Characterization of Leukocyte Mono-immunoglobulin-like Receptor 7 (LMIR7)/CLM-3 as an Activating Receptor

ITS SIMILARITIES TO AND DIFFERENCES FROM LMIR4/CLM-5*

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Here we characterize leukocyte mono-Ig-like receptor 7 (LMIR7)/CLM-3 and compare it with an activating receptor, LMIR4/CLM-5, that is a counterpart of an inhibitory receptor LMIR3/CLM-1. LMIR7 shares high homology with LMIR4 in the amino acid sequences of its Ig-like and transmembrane domains. Flow cytometric analysis demonstrated that LMIR4 was predominantly expressed in neutrophils, whereas LMIR7 was highly expressed in mast cells and monocytes/macrophages. Importantly, LMIR7 engagement induced cytokine production in bone marrow-derived mast cells (BMMCs). Although FcRγ deficiency did not affect surface expression levels of LMIR7, it abolished LMIR7-mediated activation of BMMCs. Consistently we found significant interaction of LMIR7-FcRγ, albeit with lower affinity compared with that of LMIR4-FcRγ. Our results showed that LMIR7 transmits an activating signal through interaction with FcRγ. In addition, like LMIR4, LMIR7 synergizes with TLR4 in signaling. Analysis of several chimera receptors composed of LMIR4 and LMIR7 revealed these findings: 1) the transmembrane of LMIR7 with no charged residues maintained its surface expression at high levels in the absence of FcRγ; 2) the extracellular juxtazmembrane region of LMIR7 had a negative effect on its surface expression levels; and 3) the strong interaction of LMIR4 with FcRγ depended on the extracellular juxtamembrane region as well as the transmembrane domain of LMIR4. Thus, LMIR7 shares similarities with LMIR4, although they are differentially regulated in their distribution, expression, and function.

A new family of paired immunoreceptors has been recently identified and named leukocyte mono-Ig-like receptor (LMIR)§/CMRF-35-like molecules (CLM)/myeloid-associated Ig-like receptor (MAIR)/dendritic cell-derived Ig-like receptor (DligR)/immune receptor expressed by myeloid cell (IREM)/CD300 (1–15). In mice, there exist at least eight members of the LMIR family (1–9). We and others have previously characterized LMIR1–5 (1–8). An inhibitory receptor, LMIR3/CLM-1, pairs with an activating receptor, LMIR4/CLM-5 or LMIR5/CLM-7. LMIR3 is 91 and 53% identical with LMIR4 and LMIR5, respectively, in the amino acid sequence of the Ig-like domain. In addition, LMIR4 and LMIR5 transmits an activating signal through interaction with FcRγ and DAP12, respectively (2, 3). Consistently, LMIR5 has a positively charged residue (lysine) in the transmembrane domain, which is characteristic of activating receptors interacting with adaptors containing ITAM (3). However, LMIR4 does not have a positively charged residue in the transmembrane domain; instead, it has a negatively charged residue (glutamic acid) (2, 8). Interestingly, the inhibitory receptor LMIR3 also has the potential to transmit an activating signal through interaction with FcRγ in mast cells, despite having no charged residue in the transmembrane domain (4).

FcRγ is an ITAM-bearing signal transduction subunit expressed in a variety of hematopoietic cells (15–19). It is an essential component of the high affinity receptor for IgE (FcεRI) (17), the high affinity IgG receptor (FcγRI) (18), the low affinity IgG receptor (FcγRII) (19), and the IgA receptor (FcαRI) (20, 21). In addition, FcRγ interacts with various activating receptors such as paired Ig-like receptor A (PIR-A) (22) or platelet collagen receptor glycoprotein VI (23). Although the Arg/Asp charge interaction between transmembrane domains is well characterized, recent studies have also implicated a transmembrane leucine zipper-like interaction between activating receptors and FcRγ (24, 25). However, the relevant molecular mechanism remains incompletely understood.

