.05$ vs. control, Fig. 6a).

Moreover, the mixture of P2C and the RGDS peptide showed no antitumor activity (Fig. 6b). Next, lymph node cells from mice immunized with EG7 lysate and P2C-RGDS or MALP-2 were isolated and the cytotoxicity against EG7 and B16D8 cells was measured using a ^{51}Cr release assay. P2C-RGDS specifically induced a stronger cytotoxic activity against EG7 than MALP-2, but the lymph node cells were not sufficiently cytotoxic against the negative target B16D8 cells (Fig. 6c). In addition, lymph node cells were cultured continuously with live EG7 cells for 96 h and the proportion of CD8-positive cells was analyzed by FACS. CD8-positive cells in lymph nodes derived from P2C-RGDS–treated mice remained at a level of approximately 16% of total cells, but most CD8-positive cells from MALP-2–treated mice were lost after culture with EG7 (Fig. 6d). The proportion of CD8-positive cells in a splenocyte population derived from immunized mice was also evaluated using FACS analysis. CD8-positive cells were proportionally higher among splenocytes derived from mice treated with P2C-RGDS than among splenocytes derived from mice treated with MALP-2 (Fig. 6e). Necrosis was observed on the surface of tumors after P2C-RGDS but not after MALP-2 treatment, and CD8-positive cells were detected around the necrosis tissue by immunostaining (data not shown). These data suggest that P2C-RGDS induces and activates CTLs more efficiently than MALP-2. Moreover, to analyze the mechanism underlying the strong antitumor effect of P2C-RGDS *in vivo*, the

proportions of CD11c-positive cells in draining lymph nodes were analyzed in mice treated with MALP-2 or P2C-RGDS for 24 h. P2C-RGDS induced the migration of CD11c-positive cells to the draining lymph nodes more effectively than MALP2 (P=0.055, Fig. 6f).

Discussion

Natural TLR2 ligands of bacterial origin, such as MALP-2[31, 41, 42] and FSL-1[32], have been reported to 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] Several researchers have relied on a chemobiologic strategy of producing amino acid replacements to explore which portion of lipopeptide sequence possesses effective adjuvant activity.[43] Using this strategy, Takeda Co Ltd. developed Tan-1511 analogues that show high levels of activity in the induction of granulopoiesis.[43] Another strategy has been to randomly search for sequences or peptides effective at enhancing TLR2 ligand activity.[44] The purpose of the current project was to design a TLR2 ligand with an additional function through the addition of a hydrophilic, functional peptide that could be developed as a new synthetic adjuvant (Fig. 1). In comparison to a prior developmental strategies, the present design has the advantage of allowing the selection of various functions, and because the designed lipopeptides do not exist in nature, they could show new or enhanced properties. In the present study, the TLR2 ligand was designed to possess stronger adhesive capacity through the linking of the RGDS peptide to P2C.

The compound P2C-RGDS was developed and shown to be as effective

as MALP-2 in generating BMDC 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 also activated DCs through the TLR2-MyD88 pathway (Fig. 3),[38] but P2C-RGDS activated DCs more efficiently than MALP2, and the RGDS integrin binding motif was found to be important for DCs activation over short incubation times (Fig. 4). Because DCs were treated with compounds for only 1 h, then washed and re-cultured at 37°C for 48 h in these experiments, it was predicted that P2C-RGDS might efficiently adhere to the DCs in short incubation times, and then stimulate DCs continuously at the surface or in the phagosome of DCs. Whole splenocytes stimulated with P2C-RGDS also produced more IFN-γ than MALP-2 over short incubation times (Fig. 5b), and the production of IFN-γ by splenocytes depended on CD11c-positive DCs (Fig. 5c). These data suggest that the adhesion properties of P2C-RGDS caused the efficient activation of DCs, and reflected splenocyte activation. The stronger IFN-γ induction by P2C-RGDS might also be due to its retention in the culture system by adherence to various cells among the splenocytes. Moreover, P2C-RGDS may be retained in local regions for a long time via integrin binding *in vivo*, leading to the efficient activation of immune cells such as dermal DCs. P2C-RGDS induced the migration of CD11c-positive cells into the draining lymph nodes more effectively than MALP2 in *in vivo* experiments

