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Paired Ig-Like Type 2 Receptor-Derived Agonist Ligands Ameliorate Inflammatory Reactions by Downregulating β1 Integrin Activity

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The paired immunoglobulin-like type 2 receptor (PILR) family consists of two functionally opposite members, inhibitory PILRα and activating PILRβ receptors. PILRs are widely expressed in various immune cells and interact with their ligands, especially CD99 expressed on activated T cells, to participate in immune responses. Here we investigated whether PILR-derived agonists inhibit β1 integrin activity as ligands for CD99. PILR-derived peptides as well as PILR-Fc fusion proteins prevented cell adhesion to fibronectin through the regulation of β1 integrin activity. Especially, PILRpep3, a representative 3-mer peptide covering the conserved motifs of the PILR extracellular domain, prevented the clustering and activation of β1 integrin by dephosphorylating FAK and vinculin, which are major components of focal adhesion. In addition, PILRpep3 inhibited transendothelial migration of monocytes as well as endothelial cell tube formation. Furthermore, upon intraperitoneal injection of PILRpep3 into mice with collagen-induced arthritis, the inflammatory response of rheumatoid arthritis was strongly suppressed. Taken together, these results suggest that PILR-derived agonist ligands may prevent the inflammatory reactions of rheumatoid arthritis by activating CD99.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder caused by altered cellular immunity giving rise to severe pain, swelling, stiffness, and loss of function in joints. The typical features of RA are the expansion of inflamed synovium, pannus invasion into the cartilage and bone, and irregular angiogenesis, leading to persistent progressive joint damage (Akhavani et al., 2009). It is well recognized that excessive immune response to a self-antigen by innate and adaptive immune cells lacking inflammation regulatory systems can lead to the initiation and progression of RA. In fact, several immune cell populations, such as macrophages, dendritic cells, synovial fibroblasts, osteoclasts, and infiltrating T and B cells, are abundant in the synovium of patients with RA. However, the role of these immune cells in RA pathogenesis remains unclear.

Paired immunoglobulin-like type 2 receptors (PILRs) are cell surface proteins expressed in immune cells, including natural killer cells, macrophages, and dendritic cells. The PILR receptor family has two isoforms that play opposite roles. PILRα inhibits the immune response by recruiting cytosolic phosphatase to the immunoreceptor tyrosine-based inhibitory motif on its cytoplasmic domain (Sun et al., 2014). Recently, it was shown to control monocyte infiltration via regulation of hemophilic CD99 interactions and integrin signaling (Kohyama et al., 2016). In contrast, PILRβ activates the immune response by associating with DAP12 harboring the immunoreceptor tyrosine-based activation motif (Mousseau et al., 2000; Shiratori et al., 2004; Tato et al., 2012). Therefore, it appears that the interactions of PILRs with their ligands play a critical role in regulating immune cell functions. Several PILR ligands have been identified to date, including CD99, PILR-associating neural protein (PANP), HSV-1 glycoprotein B (HSV-1 gB), neuronal proliferation and differentiation control factor-1 (NPDC1), and collectin-12 (COLEC12) (Sun et al., 2012). Especially, both PILRα and PILRβ bind to O-glycosylated transmembrane protein CD99 with different binding affinities and fast kinetics, which are typical for cell-cell recognition receptors, and transduce signals bidirectionally into PILR- as well as CD99-expressing cells (Tabata et al., 2008).

Human CD99, a 32-kDa type I transmembrane glycoprotein, is broadly expressed in various cell types and is particularly abundant in lymphocytes and several tumors (Bixel et al., 2004; Ellis et al., 1994; Schenkel et al., 2002). It is implicated in various cellular processes, including cell adhesion, apoptosis, vesicular protein transport, thymocyte differentiation, and T-cell activation and maturation (Bernard et al., 1995; 1997; Choi et al., 1998; Hahn et al., 1997; Wingett et al., 1999). Additionally, CD99 mediates the homophilic interaction between endothelial cells and leukocytes that is required for transmigration of leukocytes (Schenkel et al., 2002). A previous ***in vivo*** study demonstrated that anti-CD99 antibody inhibited transmigration of activated lymphocytes into inflammatory sites, but did not affect the

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homing of naïve lymphocytes (Bixel et al., 2004). In addition, we reported that antibody-mediated crosslinking of CD99 downregulated β1 integrin activity through SHP-2-mediated focal adhesion kinase (FAK) dephosphorylation (Lee et al., 2012; 2015). These studies suggest that CD99 may be a critical regulator of inflammatory reactions and a promising therapeutic target for inflammatory and autoimmune diseases (Watson et al., 2015; Winger et al., 2016).

Here, we investigated the potential role of PILR as a ligand for CD99 in regulating the cell-extracellular matrix (ECM) interaction and in the progression of RA characterized by infiltrating immune cells. Our results show that PILR-derived agonist ligands can function as ligands for CD99 and ameliorate the inflammatory reactions by activating CD99.

MATERIALS AND METHODS

Cell culture

Human breast adenocarcinoma MCF-7 cells and mouse monocyte WEHI 274.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.7 g sodium carbohydrate 10% (v/v) fetal bovine serum (FBS), 100 unit/ml penicillin, and 100 μg/ml streptomycin (all from Gibco-BRL, USA) at 37°C in a humidified 5% CO_2 incubator, and 1 mM sodium pyruvate was added only for MCF-7. Mouse endothelial bEnd.3 cells were cultured in DMEM supplemented with 1.5 g sodium carbohydrate, 10% v/v FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVECs) were cultured in Medium 199 supplemented with 20% v/v FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml basic fibroblast growth factor, and 5 units/ml heparin (Sigma-Aldrich Co., USA).

Preparation of PILR-human Fc fusion protein

PILR-α or -β variant cDNAs partially lacking the extracellular domain were produced by PCR. The primers used to make the mutant PILR cDNA fragments as shown in Fig. 1A were as follows: sense primer I (5′-CGGAATTCGCCATGGGTCGGCCCCTGCTGCTG-3′) and antisense primer I (5′-CGGAATTCTCCTGGGTGATGGTGAGTTTGGTCC-3′) for PILR-FcI; sense primer II (5′-CGGAATTCGTGAGAATATCCTGGAGACGGGG-3′) and antisense primer I for PILR-FcII; sense primer III (5′-CGGAATTCCAGTCTGTGTATTTCTGCCGACTTG-3′) and antisense primer I for PILR-FcIII; sense primer II and antisense primer II (5′-CGGAATTCCTGTCCTGCTTCTGCAGGTTGGAG-3′) for PILR-FcIV; sense primer III and antisense primer III (5′-GGAATTCTCCCCTGAGCTCCGTGTGTCCAG-3′ for PILR-FcV; sense primer IV (5′-CGGAATTCCAGCTCAACTCGGCAGAAATA-3′) and antisense primer IV (5′-CGGAATTCTATTTCTGCCGAGTTGAGCTG-3′) for PILR-FcVI. To obtain mutant PILR-Fc fusion constructs, each variant cDNA was subcloned into the ***Eco***RI site of the pET28a(+) vector carrying cDNA coding for the Fc domain of human IgG1. All mutant PILR-Fc constructs were confirmed by sequencing and then transformed into Escherichia coli BL21 (DE3). The transformed cells were grown in Luria-Bertani broth containing 30 μg/ml kanamycin for 4-6 h at 37°C in a shaking incubator (250 rpm). The optical density of the culture at 600 nm was allowed to reach approximately 0.5 and then, isopropyl β-D-1-thiogalactopyranoside (IPTG; 1.4 mM) was added to induce protein expression. Cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the expression of PILR-Fc protein. Recombinant proteins were purified using the Ni-NTA His-Bind Resins kit (Invitrogen Corp., USA) per the manufacturer's protocol.