In the present study, we have characterized LMIR7/CLM-3, which had not been fully analyzed. Structurally, LMIR7 is similar to LMIR4 in its Ig-like and transmembrane domains. The generation of an antibody specifically reacting with LMIR7 enabled us to delineate the differential expression profiles of

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LMIR7 and LMIR4. Importantly, LMIR7 engagement led to the activation of bone marrow-derived mast cells (BMMCs) through interaction with FcRγ, validating an activating function of LMIR7 similar to that of LMIR4. Notably, analysis of chimera receptors composed of LMIR4 and LMIR7 revealed that a short extracellular juxtamembrane region of LMIR4 played an important role in its strong interaction with FcRγ. This finding will likely lead us to uncover novel regulatory mechanisms in the interaction of a diverse array of activating receptors with FcRγ.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Other Reagents**—Rat anti-LMIR7 IgG1 monoclonal Ab (mAb) (M2) was generated by ACTGen Inc. Anti-FLAG mAb (M2), fluorescein isothiocyanate (FITC)-conjugated anti-FLAG mAb (M2), rabbit anti-FLAG Ab, mouse IgG1 mAb (MOPC21), and mouse anti-dinitrophenyl (DNP) IgE mAb (SPE-7) were purchased from Sigma-Aldrich. Mouse anti-Myc mAb (9E10) was from Roche Diagnostics. PE- or FITC-conjugated anti-CD11c, or Gr-1 mAbs, Rat IgG1 mAb, and PE-conjugated streptavidin were from eBioscience. PE-conjugated anti-mouse IgG goat F(ab')2 Ab was from Beckman Coulter. Anti-ERK Ab was from Santa Cruz Biotechnology. Rabbit anti-FcεRI-γ subunit Ab was purchased from Upstate Biotechnology. All of the phospho-specific Abs was purchased from Cell Signaling Technology. Cytokines were obtained from R&D Systems. All other reagents were from Sigma-Aldrich unless stated otherwise.

**Cell Culture and Isolation**—Murine hematopoietic cell lines and 293T cells were cultured as described (2, 3). C57BL/6 mice (Charles River Laboratories Japan Inc.) were used at 8–10 weeks of age for isolation of tissues and cells such as BM cells, peripheral blood (PB) cells, peritoneal cells, splenocytes, and thymocytes as described (2, 3). All procedures were approved by an institutional review committee. To generate BMMCs or fetal liver-derived mast cells (FLMC) with 90% purity (c-Kit+/FceRI+ by flow cytometry), BMMCs or FLMCs were cultured in the presence of 10 ng/ml IL-3 alone or with 20 ng/ml stem cell factor (SCF) as described (2–4, 26–29). To generate BMMcs derived from BMMCs (BMmDC), BM-derived macrophages (BMMφ), BM-derived myeloid dendritic cells (BMMdDC), and BM-derived plasmacytoid dendritic cells (BMPdC), BM cells were cultured in the presence of 10 ng/ml M-CSF, 20 ng/ml GM-CSF, and 50 ng/ml Flt3-ligand, respectively, as described (2–4). BMmDC or BMPdDC were sorted by using FITC-conjugated anti-CD11c Ab. BM granulocytes were prepared as described (3). The following mutant mice were used: FcRγ−/− (16), DAP10−/− (30), and DAP12−/− (31).

**Gene Expression Analysis**—Expression of LMIR7 was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) as described (2, 3). Total RNAs were extracted from each cell line and BM-derived cells with TRIzol reagents (Invitrogen), treated with deoxyribonuclease I (Invitrogen), and reverse-transcribed by using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). A fragment of LMIR7 was amplified with primers 5’-acacccacacacccacaccc-3’ and 5’-ctggagaagttttctcgcg-3’. For normalization, a fragment of β-actin was amplified with 5’-catcactatgggacagcgc-3’ and 5’-agagctcagagcagaccc-3’. Relative expression levels of LMIR7 among samples were measured by real-time RT-PCR as described (3). cDNA was amplified using a LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) under the following conditions: one cycle of 95 °C for 10 s, 40 cycles of 95 °C for 5 s, and 60 °C for 25 s. All samples were independently analyzed three times. The following primers were used: 5’-acacccacacacccacaccc-3’ and 5’-acaccaagcccatcggaca-3’ for LMIR7; and 5’-atggcgctcgctttgatcgc-3’ and 5’-ttgaagctcagagcagaccc-3’ for GAPDH. Relative gene expression levels were calculated using standard curves generated by serial dilution of cDNA and normalized by a GAPDH expression level. Product quality was checked by melting curve analysis via LightCycler software (Roche Diagnostics).

