IL4 Receptor-Targeted Proapoptotic Peptide Blocks Tumor Growth and Metastasis by Enhancing Antitumor Immunity

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Abstract

Cellular cross-talk between tumors and M2-polarized tumor-associated macrophages (TAM) favors tumor progression. Uptregulation of IL4 receptor (IL4R) is observed in diverse tumors and TAMs. We tested whether an IL4R-targeted proapoptotic peptide could inhibit tumor progression. The IL4R-binding peptide (IL4RPep-1) preferentially bound to IL4R-expressing tumor cells and M2-polarized macrophages both in vitro and in 4T1 breast tumors in vivo. To selectively kill IL4R-expressing cells, we designed an IL4R-targeted proapoptotic peptide, IL4RPep-1-K, by adding the proapoptotic peptide (KLAKLAK), to the end of IL4RPep-1. IL4RPep-1-K exerted selective cytotoxicity against diverse IL4R-expressing tumor cells and M2-polarized macrophages. Systemic administration of IL4RPep-1-K inhibited tumor growth and metastasis in 4T1 breast tumor-bearing mice. Interestingly, IL4RPep-1-K treatment increased the number of activated cytotoxic CD8+ T cells while reducing the numbers of immunosuppressive regulatory T cells and M2-polarized TAMs. No significant systemic side effects were observed. These results suggest that IL4R-targeted proapoptotic peptide has potential for treating diverse IL4R-expressing cancers. Mol Cancer Ther; 16(12); 2803–16. © 2017 AACR.

Introduction

A growing body of evidence shows that dynamic communication between tumors and M2-polarized tumor-associated macrophages (TAM) in the tumor microenvironment promotes tumor growth, survival, metastasis, and resistance to chemotherapy. Tumor cells enhance the generation of immunosuppressive M2-polarized TAMs by providing macrophage colony-stimulating factor-1 (CSF-1), a key factor for the differentiation and survival of macrophages (1–3). In turn, TAMs, unlike M1-polarized macrophages, have an increased capacity to produce protumoral growth factors, angiogenic factors, and proteases, which enhance survival and metastasis of tumor cells (1–3). TAMs also produce high levels of immunosuppressive cytokines, such as IL10 and IL4. Enhanced levels of these cytokines inhibit the infiltration of cytotoxic CD8+ T cells into tumors and suppress the effector function of T cells by increasing the recruitment of immunosuppressive regulatory T cells (Treg) in the tumor microenvironment (4–6). TAMs are characterized by low expression levels of MHCII and Ly6C (MHCIIloLy6C+) and high expression levels of CD206 and arginase (7–9). A high number of TAMs in tumor tissue is often correlated with a poor survival rate for cancer patients (10).

Targeting M2-polarized macrophages in the tumor microenvironment has been investigated as a novel strategy for tumor management. A proapoptotic peptide that selectively binds to M2-polarized macrophages reduced the number of TAMs and increased tumor survival in mice (11). Trabectedin, a chemotherapeutic agent that binds to DNA, exerts its antitumor activity by reducing the number of TAMs (12). Blockade of CSF-1 receptor (CSF-1R) by a brain-permeable peptide inhibitor reduced the population of M2-polarized macrophages and suppressed the progression of glioblastoma (13). The combination of a CSF-1R inhibitor or CSF-1-neutralizing antibody with conventional chemotherapy or radiotherapy reduced tumor growth and metastasis more effectively than the single treatment (14, 15). IL4 receptor (IL4R) is also a good marker for M2-polarized TAMs. There are two types of IL4R. Type I is composed of IL4Rα and common γ-chain and is expressed on the surface of hematopoietic stem cell origin (16, 17). Type II comprises IL4Rα and IL13Rα chains and is expressed on the surface of cells of
nonhematopoietic stem cell origin, such as tumor cells, including breast cancer and lung cancer cells (18–21). Of interest, M2-polarized macrophages express higher levels of IL4R than M1-polarized macrophages (7, 8, 15). The interaction between IL4 and IL4R plays an important role in both tumor progression and the immunosuppressive function of M2 macrophages. IL4 induces the expression of antiapoptotic proteins such as Bcl-xl in tumors and contributes to the resistance of tumor cells to chemotherapy (22, 23). On the other hand, IL4 contributes to M2 polarization of macrophages (1), upregulates the protumorigenic activity of macrophages (24), and increases cathepsin production by TAMs (25), which promotes the progression and metastasis of tumors.

Given that IL4R expression is upregulated in both tumor cells and M2-type TAMs, we aimed to inhibit tumors by targeted killing of IL4R-expressing cells. For this purpose, we exploited two types of peptides, IL4RPep-1 and (KLAKLAK)2, as an IL4R-targeting peptide and a proapoptotic peptide, respectively. We previously identified IL4RPep-1, a 9-mer peptide with the sequence CRKRLDRNC, which is homologous to the sequence of IL4, via screening of a phage-displayed peptide library (26). We previously suggested the possibility that IL4RPep-1 could specifically bind to IL4R (26) and selectively target IL4R-expressing tumors (21, 27). The amphiphilic proapoptotic peptide (KLAKLAK)2 is known to trigger mitochondrial membrane disruption and release of cytochrome c, which subsequently induces apoptotic cell death (28, 29). Previous studies have also reported that the (KLAKLAK)2 peptide linked with tumor-homing peptides exerted antitumor activity (28, 30). To selectively induce apoptosis in IL4R-expressing tumors and M2-polarized TAMs, we designed an IL4R-targeted proapoptotic peptide, IL4RPep-1-K, by adding the proapoptotic peptide (KLAKLAK)2 (in brief, K) to the end of IL4RPep-1. Despite the fact that peptides are sensitive to enzymatic degradation and are rapidly excreted through the kidney, they are attractive alternatives to antibodies as targeting ligands due to their smaller size, deep tissue penetration, susceptibility to simple chemical modification, and low chance of systemic toxicity compared with antibodies (31–33).