PILR-derived peptide synthesis

PILR-derived peptides were synthesized using an automatic peptide synthesizer (PeptrEX-R48; Peptron, Korea) according to the manufacturer's protocol. Purification and analysis of the synthesized polypeptides were carried out using reverse-phase high-performance liquid chromatography (Prominence LC-20AB; Shimadzu, Japan) and mass spectrometry (HP1100 Series LC/MSD; Hewlett-Packard, USA).

Adhesion assay

Adhesion assays were conducted as described previously (Lee et al., 2015). Briefly, MCF-7 pretreated with or without lipopolysaccharide (LPS; List Biological Laboratories, USA) or non-stimulated HUVECs were treated with each purified recombinant protein or PILR peptide. One hundred microliters of the cell suspension (5×10^5 cells/ml) was transferred to 96-well plates coated with 10 μg/ml of fibronectin and allowed to attach for 1 h at 37°C in 5% CO_2 under static conditions. Non-attached cells were removed by washing in 1× PBS and the attached cells were quantitated by manual counting under an inverted microscope (OlympusCK40; Olympus, Japan) using a hemocytometer.

Tube formation assay

A tube formation assay was used to evaluate the effect of PILR peptide on angiogenesis ***in vitro***. Matrigel (BD Biosciences, USA) was diluted to 3 mg/ml in 0.5 ml serum-free medium. A 96-well plate was coated with 50 μl of diluted matrigel per well and incubated sequentially at RT for 10 min and at 37°C for 30 h to polymerize. HUVECs were suspended in fresh Medium 199 containing 0.1% FBS, 10 ng/ml of VEGF with or without PILR peptides, seeded into each well with polymerized matrigel (5×10^4 cells/well), and incubated at 37°C in 5% CO_2 for 6, 12, or 24 h. Each culture was photographed using the inverted microscope and the total length of branched tubes was measured using the ImageJ software (NIH, USA).

siRNA preparation

siRNA duplexes were constructed as described previously (Lee et al., 2008). Target sequences were as follows: human CD99 I, sense primer I (5′-AAGATTGCAGTGGGTTCTTGCCTGTCTC-3′) and antisense primer I (5′-AACAAGAAACCCACTGCAATCCCTGTCT-3′); human CD99 II, sense primer II (5′-AACATCACTGCCTCCTTTTCCCCTGTCTC-3′) and antisense primer II (5′-AAGGAAAAGGAGGCAGTGATGCCTGTCTC-3′). Control siRNA was purchased from Bioneer Corporation (Korea); sense (5′-CCUACGCCACCAAUUUCGUTT-3′) and antisense (5′-ACGAAAUUGGUGGCGUAGGTT-3′). siRNA was transfected into cells using Lipofectamine reagent (Invitrogen Corp., USA). After transfection, the culture medium was removed and the cells were maintained in medium with 10% FBS for 24 h.

Immunofluorescence and confocal microscopy

MCF-7 cells were seeded onto laminin- or fibronectin-coated glass coverslips. After serum starvation in DMEM with 0.1% bovine serum albumin (BSA) for 16 h, the cells were treated with PILR peptide for 1 h as indicated in Figs. 2A and 3B. The cells were fixed with 400 μl of 2% paraformaldehyde in 1× cold PBS and washed with 1× PBS. Fixed cells were stained with 1 μg of primary antibody in 100 μl of serum-free medium (SFM) for 30 min at 4°C, and unbound antibody was removed by washing with SFM. The cells were incubated in 100 μl of SFM including 1

μg of secondary antibody conjugated to FITC or rhodamine in dark condition at 4°C for 40 min. Unbound secondary antibody was washed away twice with SFM. Each sample was washed with PBS and mounted onto glass slides with an aqueous mounting medium.

Additionally, MCF-7 cells were transfected with CD99 siRNA at indicated concentrations. After a 24-h incubation, the cells were seeded onto fibronectin-coated glass coverslips and treated and fixed as described above. The cells were stained with mouse anti-CD29 FITC-conjugated antibody in dark condition at 37°C for 1 h. After removing unbound antibody by washing with PBS, the samples were mounted onto glass slides with an aqueous mounting medium. Fluorescence images were acquired using a confocal microscope (Olympus FluoView FV1000; Olympus, Japan).

Western blot analysis

MCF-7 cells were seeded into 35-mm dishes (5×10^5 cells/dish) coated with fibronectin. The cells were serum-starved overnight in DMEM with 0.1% BSA and then treated with 20 μM of PILR peptide for 1 h and immediately lysed with 1% NP40 lysis buffer (1% Nonidet P40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 5 mM EDTA) with 1 mM sodium orthovanadate and protease inhibitor cocktail. The cell lysates were subjected to SDS-PAGE and the separated proteins were transferred to a polyvinylidene fluoride membrane. The membrane was incubated for 1 h at room temperature in blocking solution (3% BSA in TBS containing 0.05% Tween-20) and incubated with antibodies against phospho-FAK Y397 (Cell Signaling Technology, USA), FAK (Cell Signaling Technology, USA), phospho-vinculin Y100 (Invitrogen Corp., USA), vinculin (Merck Millipore, Germany), and actin (Santa Cruz Biotechnology, USA) in TBS buffer containing 0.05% Tween-20 for 24 h at 4°C while shaking. After the incubation with primary antibodies, the membrane was incubated with horseradish peroxidase-conjugated anti-IgG. The membrane was washed five times for 5 min with TBS-T buffer, and proteins were detected using the West-Zol plus kit (iNtRON Biotechnology, Korea).

Flow cytometry

MCF-7 cells were seeded into 35-mm dishes as described above. After serum starvation, the cells were treated with different concentrations of PILR peptide for 1 h and collected using enzyme-free cell dissociation solution (Millipore, USA). The cells were stained with Milli-Mark™ anti-integrin β1-FITC (1 μg/100 μl) for 30 min at 4°C to detect active β1 integrin and then washed with 1× cold PBS containing 0.1% sodium azide. After three washes, flow-cytometric analysis was conducted using the BD Accuri C6 system (Becton Dickinson and Co., USA).

In situ proximity ligation assay (PLA)

In situ PLA (Duolink ***In Situ*** reagents; O-LINK Bioscience, Sweden) was performed per the manufacturer's instructions. Briefly, 1×10^5 MCF-7 cells were plated onto fibronectin-coated coverslips in 24-well cell culture plates and grown in DMEM. The cells were serum-starved overnight and then treated with PILR peptides at indicated concentrations for 30 min at 37°C in 5% CO_2, followed by washing twice with 1× PBS. The cells were fixed with 2% formaldehyde in PBS for 15 min at room temperature. Subsequently, the cells were washed twice with 1× PBS, permeabilized in 0.1% Triton X-100 in PBS for 5 min, and washed twice with buffer A for 5 min. The cells were incubated with a blocking solution at 37°C for 30 min, and then washed twice with buffer A for 5 min. Protein-protein interactions were analyzed by confocal laser-scanning microscopy.