**Plasmid Constructs**—We searched the GenBankTM/European Molecular Biology Laboratory (EMBL)/DNA Data Bank of Japan (DDBJ) database by using the amino acid sequence of the Ig-like domain of mLMIR1. On the basis of the sequence data, the cDNA of mouse LMIR7 was isolated by PCR with the primers 5’-acacccacacacccacaccc-3’ and 5’-agggagggagggaggaga-3’ from a cDNA library of BMMCs derived from C57BL/6 mice and its sequence was confirmed (LMIR7/CLM-3: GenBankTM accession number AY457049) (1–3). A cDNA fragment of LMIR7 lacking the signal sequence was tagged with a FLAG or Myc epitope at the N terminus. A SLAM (signaling lymphocyte-activating molecule) signal sequence (32) (a gift from Hisashi Arase, Osaka University, Osaka, Japan), FLAG- or Myc-LMIR7, was subcloned into a pMXs-ires-puro (pMXs-IP) (33) retroviral vector to generate pMXs-FLAG-Myc-LMIR7-IP. pMXs-FLAG- or Myc-LMIR4-IP and pMXs-FcRγ-ires-blasticidin (pMXs-FcRγ-IP) were generated as described (2–4). To generate LMIR7 mutants (LMIR7-M1, -M2, and -M3) or LMIR4 mutants (LMIR4-M1 and -M2), two-step PCR mutagenesis (2–4) was performed by using pMXs-FLAG-LMIR7-IP or pMXs-FLAG-LMIR4-IP, respectively, as a template. LMIR7-M1 is the LMIR7(S189Y,V197E,V198L) mutant; and LMIR7-M2 is the LMIR7(S189Y,V197E,V198L) mutant where six amino acid residues (NSLFIW) of LMIR7 were replaced with six amino acid residues (SRPHTR) of LMIR4 in the extracellular juxtamembrane region; LMIR7-M3 is the LMIR7(S189Y,V197E,V198L) mutant where six amino acid residues (NSLFIW) of LMIR7 were replaced with six amino acid residues (SRPHTR) of LMIR4 in the extracellular juxtamembrane region. All constructs were verified by DNA sequencing.

**Transfection and Infection**—Retroviral transfection was as described (1–4, 33, 34). Briefly, retroviruses were generated by transient transfection of PLAT-E packaging cells (34) with FuGENE 6 (Roche Diagnostics). Cells were infected with retroviruses in the presence of 10 μg/ml Polybrene. Selection with puromycin or blasticidin was started 48 h after infection.

**Biochemistry**—BMMCs expressing FLAG-tagged LMIR7 or mock were stimulated by 10 μg/ml anti-FLAG mAb or mouse IgG1 mAb as control, 50 ng/ml SCF, or 10 μg/ml SPE-7 IgE for the indicated time as described (2–4, 28). To detect phosphorylation of several proteins, stimulated cells were lysed with Nonidet P-40 lysis buffer containing protease and phosphatase inhibitors.
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inhibitor mixture (Sigma-Aldrich). To detect the interaction of LMIR7 and FcRγ, 293T cells were co-transfected with two constructs of interest. Cells were lysed with digitonin lysis buffer containing protease and phosphatase inhibitor mixture. Immunoprecipitation and Western blotting were done as described (1–4).