In this study, we tested whether the IL4R-targeted proapoptotic peptide IL4RPep-1-K could inhibit tumor progression and investigated the underlying mechanism of action using a 4T1 mouse breast tumor model.

Materials and Methods

Cell cultures

4T1 cell line was purchased from the Korean Type Culture Collection in 2003. 4T1-luc cell line was purchased from PerkinElmer in 2009. Cells were cultured in DMEM (HyClone) supplemented with 10% FBS (Thermo Fisher Scientific) at 37 °C in humidified 5% CO2 atmosphere. All cells were authenticated and tested for mycoplasma using a detection kit (Intron Biotechnology) in July 2015. Expression of biomarkers was routinely tested by immunofluorescence. After thawing, cells were cultured for approximately 2 months.

Isolation of bone marrow–derived monocytes and M1 and M2 polarization

Bone marrow cells were isolated from tibias and femurs of Balb/c mice and then cultured in DMEM supplemented with 10 ng/mL macrophage-colony stimulating factor (M-CSF, Gibco) and 10% FBS for 7 days. The culture medium was changed every other day. For M1 polarization, bone marrow–derived monocytes (BMDM) were incubated with 20 ng/mL recombinant mouse interferon-γ (R&D Systems) plus 100 ng/mL lipopolysaccharide for 24 to 48 hours. For M2 polarization, BMDMs were incubated with 20 ng/mL recombinant mouse IL4 for 24 to 48 hours.

Peptide synthesis and amino acid sequences

All peptides were synthesized and were purified by high-performance liquid chromatography to >90% purity by Peptron Inc. Peptides were conjugated at the amino terminal with FITC or Flamma 675 near-infrared (NIR) fluorescence dye (BioActs). The amino acid sequences of peptides were as follows: IL4RPep-1, CRKRLDRNC; (KLAKLAK)2, KLAKLAKLAKLAKLAK; IL4RPep-1-K, CRKRLDRNCGGKLAKLAKLAK; control peptide, NSSVDK; and control-K, NSSVDDGGKGLAKLAKLAKLAK. The three glycine residues were inserted as a linker to impart peptide flexibility and minimize potential steric interactions between the targeting peptide and the proapoptotic peptide.

Peptide cell binding assays

Cells (1 × 10⁵ cells/well in a 4-well chamber) were incubated with 1% BSA at 4°C for 30 minutes for reducing nonspecific binding and then with 10 μmol/L of FITC-labeled peptide at 4°C for 1 hour. After washing, cells were fixed with 4% paraformaldehyde (PFA), followed by nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) and observed under a confocal microscope (Zeiss).

Immunofluorescence of cultured cells

Cells (1 × 10⁵ cells/well in a 4-well chamber) were incubated with 20 ng/mL of recombinant mouse IL10 (R&D Systems) for 24 hours, 20 ng/mL of recombinant mouse IL4 (R&D Systems) for 24 hours, or 5 ng/mL of recombinant mouse TGFβ (R&D Systems) plus 5% FBS for 48 hours. After treatment, cells were fixed with 4% PFA and incubated with antibodies against IL4Ra, N-cadherin, and E-cadherin (1:100 dilution, Santa Cruz Biotechnology) at 24°C for 1 hour or at 4°C for 16 hours. Cells were then incubated with fluorescence-tagged secondary antibodies at 24°C for 1 hour. Cells were stained for nucleus with DAPI, mounted with Prolong Gold anti-fade mounting reagent (Life Technologies) and observed under a confocal microscope (Zeiss).

Saturation binding assays

Cells (5 × 10⁴ cells/well in a 96-well plate) were incubated with 1% BSA at 24°C for 30 minutes and then with different concentrations of biotin-labeled peptides at 4°C for 1 hour. After washing, cells were incubated with NeutrAvidin-horse radish peroxidase (1:10,000 dilution, Thermo Fisher Scientific) at 24°C for 30 minutes. The enzyme activity was detected using 3', 5', 5'-tetramethylbenzidine substrate (Thermo Fisher Scientific), and the reaction was halted using 2 mol/L sulfuric acid as a stop solution. Absorbance was measured at 450 nm using a microplate reader. Kd values were calculated using GraphPad Prism 6 software (GraphPad Inc.).

Cytotoxicity and apoptosis assays

Cells (5 × 10⁴ cells/well in a 96-well plate) were incubated with different concentrations of peptides for 2 hours at 37°C. After
treatment, cells were incubated with a fresh culture medium containing 10% of CCK-8 reagents (Dojindo) for 1 to 4 hours. Absorbance was measured at 450 nm. IC50 values were calculated using GraphPad Prism 6 software. For apoptosis assays, cells were incubated with 15 μmol/L of peptides at 37°C for 2 hours. After incubation, cells were harvested, washed with PBS, and stained with 5 μl of Alexa Fluor 488–labeled Annexin V and 1 μl of a 100 μg/ml propidium iodide solution (Thermo Fisher Scientific). After 15 minutes of incubation, cells were analyzed using a flow cytometer (BD).