Transendothelial migration (TEM) assay

TEM of mouse monocytes through a cultured monolayer of mouse endothelial cells was assayed using Trans-well chambers (Corning Life Sciences, USA). bEnd.3 cells (5×10^4 cells/well) were plated in gelatin-coated filter inserts (24-well format, pore size 8 mm) and grown to confluence for 48 h at 37°C in a 5% CO_2 incubator. After removal of the supernatant, 5×10^5 WEHI274.1 cells pretreated with or without PILR peptide were added to the upper compartment of the transwell, and 600 μl of NIH3T3 cell-conditioned medium containing 0.005% vitamin C and 0.1% BSA was added in the bottom compartment. Mouse monocytes were allowed to transmigrate for 6 h, and migrated cells were collected and quantitated by manual counting using a hemocytometer under the inverted microscope.

In vivo TEM assay

Mouse WEHI274.1 monocytes were suspended and incubated in methionine-free medium to deplete intracellular methionine for 15 min at 37°C under 5% CO_2. Labeling medium containing 0.2 mCi/ml of [^{35}S]-methionine (PerkinElmer Inc., USA) was added, followed by incubation for 30 min at 37°C. Methionine-containing medium was added into the cell suspension. After 1 min of incubation, the cells were centrifuged and thoroughly washed twice with 1× PBS.

Twenty microliters of phorbol 12-myristate 13-acetate PMA (250 μM) was painted onto the dorsal surface of the right ear to induce skin inflammation and 20 μl of acetone/olive oil (1:4, v/v) was painted onto the dorsal surface of the left ear as a control. After 4-6 h, ^{35}S-labeled WEHI 274.1 cells were treated with PILR peptide or PBS only and intravenously administrated into the mice. After 1 h, the mice were sacrificed by cervical dislocation and the ears were separated. Samples were obtained from the tips of each ear using a 6-mm punch, and a scintillation counter (PerkinElmer Inc., USA) was used to measure the counts per minute.

CIA mouse model

Female DBA-1J mice received 200 μg of bovine type II collagen (Sigma-Aldrich, USA) in Freund's complete adjuvant (Sigma-Aldrich) by intradermal injection at the base of the tail on day 0, with a booster injection on day 14. Mice were monitored daily for signs of arthritis and each paw was scored individually as follows: 0 = normal, 1 = slight edema, 2 = increased edema with loss of landmarks, 3 = marked edema, and 4 = marked edema with ankylosis on flexion. Each mouse was assigned an arthritis score that equaled the sum of the scores for each paw, so that the possible maximum score per mouse was 16. In the prophylactic dosing model, mice were treated with PILR peptide dissolved in PBS (2.5 or 5 milligrams per kilogram body weight) daily from day 14 to day 49 by intraperitoneal injection, and monitored for disease incidence and the severity of arthritis up to day 49. CIA control mice in both experiments received injections with PBS only. On day 49, the mice were sacrificed and articular cartilage tissues were paraffinized and sectioned with a microtome. The tissue slices were stained with hematoxylin and eosin.

RESULTS

PILR-derived peptides as well as PILR-Fc fusion proteins prevent cell adhesion to the ECM through regulation of β1 integrin activity

To investigate the effects of PILR-derived agonist ligands on

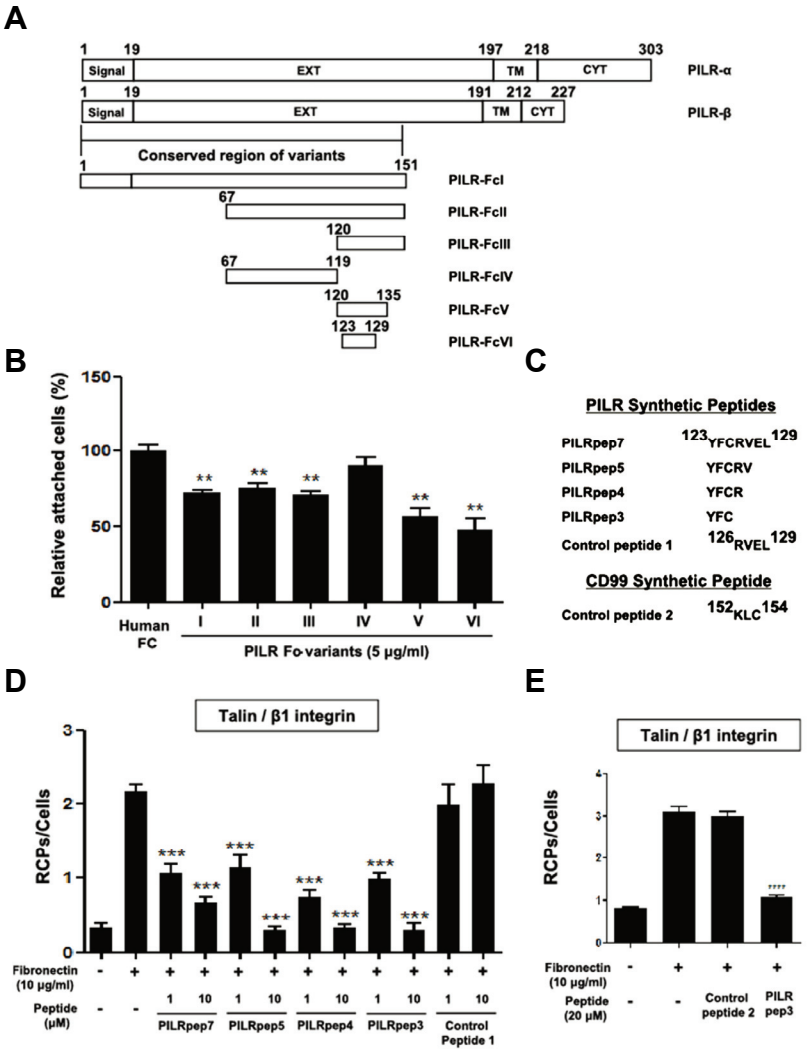

Fig. 1. Identification of PILR-derived agonistic peptides. (A) Schematic diagram of PILR-α/β-human Fc fusion variants. (B) HUVECs were suspended in fresh complete Medium 199 containing 5 μg/ml of PILR-Fc fusion protein and then seeded into a 96-well plate coated with fibronectin. After a 1-h incubation, the adhesion rate was determined by counting the number of attached cells using a hemocytometer. $^{**}P < 0.01$. (C) Amino acid sequences of the synthetic peptides. (D, E) To identify the crucial amino acid sequences, MCF-7 cells were incubated with PILR-derived peptides or control peptides for 30 min in SFM and the interaction of talin with β1 integrin was analyzed by ***in situ*** PLA. The graph indicates the intensity of the interactions. PLA signals in the cell population (n = 10) were quantified by using NIS Elements. The average number of rolling-circle products (RCPs) per cell ± SD is shown. $^{***}P < 0.001$.

CD99-mediated cellular responses, we constructed a series of PILR-Fc fusion proteins containing highly conserved regions from the extracellular domains of PILRα and PILRβ (Fig. 1A) and examined their effects on adhesion of HUVECs to fibronectin. The purified fusion proteins PILR-Fc I, II, III, V, and VI effectively reduced the attachment of HUVECs to fibronectin, whereas PILR-Fc IV did not (Fig. 1B). These results indicated that amino acid residues 123 to 129 are required for regulating cell adhesion to the ECM. To identify the amino acid sequences in the extracellular domains of PILRs critical for interaction with CD99, we prepared a series of synthetic oligopeptides derived from residues 123 to 129 (Fig. 1C) and assayed their effects on the interaction of β1 integrin with its adapter protein talin using ***in situ*** PLA. PILRpep7, PILRpep5, PILRpep4, and PILRpep3 significantly inhibited the interaction of β1 integrin with talin in a dose-dependent manner (Fig. 1D). In contrast, a tetrameric control peptide covering residues 126 to 129 had no functional effects on β1 integrin activity. Although PILRpep4 showed the strongest inhibitory effects on cell-ECM adhesion, PILRpep3 had comparable inhibitory activity. To ensure target specificity of PILRpep3, we used a trimeric peptide derived from CD99 as a negative control (Fig. 1E). PILRpep3 completely suppressed the fibronectin-induced interaction of β1 integrin with talin, whereas the PILR-unrelated trimeric peptide had no effect. These results suggested that PILRpep3, tyrosine-phenylalanine-cysteine, may function as an agonist ligand for CD99.