Flow Cytometry—Flow cytometric analysis of the stained cells was performed with a FACSCalibur (BD Biosciences) equipped with CellQuest software and Flowjo software (Tree Star) as described (2–4). Anti-LMIR7 mAb or rat IgG1 mAb as control was biotinylated by sulfo-NHS-LC-biotin (Pierce) according to the manufacturer’s instructions. Cells were incubated with 20 μg/ml biotin-anti-LMIR7 mAb or biotin-anti-rat IgG1 mAb before incubation with PE-conjugated streptavidin. The geometric mean fluorescence intensity of Myc-tagged amounts of biotinylated anti-FLAG Ab, mouse IgG1 mAb, anti-biotin MACSiBead particles loaded with equal numbers of anti-biotin MACSiBead particles (Miltenyi Biotec) were used. Briefly, we prepared equal amounts of biotinylated anti-FLAG Ab, mouse IgG1 mAb, anti-LMIR7 mAb, or rat IgG1 mAb according to the manufacturer’s instructions. Cells were stimulated by adding 2 × 10⁵ anti-biotin MACSiBead particles to 1.5 × 10⁵ cells in the presence or absence of 100 ng/ml lipopolysaccharide (LPS). After 24 h of stimulation, the concentrations of cytokines/chemokines in the supernatants were measured using enzyme-linked immunosorbent (ELISA) kits of IL-6, TNF-α, or MCP-1 from R&D Systems (2–4).

Statistical Analysis—Data are shown as the mean ± S.D., and statistical significance was determined by Student’s t test with p < 0.05 taken as statistically significant.

RESULTS

Structure of LMIR7 in Comparison with LMIR4—We originally cloned LMIR1 using a signal sequence trap based on a retrovirus-mediated signal sequence trap (1, 35). LMIR2, -3, -4, and -5 were cloned by searching the GenBankTM/EBI/DDBJ data bank using the sequence of the Ig-like domain of LMIR1 (1–3). Similarly, LMIR7 was cloned and identified from a BMMC cDNA library. LMIR7 protein from C57BL/6 mice is 245 amino acids in length. Like LMIR2, -4, and -5, LMIR7 is a type I transmembrane protein composed of an N-terminal signal peptide, an extracellular domain containing a single V-type Ig domain, a transmembrane domain, and a short cytoplasmic tail without any signaling motif. However, unlike typical activating receptors such as LMIR2 and LMIR5 (1–3), LMIR7 does not possess a positively charged residue in the transmembrane domain. An Ig-like domain of LMIR7 shares 85% identity at amino acid sequences with that of an inhibitory receptor, LMIR3/CLM-1, or an activating receptor, LMIR4/CLM-5 (Fig. 1A). In addition, LMIR7 differs from LMIR4 by only three amino acids in the transmembrane domain (Fig. 6A). The structural resemblance of LMIR7 to LMIR4 led us to analyze LMIR7 in comparison with LMIR4. First, we generated Ba/F3 cells expressing FLAG-tagged LMIR7, LMIR3, or LMIR4. Flow cytometric analysis using anti-FLAG mAb confirmed surface expression of the transduced LMIR7 as well as LMIR3 and -4 (Fig. 2A). In addition, Western blot analysis demonstrated that similar to LMIR4, LMIR7 was detected by anti-FLAG mAb as two discrete bands (of 37 and 26 kDa) irrespective of reducing or nonreducing conditions (Fig. 2B and data not shown). Notably, LMIR7 were expressed more efficiently than LMIR4 at both surface expression and total protein levels (Fig. 2, A and B). Because LMIR7 protein possesses no apparent N-linked glycosylation sites but several O-glycosylation sites within its extracellular domain, we speculated that a band (37 kDa) corresponds to an O-glycosylated form of LMIR7 expressed on the cell surface. In accordance with this, N-glycosidase F treatment did not affect the mobility of LMIR7 (data not shown).