Immunofluorescence analysis of tissues

Frozen tissue sections (8-μm thickness) were stained at 37°C for 1 hour using anti-mouse IL4Rx (Santa Cruz Biotechnology), anti-mouse F4/80 (Santa Cruz Biotechnology), anti-mouse E-cadherin (Cell Signaling Technology), anti-mouse N-cadherin (Abcam) antibodies. Alexa488- or Alexa594-conjugated antibody (Life Technologies) was used as a secondary antibody. Tissue samples were counterstained with DAPI, incubated with Prolong Gold anti-fade mounting reagent (Life Technologies), and observed under a confocal microscope (Zeiss).

Preparation of tumor-infiltrating leukocytes and single-cell suspensions from lymph nodes and spleen

To prepare tumor-infiltrating leukocytes (TIL), tumors were excised and fragmented into several pieces. The fragmented tissues were further minced into 2 to 3 mm3 pieces and incubated with collagenase D (Roche) and DNase (Sigma-Aldrich) at 37°C for 40 minutes. The tissue samples were filtered through a 100-μm cell strainer to collect digested cells. Dead cells and cellular debris were removed by Ficoll (GE Healthcare) gradient centrifugation. Collected cells were resuspended in PBS containing FBS. TILs were isolated by gating upon CD45 expression using a flow cytometer.

Flow cytometry analysis of immune cell populations

For staining of surface biomarkers, cells were incubated for 20 minutes in the dark with fluorescently labeled antibodies against CD11b, CD4, CD8, CD44, CD45, CD86, CD206, F4/80, Gr1, MHCII, and Ly6C (BioLegend). At least 10,000 events were analyzed using an LSR-Fortessa flow cytometer (BD). Data were evaluated using FlowJo software (TreeStar).

qRT-PCR

A single-cell suspension of tumor cells was prepared by homogenizing 50 mg of tumor tissues in QIAzol lysis reagent (Qiagen). RNA was isolated from the total tumor cell lysate using a miRNeasy Mini Kit (Qiagen) and subjected to qRT-PCR. Primers for Arg1, iNOS, IL10, IL4, TGFβ, IL12p40, and β-actin were obtained from Bioneer Inc. cDNA was synthesized using a PrimerScript 1st strand cDNA Synthesis Kit (Takara). qPCR using SYBR Green (Qiagen) was performed on a real-time cycler (Bio-Rad).

Animal models

Six- to 8-week-old Balb/c female mice were purchased from Orient Bio. IL4Rx-deficient Balb/c female mice were purchased from The Jackson Laboratory. Mice were caged for and maintained in conformance with the Guidelines of the Institutional Animal Care and Use Committee (IACUC) of Kyungpook National University (permission no. 2015-0017). Tumors were prepared by injecting 1 × 106 4T1 cells into the lower left mammary fat pad of Balb/c female mice.

In vivo bioluminescence and NIR fluorescence imaging

For bioluminescence imaging, mice were intraperitoneally injected with D-luciferin at a dose of 150 mg/kg and incubated for 10 minutes before imaging using an IVIS imaging system (PerkinElmer). Bioluminescence images were taken every week to monitor tumor growth. For NIR fluorescence imaging, mice were intravenously injected via the tail vein with 100 μl of a 200 μmol/L solution of Flamma 675 NIR fluorescent dye–labeled peptides and incubated for 2 hours. After incubation, the mice were anesthetized, and in vivo images were taken using the IVIS imaging system. After in vivo imaging, the mice were sacrificed, and the tumors and other organs were subjected to ex vivo imaging using the IVIS imaging system.

Antitumor treatments

Tumor-bearing mice were subjected to randomization and grouping when the size of the tumors reached approximately 100 mm3. Each peptide was intravenously injected via the tail vein of mice (14.2 μg/g of body weight, 3 times a week for 4 weeks). Paclitaxel was given through intraperitoneal injection (8 mg/g of body weight, once a week for 4 weeks) based on a previous study (34). Tumor size was measured using a digital caliper, and tumor volume was calculated using the following formula: Volume = (L × W × H)/2 (L: length, longest dimension, W: width, shorter dimension, parallel to the mouse body, and H: height, perpendicular to the length and width). Mice were checked for tumor ulceration. At the end of the treatment, the mice were sacrificed, and the lung and liver were isolated and checked for metastatic tumor masses.

Statistical analysis

Data are presented as the mean ± SD. All statistical analyses were performed using Excel and SPSS 15.0 software. Statistical significance was determined by one-way ANOVA and, when indicated, by an independent t test. P < 0.05 was considered significant. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

IL4R expression is closely related to tumor progression

In this study, we used the 4T1 mouse breast tumor, a highly malignant and metastatic tumor (35, 36), as a syngeneic and orthotopic breast tumor model. Bioluminescence imaging was performed to monitor the location and growth of tumor cells in vivo. For this purpose, 4T1-luc tumor cells that express luciferase were inoculated into the mammary gland of wild-type (WT) mice and IL4Rx-deficient (IL4Rx−/−) mice. Interestingly, the size of 4T1 tumors rapidly increased in the WT mice, while no significant increase was observed in the IL4Rx−/− mice; spontaneous regression was even observed in the IL4Rx−/− mice (Fig. 1A–C). Histologic analysis of tumor tissues showed that IL4Rx and...
N-cadherin, an epithelial–mesenchymal transition (EMT) marker, were highly expressed in the tumor tissues of WT mice, while low levels of these proteins were detected in the IL4R α⁻/⁻ mice (Fig. 1D). These findings suggest that IL4R expression is closely related to tumor growth and EMT.