PILRpep3 inhibits ECM-mediated activation of β1 integrin by dephosphorylating FAK and vinculin

Our previous results showed that CD99 activation led to a decrease in β1 integrin activity through inside-out signaling (Lee et al., 2012; 2015). Therefore, we wondered whether PILRpep3 decreases β1 integrin activity in a way similar to that of CD99 agonist ligands. As expected, PILRpep3 apparently decreased β1 integrin activity, which was increased by both fibronectin and laminin (Figs. 2A and 2B), suggesting that the active form of β1 integrin was reverted to the inactive form by PILRpep3 treatment. To verify whether the inhibitory effect of PILRpep3 on ECM-mediated activation of β1 integrin is dependent on CD99 activation, human breast cancer MCF-7 cells were transiently transfected with CD99 siRNA to achieve knockdown of CD99 expression followed by immunofluorescence assay and ***in situ***

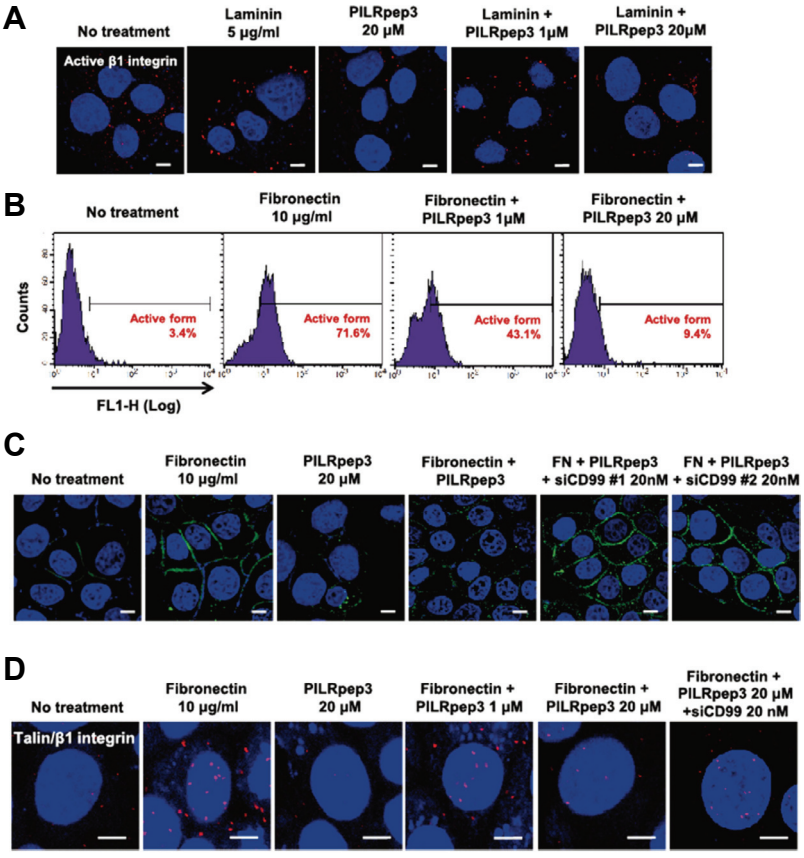

Fig. 2. PILRpep3 contributes to the inactivation of β1 integrin in a CD99-dependent manner. To detect β1 integrin activity, MCF-7 cells were seeded onto laminin- or fibronectin-coated coverslips. After a 24-h incubation, the cells were treated with PILRpep3 for 1 h and stained with mouse anti-human active β1 integrin monoclonal antibody. Active form of β1 integrin was examined by immunofluorescence (A) and flow cytometry (B). Red dots indicate active β1 integrin. (C, D) MCF-7 cells transfected with CD99 siRNA were seeded onto fibronectin-coated coverslips and treated with PILRpep3. (C) Clustering of β1 integrin was examined by immunofluorescence. (D) ***In situ*** PLA was conducted to examine talin/β1 integrin interactions. Red dots indicate the physical interactions between the indicated molecules. (A, C, and D) Nuclei were counterstained with DAPI (blue). Scale bar = 5 μm; original magnification, 600×.

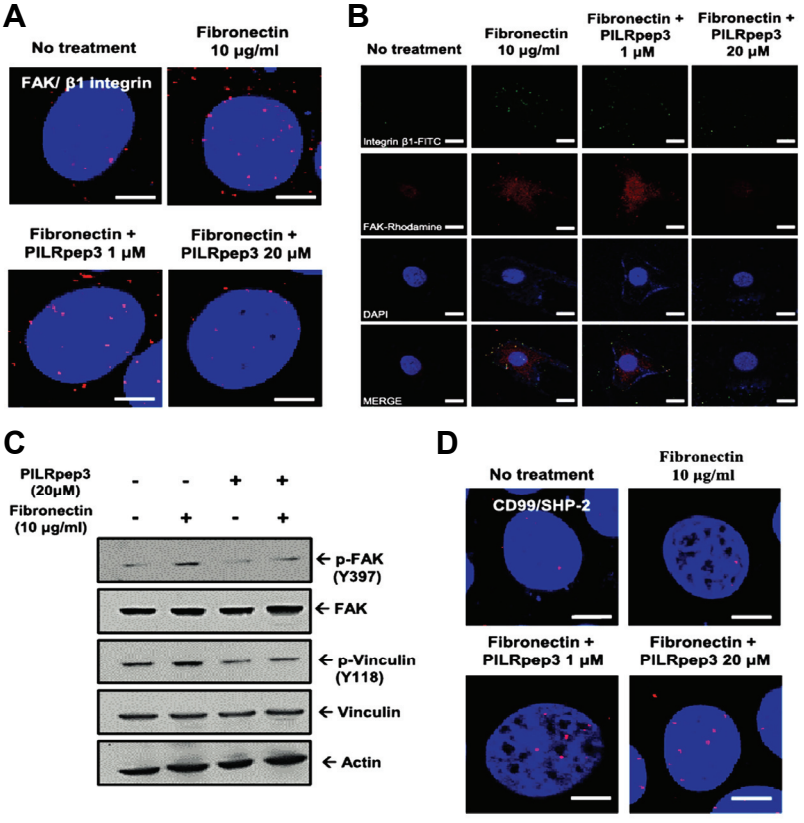

Fig. 3. PILRpep3 inhibits β1 integrin activity by dephosphorylating FAK and vinculin. MCF-7 cells were cultured in dishes or on coverslips coated with fibronectin overnight and then treated with different concentrations of PILRpep3 for 1 h. ***In situ*** PLA was conducted to analyze FAK-β1 integrin (A) and CD99-SHP2 (D) interactions, respectively. Red spots represent the proximity between the indicated molecules. (B) The cells were stained with mouse anti-human β1 integrin monoclonal antibody and rabbit anti-human FAK polyclonal antibody. Colocalization of β1 integrin and FAK was confirmed by confocal microscopy. (A, B, and D) Nuclei were stained with DAPI (blue). Scale bar = 5 μm; original magnification, 600×. (C) Whole cell lysates were subjected to immunoblotting to analyze the change in phosphorylation of FAK and vinculin. β-actin is shown as a control.