LMIR7 Is Highly Expressed in Mast Cells and Moncyte/Macrophages—To investigate the expression profile of LMIR7 in hematopoietic cells, we performed RT-PCR in a variety of hematopoietic cell lines. As a result, high expression levels of LMIR7 were observed in macrophage cell lines J774-1 and RAW264.7 and mast cell line MC/9 (Fig. 1B). In addition, we found detectable expression of LMIR7 in B-lineage cell lines.
WEHI-231 and A20 and in a DC line, DC2.4, but not in other myeloid cell lines or T-lineage cell lines (Fig. 1B). On the other hand, real-time PCR analysis using BM-derived cells showed that expression levels of LMIR7 transcripts were specifically higher in BMMC and BMmDC, BMpDC, or BM granulocytes (Fig. 1C, BMG). To examine the expression profiles of LMIR7 at protein levels, next we generated anti-LMIR7 mAb. As depicted in Fig. 2A, anti-LMIR7 mAb efficiently detected LMIR7 expressed on the surface of Ba/F3 cells transduced with FLAG-tagged LMIR7. This Ab did not detect any LMIR1, LMIR2, LMIR3, LMIR4, or LMIR5 transduced into Ba/F3 cells (Fig. 2A). Moreover, similar to anti-FLAG mAb, anti-LMIR7 mAb detected only FLAG-tagged LMIR7 alone, but not LMIR3 and LMIR4, as two discrete bands in the lysates of transduced Ba/F3 cells (Fig. 2B). However, unlike anti-FLAG Ab, anti-LMIR7 mAb more strongly detected a band with high molecular mass (37 kDa) compared with another band (23 kDa) (Fig. 2B), suggesting that anti-LMIR7 mAb reacted preferentially with the glycosylated form of LMIR7 expressed on the cell surface. We then stained hematopoietic cells using anti-LMIR7 mAb. Flow cytometric analysis demonstrated that LMIR7 was not expressed in B cells (B220+) in BM or spleen, in T-lineage cells (Thy-1.2+ or CD3+) in PB, spleen, or thymus, or in NK cells (NK1.1+) in spleen (Fig. 2D and E, and data not shown). On the other hand, immature to mature neutrophils (CD11bhigh) in BM or mature neutrophils (CD11bhigh) in PB were LMIR7dull/low, and monocytes (Ly6chigh) in PB were LMIR7high (Fig. 2D). Interestingly, a population of CD11c+ cells in PB, but not in spleen, were LMIR7high (Fig. 2D and data not shown). Notably, peritoneal macrophages (F4/80+) displayed high expression levels of

FIGURE 2. Cell surface expression of LMIR7 in hematopoietic cells. A and B, the sensitivity and specificity of anti-LMIR7 mAb were confirmed by flow cytometry (A) and Western blot (B). A, Ba/F3 cells were transduced with FLAG-tagged LMIR1, -2, -3, -4, -5, -7, or mock. The cells were stained with rat anti-LMIR7 mAb (upper panel) or mouse anti-FLAG mAb (lower panel) followed by PE-conjugated anti-rat IgG, Ab or anti-mouse IgG, Ab, respectively. B, lysates of Ba/F3 cells expressing FLAG-tagged LMIR3, -4, -7, or mock were immunoprecipitated with anti-LMIR7 mAb or rabbit anti-FLAG Ab and then immunoblotted with anti-LMIR7 mAb (top panel) or mouse anti-FLAG mAb (third panel), respectively. Total cell lysates were also immunoblotted with anti-LMIR7 mAb (second panel) or anti-α-tubulin Ab (bottom panel). IB and IP indicate immunoblot and immunoprecipitation, respectively. C, total cell lysates of BMmDC or BMMC were immunoblotted with anti-LMIR7 mAb (upper panel) or anti-α-tubulin Ab (lower panel). D, analysis of LMIR7 expression in hematopoietic cells derived from C57 BL/6 mice. Single cell suspensions were prepared from BM, PB, peritoneal cavity, thymus, and spleen. Cells were stained with biotin anti-LMIR7 mAb or biotin rat IgG1 mAb followed by PE-conjugated streptavidin and FITC-conjugated Abs as indicated. FSClowSSClow populations in granulocytes or macrophages, FSChighSSChigh populations in lymphocytes cells, NK cells, or DC, or FSCintSSCint populations in monocytes were gated and analyzed for LMIR7 expression. E, BMMC, BMmDC, BMpDC, or NK1.1+ spleen cells were stained with biotin anti-LMIR7 mAb or biotin rat IgG1 mAb followed by PE-conjugated streptavidin. The result of control or LMIR7 staining is shown as a filled or bold line histogram, respectively. NK1.1+ cells were sorted from spleen cells by using FITC-conjugated anti-NK1.1 Ab.