(Fig. 6f). These DCs might activate CD8-positive cells, enhance cytotoxicity (Fig. 6c), and lead to retardation of tumor growth (Fig. 6a). These data suggest that the greater activation of DCs by P2C-RGDS compared to MALP2 influences IFN-γ production by splenocytes, thereby resulting in increased cytotoxicity and antitumor effects *in vivo*.

The induction of IFN-γ production by BCG-CWS treatment is one of the indexes for continuing treatment in clinical applications[14, 19], and the response can be confirmed in mouse experiments. IFN-γ stimulation up-regulates the expression of MHC class I in tumor cells,[45] presumably improving tumor recognition by immune cells, and leading to increased suppression of tumor growth. With short stimulation periods, P2C-RGDS induced as much IFN-γ as BCG-CWS. Although the present compound was designed without considering IFN-γ induction, results show that CD8-positive cells produced IFN-γ in splenocytes stimulated with P2C-RGDS alone in the absence of antigen peptide (Fig5). The mechanism of IFN-γ induction by P2C-RGDS should be analyzed in the future. The tumor volume of the BCG-CWS treatment group was about 60% of control on day 22 (data not shown), and the therapeutic effects of P2C-RGDS were almost equivalent to those of BCG-CWS. Because BCG-CWS must be emulsified with drakeol, the use of P2C-RGDS has significant advantages.

The integrin binding sequence has served as the basis for the design of drugs that depend on adhesive activity. In the present work, this adhesive function was applied to TLR ligands to enhance immunoadjuvant activity. Cilengitide, a cyclic RGD peptide, was developed as an integrin αV antagonist, which impair angiogenesis, tumor growth and metastasis[46] because integrins are expressed on various tumor cells.[47] Although EG7 expresses integrin αV, β1, and β3, P2C-RGDS and RGDS peptide did not show direct cytotoxic activity against EG7 cells at concentrations up to 100 nM (data not shown). Furthermore, the adjuvant activities of P2C-RGDS were compared to those of a mixture of P2C and RGDS peptide. P2C had no adjuvant activity, such as the activation of DCs and splenocytes *in vitro* or antitumor effect *in vivo* by lipopeptides (Figs. 2b, 5a, 6a). The mixture did not show any effects *in vivo* such as appreciable antitumor activity (Fig. 6b). Based on these results, the stronger antitumor activity of P2C-RGDS compared to that of MALP-2 is thought to occur through an increase in cell adhesive ability, but not through the inhibition of angiogenesis.

Concerning the relationship between TLR and its ligand, it is suggested that a co-receptor plays a key role for TLR binding and signaling[1, 7, 48] as observed previously for CD14 in the LPS-TLR4 signaling pathway. Although integrin binding is predicted to support the capture and phagocytosis of ligands by DCs, integrin signaling in addition to TLR signaling might influence

adjuvant activity. Stronger adjuvants will be developed by selecting for other properties in addition to TLR signaling that are essential for adjuvant activity, and the integrin signal could be one of the candidates.

In the present work, the inclusion of an integrin-binding motif in a TLR2 ligand was examined for its effect in increasing the activity of the compound as an antitumor adjuvant by enhancing adhesion of the ligand to DCs and other cells. DCs efficiently recognized P2C-RGDS, which they adhered to and maintained around cells, and P2C-RGDS showed stronger antitumor activity than MALP-2 *in vivo*. The present adjuvant-engineering project is a new strategy to incorporate biological findings into drug design. 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. More than 20 TLR2 ligands with 10 alternative functions have already been synthesized by adjuvant engineering and our group is working to develop the strongest adjuvant through continued evaluation and improvements.

For Review

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For Review
For Review