PLA with PILRpep3 treatment. The immunofluorescence assay showed that knockdown of CD99 attenuated the inhibitory effects of PILRpep3 on fibronectin-induced β1 integrin clustering (Fig. 2C). Correspondingly, ***in situ*** PLA showed that PILRpep3 inhibits the fibronectin-induced interaction between β1 integrin and talin, which was completely abrogated by CD99 silencing (Fig. 2D).

Previous studies demonstrated that FAK acts as a key modulator of focal adhesion (FA) formation and consequent cell migration (Mitra and Schlaepfer, 2006; Zhang and Zou, 2015). Indeed, β1 integrin clustering recruits downstream FAK and induces its autophosphorylation at Y397 and subsequent recruitment of talin, vinculin, and paxillin, leading to the activation of the integrin-stimulated signaling pathway. These findings prompted us to investigate whether PILRpep3-induced inactivation of β1 integrin was due to changes in the phosphorylation of FAK and disruption of FA. Figure 3A shows that cell adhesion to fibronectin increased the interaction of β1 integrin with FAK. Additionally, co-immunofluorescence staining showed marked colocalization of β1 integrin and FAK (Fig. 3B). In contrast, treatment with PILRpep3 prevented this fibronectin-mediated interaction between β1 integrin and FAK in a dose-dependent manner. In addition, PILRpep3 inhibited the phosphorylation of FAK at Y397 as well as of vinculin at Y118, which were induced by binding of the cells to fibronectin (Fig. 3C). In our previous study, CD99 activation led to SHP2 recruitment, which enhances MAPK signaling and induces dephosphorylation of FAK at Y397, causing a weaker binding capacity of β1 integrin (Lee et al., 2012; 2015). To determine whether SHP2 is also involved in the PILR peptide-initiated CD99 signaling pathway, we examined the physical proximity of SHP2 to CD99. ***In situ*** PLA showed that PILRpep3 recruited SHP2 to CD99 with added fibronectin in a dose-dependent manner (Fig. 3D).

PILRpep3 ameliorates RA by inhibiting TEM of leukocytes and endothelial cell tube formation

In RA, inflammatory damages of synovial tissue are mediated by angiogenesis and infiltration of leukocytes into the synovium (Mellado et al., 2015; Rabquer et al., 2008). We compared the inhibitory effect of PILRpep3 on adhesion of mouse monocytes to fibronectin under normal and inflammatory conditions. Treatment with LPS induced a significant increase in the adhesion of monocytes (Fig. 4A). PILRpep3 remarkably inhibited cell adhesion under both normal and inflammatory conditions in a concentration-dependent manner. At 1 μM, PILRpep3 significantly inhibited cell adhesion under inflammatory condition as well as under normal condition. These results showed that the effect of PILRpep3 on cell adhesion was significantly higher under inflammatory condition. To determine whether PILRpep3 regulates TEM of monocytes, we investigated the migration of mouse monocytes through endothelial cells ***in vitro*** and ***in vivo***. PILRpep3 dose-dependently interfered with the TEM of mouse monocytes ***in vitro*** as well as the recruitment of ^{35}S-labeled mouse monocytes into inflamed ear ***in vivo*** (Figs. 4B and 4C). On the other hand, no difference in monocyte TEM was observed under normal conditions. Additionally, we examined whether this peptide affects the formation of new blood vessels. PILRpep3 effectively inhibited HUVEC adhesion to fibronectin at concentrations $\geq$ 100 μM and showed maximum inhibitory efficiency at 500 μM (Fig. 4D). Consistent with these results, the peptide dose-dependently inhibited VEGF-induced tube formation in HUVECs (Fig. 4E).

Finally, we examined the effect of PILRpep3 on the pathological progression of RA in DBA-1J mice. The average index scores at the initiation of PILRpep3 injections were similar across all groups. Intraperitoneal injection of PBS had no apparent effect on the development of collagen-induced arthritis, so that the swelling of the inflamed paw was gradually aggravated (Fig. 5A). In contrast, PILRpep3 injection resulted in dose-dependent inhibition of collagen-induced arthritis. Correspondingly, histological evaluation showed that PILRpep3 suppressed tissue inflammation and bone deformity (Fig. 5B). The inhibitory efficacy of PILRpep3 (5 mg/kg) on the CIA was comparable to that of methotrexate (MTX; 5 mg/kg). These results suggested that PILR-derived agonist peptides could therapeutically regulate the progression of immune cell-mediated inflammatory diseases, such as RA, by inhibiting leukocyte TEM and angiogenesis.

DISCUSSION

Here, we demonstrated that PILR-Fc fusion proteins and PILR-derived peptides can function as agonist ligands for CD99. PILR-derived agonist ligands prevent cell adhesion to the ECM through regulation of β1 integrin activity. Especially, PILRpep3 prevented the clustering and activation of β1 integrin by dephosphorylating FAK and vinculin. In addition, PILRpep3 regulated monocyte infiltration and endothelial cell tube formation. Furthermore, intraperitoneal injection of PILRpep3 into mice strongly suppressed collagen-induced arthritis.

It is known that PILR family members interact with CD99 (Kogure et al., 2011; Lee et al., 2012; Nam et al., 2013; Sun et al., 2012; Tabata et al., 2008). As we have reported before, CD99 activation with monoclonal antibody recruited SHP2 and allowed it to dephosphorylate FAK at Y397 to regulate the FA complex, resulting in a decrease in β1 integrin activity (Lee et al., 2012; 2015). Consistently, PILR-Fc fusion proteins and PILR-derived peptides effectively reduced the adhesion of HUVECs to the ECM and stimulated the recruitment of SHP2, subsequently inducing dephosphorylation of FAK and vinculin. These inhibitory effects of PILR-derived agonist ligands on β1 integrin activity were disturbed by CD99 downregulation by CD99 siRNA transfection. These results indicate that PILR-Fc proteins and PILR-derived peptides function as agonist ligands for CD99.

Sun et al. (2012) demonstrated that the critical region in a highly conserved domain of PILR is essential for activating interacting molecules. Correspondingly, our deletion-based approach using Fc fusion proteins and synthetic peptides revealed that PILRpep7 covering amino acids 123 to 129 of hPILRα and hPILRβ suppress β1 integrin activity. Especially, the arginine at position 126 is known to be required for the interaction of hPILRα with its ligands (Sun et al., 2012). Interestingly, our results show that PILRpep3, tyrosine-phenylalanine-cysteine, appears to be sufficient for interaction with CD99, and is comparable to PILRpep4 containing the arginine residue with regard to the inhibitory effect on fibronectin-mediated cell adhesion. On the other hand, the tetrameric control peptide comprising residues 126 to 129 had not any inhibitory effect. Not only arginine at 126 but also tyrosine at 123 is evolutionarily conserved in hPILRs. These results suggest that both residues are functionally important, but the arginine may not be required for the interaction with ligands. In addition, we determined the target specificity of PILRpep3 by using an unrelated trimeric peptide as a control. Since the trimeric control peptide is derived from cytoplasmic domain of CD99, it cannot functionally interact with the extracellular domain of CD99 to inactivate β1 integrin. This result suggests that the peptide sequence composed of