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When BMMC, BMmDC, BMpDC, and BMM/H9021 were stained with anti-LMIR7 mAb, we found that BMmDC and BMpDC expressed scarcely detectable levels of LMIR7 (Fig. 2E). Remarkably, LMIR7 was expressed at high levels in BMMC as well as in BMM/H9021 (Fig. 2E). Taken together with the results on LMIR7 expression at transcript levels, these findings indicated that LMIR7 was not expressed in lymphoid-lineage cells, except for a few B-lineage cell lines, but was broadly expressed in myeloid-lineage cells. Importantly, LMIR7 was highly expressed in mast cells, monocytes/macrophages, and a small subset of CD11c⁺ cells in PB. High expression levels of endogenous LMIR7 in BMMC and BMMΦ were also confirmed by Western blot analysis (Fig. 2C). Because LMIR4 is predominantly expressed in neutrophils, the present results indicated that LMIR7 and LMIR4 were differentially distributed in hematopoietic cells.

LMIR7 Engagement Induced the Activation of BMMC—In view of expression profiles of LMIR7, we examined the functions of LMIR7 in mast cells. BMMCs were retrovirally transduced with either FLAG-tagged LMIR7 or mock. We found comparable expression levels of c-Kit and FcεR1 in both BMMCs and confirmed the expression of transduced LMIR7 by staining with anti-FLAG mAb (Fig. 3A). In addition, transduction with LMIR7 did not affect the differentiation or the growth of BMMCs (data not shown). Although stimulation with IgE induced equivalent levels of ERK phosphorylation in both BMMCs, stimulation with anti-FLAG mAb led to ERK activation only in LMIR7-transduced BMMCs (Fig. 3B). Accordingly, stimulation with anti-FLAG mAb-loaded beads resulted in robust cytokine/chemokine (IL-6, TNF-α, and MCP-1) production of BMMCs expressing FLAG-LMIR7 (Fig. 3C). We confirmed that stimulation with control Ab-coated beads did not induce significant levels of cytokine/chemokine production in these cells (Fig. 3C). On the other hand, a similar stimulation did not cause degranulation, characterized by β-hexosaminidase release, of the LMIR7-transduced BMMCs (data not shown). Interestingly, cross-linking of LMIR7 synergistically enhanced the cytokine/chemokine production in these cells (Fig. 3C). On the other hand, a similar stimulation did not cause degranulation, characterized by β-hexosaminidase release, of the LMIR7-transduced BMMCs (data not shown). Interestingly, cross-linking of LMIR7 synergistically enhanced the cytokine/chemokine production of LMIR7-transduced BMMC stimulated by LPS through TLR4 (Fig. 3D). Importantly, BMMC or FLMC produced significant levels of IL-6 when endogenous LMIR7 was engaged with anti-LMIR7 mAb-loaded beads but not control Ab-loaded beads (Fig. 3E). Collectively, our results show that LMIR7 cross-linking alone induced the activation of mast cells leading to cytokine/chemokine production, demonstrating that LMIR7 is an activating receptor.