M2-polarized macrophages produce immunosuppressive molecules, which enhance IL4R expression and tumor progression. Previous studies have suggested that M2-type (MHCII low, Ly6C low) macrophages express higher levels of IL4R than M1-type (MHCII high, Ly6C intermediate) macrophages (7, 8, 15). We tested whether this also occurs in the 4T1 tumor model. WT tumor-bearing mice were sacrificed 10 to 24 days after tumor implantation, and then, the population of M1- or M2 macrophages was analyzed in CD11b⁺ F4/80⁺ gated macrophages. Flow cytometry analysis showed that, compared with WT healthy mice, tumor-bearing mice exhibited a significant increase in M2-type macrophages in the spleen (Fig. 2A) and in TILs (Fig. 2B) as tumors developed. The balanced ratio of M1/M2 macrophages in the spleens of normal healthy mice was shifted to M2-type macrophages as tumors developed (Fig. 2A). In the TILs, the enhanced

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**Figure 1.**
The level of IL4R is closely related to the progression of 4T1 tumors. A. Bioluminescence imaging and monitoring of tumor growth. Images were taken 10 minutes after injection of D-luciferin into WT and IL4R α⁻/⁻ mice after inoculation of 4T1-luc tumors into a mammary gland. B. Quantification of total photon flux (the number of photons/second, p/s) at tumor regions shown in A. C. Tumor volume changes after tumor inoculation in WT and IL4R α⁻/⁻ mice (n = 3). D. Staining of IL4Rα, E-cadherin, and N-cadherin (green) in cultured 4T1 cells and tumor tissues of WT and IL4Rα⁻/⁻ mice. Nuclei were counterstained with DAPI (blue). Scale bars, 20 µm.
Increased IL4R expression is closely correlated with an immunosuppressive tumor microenvironment. Flow cytometry analysis of MHCII<sup>low</sup>Ly6C<sup>low</sup> M2 macrophages among splenocytes (A), and TILs (B), isolated from the spleens of normal and 4T1 tumor-bearing WT mice (n = 6) and tumor tissues at days 10 and 24 after tumor inoculation. The populations of IL4R<sup>hi</sup> or IL4R<sup>lo</sup> macrophages among splenocytes (C), and TILs (D), isolated from the spleens of normal and tumor-bearing WT mice and tumor tissues at day 14 after tumor inoculation (n = 6). E, Immunostaining of CD86<sup>+</sup> and CD206<sup>+</sup> macrophages (green) in tumor tissues. Scale bars, 20 μm. The population of total T cells (F), or activated CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells (G), among the TILs. The population of CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs (H), among TILs or CD11b<sup>+</sup>Gr1<sup>+</sup> MDSCs (I), in tumor dLN of WT and IL4Rα<sup>-/-</sup> mice (n = 3). Statistical significance was determined by an independent t test.
M2 macrophages continuously maintained their M2 phenotypes during cancer progression (Fig. 2B). Next, we analyzed whether enhanced M2-type macrophages in the tumor-bearing mice expressed high levels of IL4R. Most of the macrophages isolated from the spleens of tumor-bearing mice and in the TILs expressed IL4R (Fig. 2C and D, respectively). We also tested whether the increased tumor progression in WT mice compared with IL4R−/− mice is related to infiltration of M2-type TAMs. Indeed, WT mice showed an enhanced population of CD206+ M2-type TAMs (Fig. 2E). No difference was observed in CD86+ M1-type macrophages between the groups (Fig. 2E).

As an increase in M2-type macrophages in tumor-bearing WT mice likely induces an immunosuppressive microenvironment (1–6), we analyzed the phenotypes of the immune cells. Flow cytometry analysis of TILs showed that, compared with IL4R−/− mice, WT tumor-bearing mice had much lower populations of CD4+ and CD8+ T cells in the TILs (Fig. 2F). Moreover, these T cells showed a less activated (CD44low) phenotype, especially in the CD8+ T cells, while the CD8+ T cells of IL4R−/− mice showed a highly activated (CD44high) phenotype (Fig. 2G). Interestingly, WT tumor-bearing mice showed a significant increase in immunosuppressive CD4+Foxp3+ Tregs (Fig. 2H) and CD11b+Gr1+ myeloid-derived suppressor cells (MDSC) compared with IL4R−/− mice (Fig. 2I).