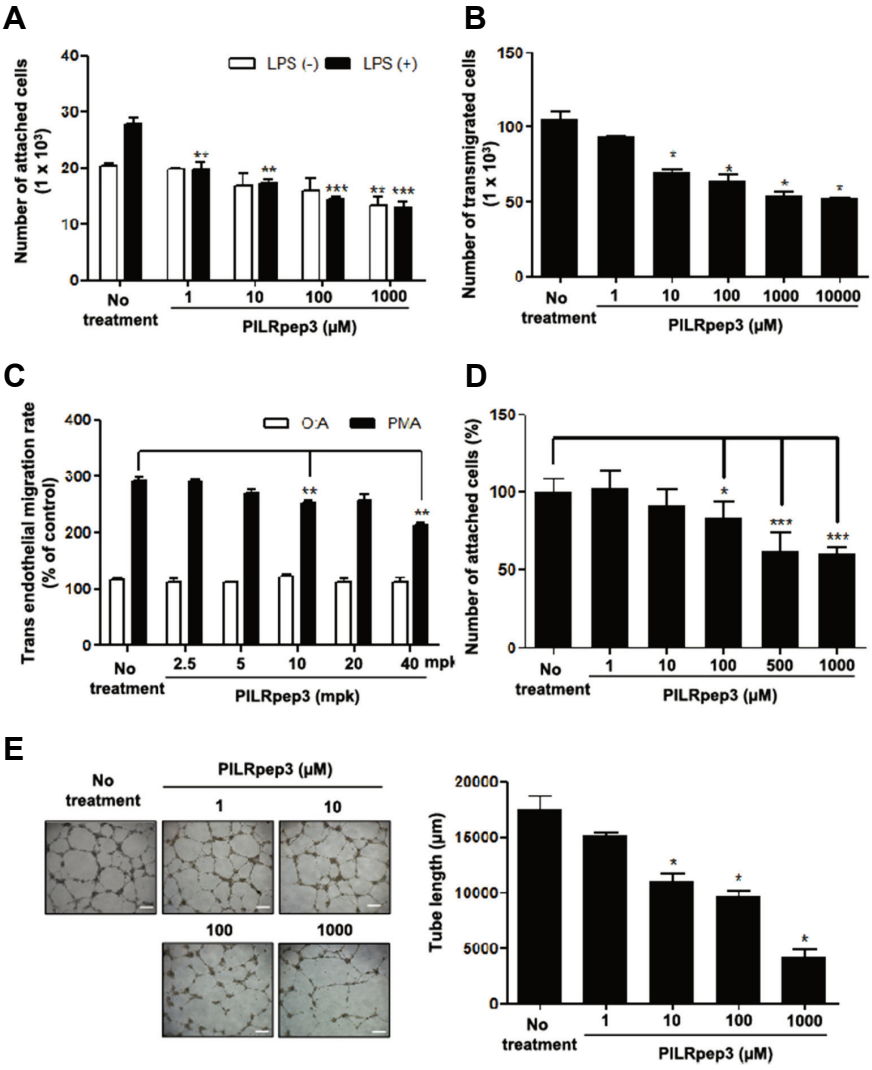

Fig. 4. PILRpep3 suppresses monocyte transendothelial migration as well as HUVEC tube formation. (A) WEHI274.1 monocytes were treated with PILRpep3 in the presence or absence of 10 μg/ml of LPS and subjected to adhesion assay to determine the attachment of the cells to fibronectin. $^{**}P < 0.01$, $^{***}P < 0.001$. (B) WEHI274.1 monocytes pretreated with PILRpep3 or control peptide were allowed to migrate through a monolayer of bEnd.3 cells grown on transwell filters in response to the chemoattractant in fibroblast-conditioned medium in the lower chambers. The efficiency of TEM was assessed by counting the number of transmigrated cells using a hemocytometer. $^{*}P < 0.05$. (C) ^{35}S-labeled WEHI274.1 cells with or without PILRpep3 were administrated at the base of the tail 4-6 h after vehicle or PMA application to the ear skin. Transmigration of mouse monocytes into inflamed ear was measured using a liquid scintillation counter. $^{**}P < 0.01$. (D) HUVECs were treated with PILRpep3 and subjected to an adhesion assay to evaluate the attachment of the cells to fibronectin. $^{*}P < 0.05$, $^{***}P < 0.001$. (E) Tube formation by HUVECs on matrigel was quantified by measuring the length of tubes in three randomly selected microscopic fields (bar graphs). Representative photographs are shown at magnification 200× (scale bar = 25 μm). $^{*}P < 0.05$.

tyrosine, phenylalanine, and cysteine confers receptor specificity to PILRpep3.

PILRpep3 inhibited TEM of monocytes as well as endothelial cell tube formation. RA is a chronic inflammatory disease involving uncontrolled infiltration of various immune cells into the synovial tissues. The formation of new blood vessels to the synovium is another key event in RA progression. The importance of β1 integrin functions in these processes has been well documented (Azcutia et al., 2013; Choy, 2012; Davignon et al., 2013; Kim et al., 2000; Liote et al., 1996; Paleolog, 2009; Parsons et al., 2012; Ucuzian et al., 2010). We reasoned that PILR peptide-induced activation of CD99 could inhibit TEM of leukocytes and angiogenesis via suppression of β1 integrin activity. Indeed, PILRpep3 treatment inhibited transmigration of monocytes through an endothelial monolayer and tube formation induced by VEGF in a dose-dependent manner. In addition, peptide injection significantly suppressed monocyte infiltration into locally inflamed ear skin induced by topical application of PMA. The influence of PILR-derived peptides on the inflammatory response was confirmed in CIA mice showing typical histological aspects of arthritis, severe monocyte/macrophage infiltration and joint deformity, and was comparable to that of MTX. MTX is widely used for treating certain types of cancer, psoriasis, and inflammatory diseases including RA (Tian and Cronstein, 2007). It was developed as a folic acid analogue, interfering with the growth of certain cells such as cancer and bone marrow cells, by inhibiting purine and pyrimidine synthesis. However, our study showed that PILRpep3 specifically interacted with CD99 expressed on the surface of monocytes, and subsequently interfered with cellular adhesion and TEM, giving rise to the suppression of immune responses. Thus, simultaneous use of MTX and PILRpep3 might be more powerful and effective for the treatment of RA. These results suggest that PILRpep3-induced decrease in affinity of β1 integrin might suppress inflammatory reactions by reducing integrin-mediated leukocyte infiltration and inflammation-associated angiogenesis.