FcγR Deficiency Did Not Affect Surface Expression Levels of LMIR7 in Mast Cells or Macrophages but Dampered Cytokine Production of Mast Cells Stimulated by LMIR7 Cross-linking—LMIR4 transmits an activating signal by interacting with FcγR.
among adaptor molecules containing ITAM or the related activating motif-bearing molecules (2). We asked which adaptor molecule plays an important role in LMIR7-mediated activating signal. Flow cytometric analysis demonstrated that surface expression levels of LMIR7 did not differ among wild type (WT) and DAP10, DAP12, or FcRγ-deficient peritoneal Mφ or BMMC (Fig. 4A). We confirmed that surface expression levels of FceRI and c-Kit among these BMMCs were comparable and that those of FceRI in FcRγ-deficient BMMC were not detectable (Fig. 4A), as reported (2, 16). In addition, neither DAP10/DAP12 nor DAP12/FcRγ double deficiency affected surface expression levels of LMIR7 (data not shown). Moreover, when LMIR7 was transduced into DAP10−/−, DAP12−/−, or FcRγ-deficient or WT BMMC, the surface expression levels of transduced LMIR7 as well as FceRI and c-Kit were comparable among these transfectants (Fig. 4B). We confirmed that FceRI expression was not detectable in FcRγ-deficient BMMC (Fig. 4B) as reported (16). Taken together, these results indicated that DAP10, DAP12, or FcRγ did not affect surface expression levels of LMIR7. To examine whether adaptor molecules are involved in LMIR7 functions, we stimulated these transfectants with anti-FLAG mAb- or control mAb-loaded beads. Strikingly, LMIR7-mediated cytokine production was abolished by a deficiency in FcRγ but not DAP10 or DAP12, although we found comparable levels of cytokine production among these cells stimulated using phorbol 12-myristate 13-acetate as control (Fig. 5A). Consistently, ERK activation of LMIR7-transduced BMMC stimulated by LMIR7 cross-linking, but not by SCF as control, was absent in FcRγ-deficient cells (Fig. 5B). We then asked whether LMIR7 physically associated with FcRγ. To this end, 293T cells were co-transfected with FcRγ or a control construct together with a FLAG-tagged LMIR7 or a control construct. Co-immunoprecipitation experiments demonstrated that FcRγ interacted significantly with LMIR7 (Fig. 5C). Collectively, these results indicated that FcRγ was required for LMIR7-mediated activation signaling but was dispensable for surface expression of LMIR7.