Because the generation of Tregs and MDSCs in the tumor microenvironment requires an immunosuppressive cytokine milieu, we tested whether M2-type TAMs contribute to the secretion of immunosuppressive cytokines, such as IL10 and TGFβ (37–39). Indeed, tumor-bearing WT mice expressed much higher levels of immunosuppressive cytokines (IL10, IL4, and TGFβ) and arginase1 (Arg1) and inducible NO synthase (iNOS), while the CD8+ T cells of IL4R−/− mice showed a less activated (CD44low) phenotype, especially in the CD8+ T cells, while the CD8+ T cells of IL4R−/− mice showed a highly activated (CD44high) phenotype (Fig. 2G). Interestingly, WT tumor-bearing mice showed a significant increase in immunosuppressive CD4+Foxp3+ Tregs (Fig. 2H) and CD11b+Gr1+ myeloid-derived suppressor cells (MDSC) compared with IL4R−/− mice (Fig. 2I).

The IL4R-targeted proapoptotic peptide IL4RPep-1 preferentially binds to IL4R-expressing cancer cells and M2-polarized macrophages

The data described above collectively suggest that targeted killing of IL4R-expressing cells can inhibit tumor progression. We tested whether the IL4R-targeting peptide IL4RPep-1 specifically binds to IL4R-expressing cells. IL10 treatment significantly increased the levels of IL4R in 4T1 cells (Fig. 4A) and Py2t murine breast tumor cells (Supplementary Fig. S2A; Supplementary Table S1). IL4RPep-1 preferentially bound to IL10-treated 4T1 tumor cells compared with untreated 4T1 cells (Fig. 4A) with enhanced binding affinity (lower Kd values) (Fig. 4B and C). In contrast, the control peptide did not show specific binding to cells. After binding, IL4RPep-1 was efficiently internalized into IL10-treated 4T1 cells (Supplementary Fig. S3). In addition, IL4RPep-1 also showed an enhanced binding affinity to in vitro generated M2-polarized macrophages compared with M1 macrophages (Fig. 4D–F). In M2-type macrophages, IL4R levels were particularly high compared with M1-type macrophages (Fig. 4D). Supplementary Fig. S2B; Supplementary Table S1). We also found that IL4RPep-1 could target other IL4R+ tumors (MDAMB231 and A549) and human monocyte-derived M2 macrophages (Supplementary Fig. S4; Supplementary Table S1). However, as expected, IL4RPep-1 did not bind to IL4R− tumors (MCF7 and HCT8), HEK293 cells, or Jurkat human T cells (Supplementary Fig. S4; Supplementary Table S1). These results indicated that IL4RPep-1 could selectively target diverse IL4R+ tumor cells and M2-type macrophages.

To further examine whether IL4RPep-1 could target 4T1 breast tumors in vivo, IL4RPep-1 was labeled with a NIR fluorescent dye and injected intravenously into WT and IL4R−/− mice bearing 4T1-luc tumors of similar size (bioluminescence intensity, Fig. 5A). NIR fluorescence imaging showed strong fluorescence signals of IL4RPep-1 at tumors in WT mice, which was well matched with the location of the tumors (Fig. 5B). Compared with WT mice, IL4Rx−/− mice showed much lower fluorescence signals at tumors and higher background signals throughout the body (Fig. 5B). Quantitative analysis of ex vivo images of tumors and diverse organs showed that fluorescence signals, indications of the accumulation of IL4RPep-1, in tumors of WT tumor-bearing mice were much higher than those in the liver and lung (Fig. 5C; Supplementary Fig. S5). IL4Rx−/− mice showed higher levels of fluorescence signals in the liver and kidney than in the tumors (Fig. 5C; Supplementary Fig. S5). The signals in the kidney are probably due to the excretion of unbound peptides through the kidney. Histologic analysis of tumor tissues confirmed that IL4RPep-1 preferentially targeted IL4R-expressing cells. The signals of IL4RPep-1 and IL4R at tumor tissues were colocalized in WT tumor-bearing mice (Fig. 5D). Flow cytometry analysis of the cells prepared from the tumor tissue further confirmed that approximately 80% of cells were cotained with IL4RPep-1 and IL4R (Fig. 5E). These results indicated that IL4RPep-1 could selectively target IL4R-expressing cells in the tumor microenvironment.

The IL4R-targeted proapoptotic peptide IL4RPep-1-K suppresses tumor growth and metastasis