Both β1 and β2 integrin families play a central role in the adhesion of monocytes/macrophages to the endothelium or ECM and extravasation to the inflamed tissues (McNally and Anderson, 2002). The basal expression of β1 integrin molecules

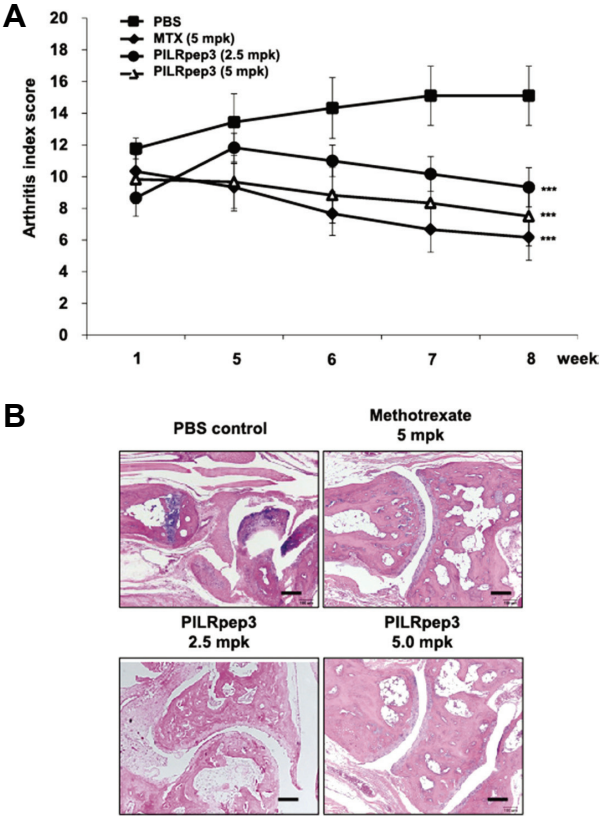

Fig. 5. PILRpep3 inhibits disease progression in the collagen-induced arthritis mouse model. (A) DBA/1J Mice (n = 5 per group) were immunized with bovine collagen II in complete Freund's adjuvant, followed by secondary immunization at day 14 with bovine collagen II in incomplete Freund's adjuvant. Thereafter, mice received PBS, 5 milligrams per kilogram body weight of MTX, or PILRpep3 daily and paw swelling in each mouse was clinically scored as described in the Materials and Methods. Data are the means (± SEMs) of 5 scores/group at each time point. ***P < 0.001. (B) At day 35 after CIA onset, articular cartilage was stained with hematoxylin and eosin to evaluate architectural erosion under a light microscope. Scale bar = 100 μm; original magnification, 40×.

(VLA-1 to VLA-6) in monocytes is merely detectable, but expression is upregulated when monocytes are stimulated by inflammation-mediated cytokines such as TNF, IFN, or interleukins, or by crosslinking of β2 integrin (McNally and Anderson, 2002; Rubio et al., 1995). It has been known that β1 integrin molecules are upregulated on the surface of the cells in response to LPS (Kang et al., 1995; McNally and Anderson, 2002). Consistently, our results showed that adhesion of LPS-treated monocytes to the ECM was significantly increased as compared to that of non-treated monocytes, and PILRpep3 more dramatically inhibited β1 integrin-mediated adhesion of monocytes treated with LPS. This might be due to upregulation of the surface expression of β1 integrin molecules. Of note, PILRpep3 did not affect monocyte infiltration in the non-inflamed skin. For several decades, it has been considered that macrophages are major mediators of inflammation in patients with RA (Bauer et al., 2009; Kraan et al., 2001). Massive amounts of monocytes are continuously recruited into the synovium and subsequently stimulate monocyte-to-macrophage differentiation, leading to the accumulation of macrophages. Macrophages accumulate various inflammatory mediators, causing eventual destruction of the cartilage and bone. However, monocytes, macrophages, and dendritic cells also contribute to normal immune surveillance to provide efficient immunological defense to protect the host against infection by microbes and to maintain tissue homeostasis. Thus, it is clinically significant that PILRpep3 affects only inflammatory immune response but not normal innate immunity, suggesting that it would not cause any significant immunodeficiency.

In conclusion, our findings demonstrate that PILR-derived agonist ligands activate CD99, which suppresses inflammatory responses via the inactivation of β1 integrin. Accordingly, these PILR-derived agonist ligands can be novel therapeutic drug candidates for inflammatory diseases.

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REFERENCES

Akhavani, M.A., Madden, L., Buysschaert, I., Sivakumar, B., Kang, N., and Paleolog, E.M. (2009). Hypoxia upregulates angiogenesis and synovial cell migration in rheumatoid arthritis. Arthritis Res. Ther. ***3***, R64.

Azcutia, V., Routledge, M., Williams, M.R., Newton, G., Frazier, W.A., Manica, A., Croce, K.J., Parkos, C.A., Schmider, A.B., Turman, M.V., et al. (2013). CD47 plays a critical role in T-cell recruitment by regulation of LFA-1 and VLA-4 integrin adhesive functions. Mol. Biol. Cell ***21***, 3358-3368.

Bauer, M., Brakebusch, C., Coisne, C., Sixt, M., Wekerle, H., Engelhardt, B., and Fassler, R. (2009). β1 integrins differentially control extravasation of inflammatory cell subsets into the CNS during autoimmunity. Proc. Natl. Acad. Sci. USA ***6***, 1920-1925.

Bernard, G., Zoccola, D., Deckert, M., Breittmayer, J.P., Aussel, C., and Bernard, A. (1995). The E2 molecule (CD99) specifically triggers homotypic aggregation of CD4+ CD8+ thymocytes. J. Immunol. ***1***, 26-32.

Bernard, G., Breittmayer, J.P., de Matteis, M., Trampont, P., Hofman, P., Senik, A., and Bernard, A. (1997). Apoptosis of immature thymocytes mediated by E2/CD99. J. Immunol. ***6***, 2543-2550.

Bixel, G., Kloep, S., Butz, S., Petri, B., Engelhardt, B., and Vestweber, D. (2004). Mouse CD99 participates in T-cell recruitment into inflamed skin. Blood ***10***, 3205-3213.

Choi, E.Y., Park, W.S., Jung, K.C., Kim, S.H., Kim, Y.Y., Lee, W.J., and Park, S.H. (1998). Engagement of CD99 induces up-regulation of TCR and MHC class I and II molecules on the surface of human thymocytes. J. Immunol. ***2***, 749-754.

Choy, E. (2012). Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. Rheumatology (Oxford) ***51***, v3-11.

Davignon, J.L., Hayder, M., Baron, M., Boyer, J.F., Constantin, A., Apparailly, F., Poupot, R., and Cantagrel, A. (2013). Targeting monocytes/macrophages in the treatment of rheumatoid arthritis. Rheumatology (Oxford) ***4***, 590-598.

Ellis, N.A., Ye, T., Patton, S., German, J., Goodfellow, P.N., and Weller, P. (1994). Cloning of PBDX, an MIC2-related gene that spans the pseudoautosomal boundary on chromosome Xp. Nat. Genet. ***4***, 394-400.

Hahn, J.H., Kim, M.K., Choi, E.Y., Kim, S.H., Sohn, H.W., Ham, D.I., Chung, D.H., Kim, T.J., Lee, W.J., Park, C.K., et al. (1997). CD99 (MIC2) regulates the LFA-1/ICAM-1-mediated adhesion of lymphocytes, and its gene encodes both positive and negative regulators of cellular adhesion. J. Immunol. ***5***, 2250-2258.

Kang, Y.H., Lee, C.H., Brummel, S.E., Newball, H.H., and Forrester,

J. (1995). Effects of endotoxin on expression of VLA integrins by human bronchoalveolar lavage macrophages. J. Leukoc. Biol. ***4***, 624-634.

Kim, S., Bell, K., Mousa, S.A., and Varner, J.A. (2000). Regulation of angiogenesis in vivo by ligation of integrin α5β1 with the central cell-binding domain of fibronectin. Am. J. Pathol. ***4***, 1345-1362.

Kogure, A., Shiratori, I., Wang, J., Lanier, L.L., and Arase, H. (2011). PANP is a novel O-glycosylated PILRα ligand expressed in neural tissues. Biochem. Biophys. Res. Commun. ***3***, 428-433.

Kohyama, M., Matsuoka, S., Shida, K., Sugihara, F., Aoshi, T., Kishida, K., Ishii, K.J., and Arase, H. (2016). Monocyte infiltration into obese and fibrilized tissues is regulated by PILRα. Eur. J. Immunol. ***46***, 1214-1223.