To selectively kill IL4R-expressing tumor cells and M2-polarized macrophages, we designed the IL4R-targeted proapoptotic peptide IL4RPep-1-K. We tested whether treatment with IL4RPep-1-K could selectively induce cell death in IL4R-expressing 4T1 tumor cells and M2-polarized macrophages. IL4RPep-1-K exerted preferential cytotoxicity to IL10-treated, IL4R-expressing 4T1 cells over naive 4T1 cells, resulting in a lowering of IC50 values, 18.8 versus 371.6 mol/L, respectively (Fig. 6A). Compared with the untreated proapoptotic peptide (KLAKLAK)2, IL4RPep-1-K induced apoptosis in the IL10-treated 4T1 cells more effectively (Fig. 6B). In addition, IL4RPep-1-K showed selective cytotoxicity to M2-polarized macrophages over M1-polarized macrophages, resulting in lower IC50 values, 18.8 versus 371.6 mol/L, respectively (Fig. 6C), and effectively induced apoptosis in M2 macrophages (Fig. 6D). In addition, IL4RPep-1-K exerted
Figure 3.
Immunosuppressive factors from TAMs induce EMT and IL4R expression. A, qRT-PCR expression analysis of IL10, IL4, TGFβ, Arg1, iNOS (NOS2), and IL12p40 in the tumor tissues of WT and IL4Rα-deficient mice (n = 3). B, Expression of N-cadherin and IL4Rα in 4T1 tumor cells treated with the indicated cytokines for 24 to 48 hours and stained with anti-N-cadherin (green) and anti-IL4Rα antibodies (red). Nuclei were counterstained with DAPI (blue). Scale bars, 20 μm. C, Cytokine levels determined by ELISA in the culture supernatants of bone marrow–derived M1- and M2-polarized macrophages. The data are presented as the mean ± SD of three separate experiments. D, Analysis of EMT markers in 4T1 cells treated with conditioned medium (CM) of M1- or M2-polarized macrophages. Cells were stained with antibodies against E-cadherin, vimentin, N-cadherin, and IL4Rα (red). Nuclei were counterstained with DAPI. Scale bars, 20 μm. molcancerthermol.org:8080/molcancerthermol.org/content/16/12/2809.full.pdf
Figure 4.
IL4RPep-1 selectively targets IL4R-expressing tumor cells and M2-polarized macrophages. A, Cells were incubated with FITC-labeled IL4RPep-1 or control peptide (green) and then incubated with anti-IL4Rα antibody (red). Nuclei were counterstained with DAPI (blue). Scale bars, 20 μm. The K_d values of IL4RPep-1 binding to 4T1 cells in the absence (B) or presence (C) of IL10.
D, Cells were incubated with FITC-labeled IL4RPep-1 or control peptide (green) and then incubated with anti-IL4Rα antibody (red). Nuclei were counterstained with DAPI (blue). Scale bars, 20 μm. The K_d values of IL4RPep-1 binding to M1-polarized macrophages (E) or M2-polarized macrophages (F). The K_d values were calculated from saturation binding assays using GraphPad Prism 6 software.
selective cytotoxicity to other IL4Rα<sup>low</sup> cells over IL4Rα<sup>high</sup> cells (Supplementary Fig. S6; Supplementary Table S1).

Next, we tested the antitumor efficacy of IL4RPep-1-K in the 4T1 tumor model. IL4RPep-1-K was stable without degradation up to 24 hours in the presence of serum (Supplementary Fig. S7). Systemic administration of IL4RPep-1-K significantly inhibited primary mammary tumor growth (Fig. 6E). Combined treatment of IL4RPep-1-K with paclitaxel (P) (IL4RPep-1-K+P) further inhibited tumor growth (Fig. 6E). On the other hand, treatment with a mixture of IL4RPep-1 and (KLAKLAK)<sub>2</sub> (K) (IL4RPep-1+K), paclitaxel alone, or their combination (IL4RPep-1+K+P) slightly inhibited 4T1 tumor growth compared with the saline-treated group (Fig. 6E). Interestingly, treatment with IL4RPep-1-K or IL4RPep-1-K+P significantly blocked the metastasis of 4T1 tumors to the lung and liver (Fig. 6E) and reduced the levels of N-cadherin–expressing cells (Supplementary Fig. S8A) by inducing apoptosis in 4T1 tumor cells (Supplementary Fig. S8B).

The IL4R-targeted proapoptotic peptide IL4RPep-1-K enhances antitumor immunity by altering immune phenotypes in the tumor microenvironment

We tested the possibility that the anticancer effect of IL4RPep-1-K is also accompanied by an enhancement of antitumor immunity in the tumor microenvironment. Treatment with
Figure 6.
IL4RPep-1-K induces selective cytotoxicity in IL4R-expressing tumor cells and M2-polarized macrophages and inhibits 4T1 tumor growth and metastasis. 
A, IC50 of IL4RPep-1-K in 4T1 cells untreated or treated with IL10. The cell survival rate was analyzed using CCK-8 assays. IC50 of IL4RPep-1-K was determined using GraphPad Prism 6 software.
B, The percentage of apoptotic cells (Annexin V+/PI+) in the 4T1 cells was determined by flow cytometry after treatment with the indicated peptides. The data are presented as the mean ± SD of three separate experiments.
C, IC50 of IL4RPep-1-K in M1- and M2-polarized macrophages after treatment with IL4RPep-1-K. D, The percentage of apoptotic cells (Annexin V+/PI+) in the M1- and M2-polarized macrophage populations was determined by flow cytometry after treatment with the indicated peptides. The data are presented as the mean ± SD of three separate experiments.
E, Tumor volume changes after the indicated treatments. Each peptide was intravenously injected into 4T1 tumor-bearing mice (n = 10; 14.2 μg/gram of body weight, three times a week for 4 weeks). Paclitaxel was administered through intraperitoneal injection (8 μg/gram of body weight, once a week for 4 weeks). F, The number of metastatic tumor nodules in the lung and liver at the end of treatments (n = 10). K, (KLAKLAK)2; IL4RPep-1+K, mixture of IL4RPep-1 and K; PTX or P, paclitaxel; N.D., not detected.
IL4RPep-1-K or IL4RPep-1-K+P significantly increased the populations of M1-polarized macrophages (Fig. 7A) and activated (CD44high) types of lymphocytes (Fig. 7B and C). Interestingly, treatment with IL4RPep-1-K or IL4RPep-1-K+P reduced the population and number of immunosuppressive Tregs (Fig. 7D) and the levels of immunosuppressive molecules, such as IL10, IL4, TGFβ, Arg1, and iNOS, while increasing the expression of immunostimulatory IL12 (Fig. 7E). In addition, treatment with IL4RPep-1-K or IL4RPep-1-K+P significantly reduced the number of total splenocytes and M2-type macrophages in the spleen of tumor-bearing mice, while increasing the number of M1-type macrophages (Supplementary Fig. S9A–S9C). In dLNs, IL4RPep-1-K or IL4RPep-1-K+P significantly reduced the population of total CD11b+F4/80+ macrophages, M2-type macrophages, Tregs, and MDSCs, while increasing M1-polarized macrophages (Supplementary Fig. S10A–S10D). To examine whether the antitumor activity of IL4RPep-1-K is mediated through CD8+ T cells, CD8– T cells were depleted in vivo by treatment with an mAb against CD8α (Supplementary Fig. S11A). The antitumor activity of IL4RPep-1-K was partially reduced after the CD8– T-cell depletion (Supplementary Fig. S11B). Collectively, these results suggested that the IL4R-targeted proapoptotic peptide inhibited tumor progression by reprogramming the tumor microenvironment from immunosuppressive to immunostimulatory conditions.

We also analyzed whether treatment with IL4RPep-1-K or IL4RPep-1-K+P induced systemic side effects by measuring hemato logic parameters and liver and kidney function. After IL4RPep-1-K or IL4RPep-1-K+P treatment, the total white blood cell count was decreased close to the value of the healthy controls (Supplementary Fig. S11A). No significant side effects were observed in the hematologic parameters (Supplementary Fig. S11B–S11N). The serum levels of liver function enzymes, such as aspartate transferase and alanine transferase, as well as kidney function indicators, such as blood urea nitrogen and creatinine, were close to those of healthy controls after the treatments (Supplementary Fig. S11O and S11R), indicating that no significant toxicities to the liver or kidney were induced by the treatments. In addition, no death of animals occurred during the treatment period.

Discussion

Dynamic interactions between tumor cells and M2-type TAMs continuously reinforce each other in the tumor microenvironment. Because many types of tumors and M2-type macrophages express IL4R, we developed a IL4R-targeted proapoptotic peptide, IL4R-Pep-1-K, to induce targeted killing of IL4R-expressing cells. IL4R-Pep-1-K treatment suppressed tumor growth and metastasis through selective cytotoxicity toward IL4R-expressing tumor cells and M2 macrophages in the 4T1 breast tumor, which was accompanied by an enhanced antitumor immunity. Our results suggest a potential application of IL4R-Pep-1-K for treating diverse IL4R-expressing tumors.

The rationale and importance of targeting IL4R has been previously reported. Compared with M1-type macrophages, M2-polarized macrophages express higher levels of IL4R in MMTV-PyMT and T5A mouse mammary tumor models (7, 8, 15). IL4R is upregulated in human primary breast cancer tissues (18). We also found that as a 4T1 tumor develops, there was a significant increase in IL4R-expressing cells, such as M2-type macrophages. IL4R-expressing cells may contribute to the progression of tumors via a positive feedback mechanism between IL4R and immunosuppressive factors. TAMs produce high levels of immunosuppressive TGFβ and IL10, as shown in previous studies (37–39) and the results in this study, which in turn enhance IL4R expression in tumor cells. TAM-derived IL10, in particular, has been known to recruit Tregs and inhibit maturation and antigen presentation by dendritic cells (5). When 4T1 tumor cells were implanted in the mammary glands of WT mice, they began to express high levels of IL4R and underwent EMT. In contrast, when 4T1 tumor cells were implanted in IL4R−deficient mice, they maintained an epithelial phenotype, slowly progressed, and even showed spontaneous regression within 2 to 3 weeks of onset. Similar to our results, 4T1 cells implanted into IL4R−deficient mice or IL4R−knockdown 4T1 tumor cells implanted into wild-type mice showed attenuated metastatic colonization (20, 40). IL4R−deficient mice also exhibited reduced colon tumor progression (41). These findings collectively suggest that selective inhibition of IL4R can suppress tumor progression. Indeed, several approaches have been used to treat tumors by blocking IL4 activity or IL4R using antibodies (15) or RNA aptamers (42). In this study, we developed an IL4R-targeted proapoptotic peptide, IL4R-Pep1-K, and demonstrated that administration of IL4R-Pep-1-K could suppress 4T1 tumor growth and metastasis by selectively binding and exerting cytotoxicity toward tumor cells and M2-type macrophages in vivo. This antitumor effect of IL4R-Pep-1-K was accompanied by a reprogramming of the immunosuppressive tumor microenvironment into an immunostimulatory microenvironment, as evidenced by increases in M1-polarized macrophage activity and CD8+ T cells as well as decreases in M2-polarized TAMs, Tregs, and MDSCs as well as immunosuppressive molecules. In support of our results, it has been reported that depletion of TAMs using a CSF-1R kinase inhibitor or CSF-1–neutralizing antibody stimulates infiltration of CD8+ T cells into tumors (14). TAMs contribute to the depletion of T cells by inducing apoptosis through STAT1 signaling (43). Tumor cells cooperate with TAMs to block the recruitment of cytotoxic CD8+ T cells through nitration of the chemokine CCL2 (44). As MDSCs also secrete immunosuppressive Arg1 and iNOS (45), the reduction in MDSCs after IL4RPep-1-K treatment may also contribute to the reprogramming of immunosuppressive conditions. In addition, we found that the depletion of CD8+ T cells reduced the antitumor growth activity of IL4RPep-1-K. In a previous study, a cytotoxic peptide, KL1KLkkLkKLkkKK, fused with a sequence of IL4, 77KQLRFKLRLDRNG69, has been shown to exert antitumor activity in nude mice in which T cells were defective (46, 47). Taken together, these results suggest a rationale and highlight the potency of IL4R-targeted anticancer therapy, which may work through both T cell–dependent and T cell–independent mechanisms.