Kraan, M.C., Patel, D.D., Haringman, J.J., Smith, M.D., Weedon, H., Ahern, M.J., Breedveld, F.C., and Tak, P.P. (2001). The development of clinical signs of rheumatoid synovial inflammation is associated with increased synthesis of the chemokine CXCL8 (interleukin-8). Arthritis Res. ***1***, 65-71.

Lee, K.J., Ha, E.S., Kim, M.K., Lee, S.H., Suh, J.S., Lee, S.H., Park, K.H., Park, J.H., Kim, D.J., Kang, D., et al. (2008). CD36 signaling inhibits the translation of heat shock protein 70 induced by oxidized low density lipoprotein through activation of peroxisome proliferators-activated receptor γ. Exp. Mol. Med. ***6***, 658-668.

Lee, K.J., Lee, S.H., Yadav, B.K., Ju, H.M., Kim, M.S., Park, J.H., Jeoung, D., Lee, H.S., and Hahn, J.H. (2012). The activation of CD99 inhibits cell-extracellular matrix adhesion by suppressing β 1 integrin affinity. BMB Rep. ***3***, 159-164.

Lee, K.J., Yoo, Y.H., Kim, M.S., Yadav, B.K., Kim, Y., Lim, D., Hwangbo, C., Moon, K.W., Kim, D.J., Jeoung, D., et al. (2015). CD99 inhibits CD98-mediated 1 integrin signaling through SHP2-mediated FAK dephosphorylation. Exp. Cell Res. ***2***, 211-222.

Liote, F., Boval-Boizard, B., Weill, D., Kuntz, D., and Wautier, J. (1996). Blood monocyte activation in rheumatoid arthritis: increased monocyte adhesiveness, integrin expression, and cytokine release. Clin. Exp. Immunol. ***1***, 13-19.

McNally, A.K., and Anderson, J.M. (2002). β1 and β2 integrins mediate adhesion during macrophage fusion and multinucleated foreign body giant cell formation. Am. J. Pathol. ***2***, 621-630.

Mellado, M., Martínez-Muñoz, L., Cascio, G., Lucas, P., Pablos, J.L., and Rodríguez-Frade, J.M. (2015). T cell migration in rheumatoid arthritis. Front. Immunol. ***6***, 384.

Mitra, S.K., and Schlaepfer, D.D. (2006). Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr. Opin. Cell Biol. ***5***, 516-523.

Mousseau, D.D., Banville, D., L'Abbé, D., Bouchard, P., and Shen, S. (2000). PILRα, a novel immunoreceptor tyrosine-based inhibitory motif-bearing protein, recruits SHP-1 upon tyrosine phosphorylation and is paired with the truncated counterpart PILRβ. J. Biol. Chem. ***6***, 4467-4474.

Nam, G., Lee, Y.K., Lee, H.Y., Ma, M.J., Araki, M., Araki, K., Lee, S., Lee, I.S., and Choi, E.Y. (2013). Interaction of CD99 with its paralog CD99L2 positively regulates CD99L2 trafficking to cell surfaces. J. Immunol. ***11***, 5730-5742.

Paleolog, E.M. (2009). The vasculature in rheumatoid arthritis: cause or consequence? Int. J. Exp. Pathol. ***3***, 249-261.

Parsons, S.A., Sharma, R., Roccamatisi, D.L., Zhang, H., Petri, B., Kubes, P., Colarusso, P., and Patel, K.D. (2012). Endothelial paxillin and focal adhesion kinase (FAK) play a critical role in neutrophil transmigration. Eur. J. Immunol. ***2***, 436-446.

Rabquer, B.J., Pakozdi, A., Michel, J.E., Gujar, B.S., Haines, G.K., Imhof, B.A., and Koch, A.E. (2008). Junctional adhesion molecule C mediates leukocyte adhesion to rheumatoid arthritis synovium. Arthritis Rheum. ***10***, 3020-3029.

Rubio, M.A., Sotillos, M., Jochems, G., Alvarez, V., and Corbií, A.L. (1995). Monocyte activation: rapid induction of α1/β1 (VLA-1) integrin expression by lipopolysaccharide and interferon–γ. Eur. J. Immunol. ***9***, 2701-2705.

Schenkel, A.R., Mamdouh, Z., Chen, X., Liebman, R.M., and Muller, W.A. (2002). CD99 plays a major role in the migration of monocytes through endothelial junctions. Nat. Immunol. ***2***, 143-150.

Shiratori, I., Ogasawara, K., Saito, T., Lanier, L.L., and Arase, H. (2004). Activation of natural killer cells and dendritic cells upon recognition of a novel CD99-like ligand by paired immunoglobulin-like type 2 receptor. J. Exp. Med. ***4***, 525-533.

Sun, Y., Senger, K., Baginski, T.K., Mazloom, A., Chinn, Y., Pantua, H., Hamidzadeh, K., Ramani, S.R., Luis, E., Tom, I., et al. (2012). Evolutionarily conserved paired immunoglobulin-like receptor alpha (PILRα) domain mediates its interaction with diverse sialylated ligands. J. Biol. Chem. ***19***, 15837-15850.

Sun, Y., Caplazi, P., Zhang, J., Mazloom, A., Kummerfeld, S., Quinones, G., Senger, K., Lesch, J., Peng, I., Sebrell, A., et al. (2014). PILRα negatively regulates mouse inflammatory arthritis. J. Immunol. ***2***, 860-870.

Tabata, S., Kuroki, K., Wang, J., Kajikawa, M., Shiratori, I., Kohda, D., Arase, H., and Maenaka, K. (2008). Biophysical characterization of O-glycosylated CD99 recognition by paired Ig-like type 2 receptors. J. Biol. Chem. ***14***, 8893-8901.

Tato, C.M., Joyce-Shaikh, B., Banerjee, A., Chen, Y., Sathe, M., Ewald, S.E., Liu, M., Gorman, D., McClanahan, T.K., Phillips, J.H., et al. (2012). The myeloid receptor PILRβ mediates the balance of inflammatory responses through regulation of IL-27 production. PLoS One ***3***, e31680.

Tian, H., and Cronstein, B.N. (2007). Understanding the mechanisms of action of methotrexate. Bull. N.Y.U. Hosp. Jt. Dis. ***3***, 168-173.

Ucuzian, A.A., Gassman, A.A., East, A.T., and Greisler, H.P. (2010). Molecular mediators of angiogenesis. J. Burn Care. Res. ***1***, 158-175.

Watson, R.L., Buck, J., Levin, L.R., Winger, R.C., Wang, J., Arase, H., and Muller, W.A. (2015). Endothelial CD99 signals through soluble adenylyl cyclase and PKA to regulate leukocyte transendothelial migration. J. Exp. Med. ***7***, 1021-1041.

Winger, R.C., Harp, C.T., Chiang, M.Y., Sullivan, D.P., Watson, R.L., Weber, E.W., Podojil, J.R., Miller, S.D., and Muller, W.A. (2016). Cutting edge: CD99 is a novel therapeutic target for control of T Cell-Mediated central nervous system autoimmune disease. J. Immunol. ***4***, 1443-1448.

Wingett, D., Forcier, K., and Nielson, C.P. (1999). A role for CD99 in T cell activation. Cell. Immunol. ***1***, 17-23.

Zhang, L., and Zou, W. (2015). Inhibition of integrin β1 decreases the malignancy of ovarian cancer cells and potentiates anticancer therapy via the FAK/STAT1 signaling pathway. Mol. Med. Rep. ***6***, 7869-7876