Here, we present the possibility that IL4R-Pep-1-K could be used as a therapeutic modality for the treatment of triple-negative breast cancer (TNBC). 4T1 breast tumor, used in this study as a model, is a highly malignant and invasive tumor that can spontaneously metastasize to distant sites, including the liver and lung (35, 36). The 4T1 breast tumor is considered to be a mouse model, is a highly malignant and invasive tumor that can spontaneously metastasize to distant sites, including the liver and lung (35, 36). 4T1 breast tumor is considered to be a mouse model, which is frequently resistant to chemotherapy and currently has no targeted therapeutics (35, 36). Paclitaxel, a microtubule-stabilizing drug (48), has been used for chemotherapy in TNBC (49). We found that paclitaxel alone showed only weak antitumor activity toward 4T1 tumors. Notably, single treatment with IL4RPep-1-K efficiently inhibited tumor growth...
Figure 7.
IL4RPep-1-K treatment enhances antitumor immunity. The population of CD11b<sup>+</sup>F4/80<sup>+</sup>MHCII<sup>high</sup>Ly6C<sup>intermediate</sup>M1-polarized macrophages (A), total CD4<sup>+</sup> or CD8<sup>+</sup> T cells (B), CD44<sup>+</sup> activated T cells in CD4<sup>+</sup> and CD8<sup>+</sup> T cells (C), or CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs (D), among the TILs after the indicated treatments. E, qRT-PCR analysis of relative mRNA levels of IL10, IL4, TGFβ, Arg1, iNOS (NOS2), and IL12p40 in tumor tissues after the indicated treatments. PTX or P, paclitaxel.
and metastasis in the 4T1 mouse TNBC model, and combined treatment with paclitaxel further enhanced the antitumor therapeutic effect. In addition, we showed that IL4RPep-1 could bind to both mouse and human tumor cells that highly express IL4R. As previously described by us and other groups (21, 26, 47, 50), three arginine residues on IL4RPep-1 (CRKRLDRNC) are homologous to those residues on human IL4 (RFLKLRDRNLW) and mouse IL4 (QRLFRAFR). This may explain the cross-species specificity of IL4RPep-1 binding. Previously, an immunotoxin composed of a fragment of IL4 and Pseudomonas exotoxin has shown a therapeutic efficacy in breast tumor (18). However, there are concerns of systemic side effects, such as immunogenicity and hepatotoxicity, by treatment with immunotoxins (51, 52). Treatment with IL4RPep-1-K did not show significant side effects such as hepatotoxicity. In addition, IL4RPep-1-K is a smaller peptide and is expected to more efficiently penetrate into tumor tissues compared with immunotoxins (31–33). It may still be necessary to consider that a possible difference in peptide clearance depending on individuals can affect treatment outcome and doses for treatments. Collectively, these findings indicate the feasibility of using IL4RPep-1-K as a therapeutic agent for the treatment of patients with TNBC.

Whether there is a functional relationship between EMT and IL4R expression remains a subject for future study; specifically, such a study should determine whether IL4R induces the expression of IL4 or IL4R promotes EMT progression in 4T1 tumor cells. In addition, it would be interesting to determine whether IL4RPep-1-K holds potential as an anticancer drug against tumor cells undergoing EMT, as IL4RPep-1-K treatment reduced IL10, IL4, and TGFβ, as well as the population of N-cadherin-expressing cells in tumor tissues. In addition, IL4RPep-1 is efficiently internalized into cells, indicating that IL4RPep-1 could be a useful vehicle for intracellular delivery of diverse therapeutic agents into IL4R-expressing cells.

In summary, our data suggest that the IL4R-targeted proapoptotic peptide IL4RPep-1-K might be beneficial for the treatment of IL4R-expressing tumors because it blocks dynamic communication between tumor cells and TAMs by enhancing antitumor immunity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.M.P. Vadevoo, J.-E. Kim, G.R. Gunassekaran, S.-H. Im, B. Lee Development of methodology: S.M.P. Vadevoo, J.-E. Kim, G.R. Gunassekaran Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M.P. Vadevoo, J.-E. Kim, G.R. Gunassekaran, H.-K. Jung, L. Chi, S.-H. Im, B. Lee Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.M.P. Vadevoo, J.-E. Kim, G.R. Gunassekaran, S.-H. Im, B. Lee Writing, review, and/or revision of the manuscript: S.M.P. Vadevoo, J.-E. Kim, G.R. Gunassekaran, S.-H. Im, B. Lee Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.M.P. Vadevoo, J.-E. Kim, G.R. Gunassekaran, D.E. Kim, S.-H. Lee, S.-H. Im, B. Lee Study supervision: S.-H. Im, B. Lee

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IL4 Receptor–Targeted Proapoptotic Peptide Blocks Tumor Growth and Metastasis by Enhancing Antitumor Immunity

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