Strong Interaction of LMIR4 with FcRγ in Comparison with LMIR7 Depended on the Extracellular Juxtamembrane Region and the Transmembrane Domain of LMIR4—In the course of this study, we found that FcRγ was less efficiently co-immunoprecipitated with LMIR7 in comparison with LMIR4 (Fig. 6C and data not shown). We next sought to dermine the molecular mechanism by which LMIR4-FcRγ interaction becomes stronger than LMIR7-FcRγ interaction. As shown in Fig. 6A, LMIR7 differed from LMIR4 by only three amino acid residues in the transmembrane domain. As it is generally accepted that a transmembrane structure plays a critical role in receptor interaction (15, 24, 25), we generated a chimera receptor composed of an extracellular domain, LMIR7, and an intracellular domain, LMIR4, designated LMIR7-M1. When Ba/F3 cells were transduced with Myc-tagged LMIR4, LMIR4, and an intracellular domain, LMIR7, designated LMIR7-M1. When Ba/F3 cells were transduced with Myc-tagged LMIR4 or mock, flow cytometric analysis using anti-Myc mAb showed that surface expression levels of LMIR7-M1 were equivalent to those of LMIR4 and lower than those of LMIR7 (Fig. 6B). These results indicated that the transmembrane domain of LMIR7 was indispensable for maintaining surface expression of LMIR7 at high levels. Interestingly, further transduction with FcRγ into these Ba/F3 cells weakly or moderately increased the surface expression levels of LMIR7-M1 or LMIR4, respectively, although it did not affect those of LMIR7 (Fig. 6B). We also performed co-immunoprecipitation experiments similar to those described in Fig. 5C. Notably, FcRγ was efficiently co-immunoprecipitated with LMIR7-M1 in comparison with LMIR7, whereas the amount of FcRγ co-immunoprecipitated with LMIR7-M1 was still lower than that co-immunoprecipitated with LMIR4 (Fig. 6C). These results led us to ask whether a structural domain other than the transmembrane domain of LMIR4 was involved in the tight interaction between LMIR4 and
We paid attention to the difference of six amino acid residues (SRPHTR versus NSLFIW) in the extracellular juxtamembrane domain of LMIR4 versus LMIR7 (Fig. 6A). We additionally generated two types of chimera receptors: LMIR7-M2, where an extracellular juxtamembrane region of LMIR7 was replaced with that of LMIR4, and LMIR7-M3, where both an extracellular juxtamembrane region and a transmembrane domain were replaced with those of LMIR4 (Fig. 6A). Myc-tagged LMIR7-M2 or LMIR7-M3 was transduced into Ba/F3 cells, demonstrating that surface expression levels of LMIR7-M2 were higher than those of LMIR7-M1 or LMIR4 (respectively) (Fig. 6B). On the other hand, the presence of FcRγ weakly or moderately increased the surface expression levels of LMIR7-M2 or LMIR7-M3, respectively (Fig. 6B). Intriguingly, the amount of FcRγ co-immunoprecipitated with LMIR7-M2 or LMIR7-M3 was comparable with that co-immunoprecipitated with LMIR7-M1 or LMIR4, respectively (Fig. 6C). Collectively, these results indicated that the extracellular juxtamembrane region of LMIR4 had a positive effect on its surface expression levels, whereas the transmembrane domain of LMIR4 had a negative effect. In addition, the extracellular juxtamembrane region as well as the transmembrane domain of LMIR4 played a critical role in the strong interaction between LMIR4 and FcRγ. For further analysis, we generated two chimeric receptors: LMIR4-M1, where the transmembrane domain of LMIR4 was replaced with that of LMIR7, and LMIR4-M2, where the extracellular juxtamembrane region was replaced with that of LMIR7 (Fig. 6A). Myc-tagged LMIR4-M1 or LMIR4-M2 was transduced into Ba/F3 cells, demonstrating that surface expression levels of LMIR4-M1 or LMIR4-M2 were higher or lower, respectively, than those of LMIR4 (Fig. 6D). These results indicated the negative effect of the extracellular juxtamembrane region of LMIR7 on its surface expression levels, as well as the positive effect of the transmembrane domain of LMIR7. The presence of FcRγ did not significantly increase the surface expression levels of LMIR4-M1 or LMIR4-M2 (Fig. 6D). We found that FcRγ co-immunoprecipitated with LMIR4-M1 or LMIR4-M2, but the amount of FcRγ co-immunoprecipitated with LMIR4-M1 or LMIR4-M2 was lower than that co-immunoprecipitated with LMIR4 (Fig. 6E). It should be noted that the amount of FcRγ co-immunoprecipitated with LMIR4-M1 was lower than that co-immunoprecipitated with LMIR4-M2, notwithstanding the higher expression levels of LMIR4-M1 in comparison with LMIR4-M2 (Fig. 6E). Taken together, these results suggested that both the transmembrane domain and the extracellular juxtamembrane.
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region of LMIR4 were required, but in different ways, for the efficient up-regulation of surface LMIR4 by the LMIR4-FcRγ interaction.

**DISCUSSION**

In the present study, we have characterized LMIR7 as an activating receptor closely related to LMIR4 among the LMIR family receptors. Homology research has indicated that LMIR7 is similar to LMIR4 in structure: between them, 85% of the Ig-like domain and 87% of the transmembrane domain are identical in amino acid sequences. Interestingly, neither LMIR7 nor LMIR4 possesses a positively charged residue in the transmembrane domain that is thought to be required for the interaction with ITAM or related activating motif-bearing adaptor proteins. However, our conclusion that, like LMIR4, LMIR7 interacts with FcRγ and thereby transmits an activating signal is based on the following findings: cross-linking of LMIR7 induces ERK activation and cytokine production in BMMC; these activation events are dampened by FcRγ deficiency; and FcRγ is co-immunoprecipitated with LMIR7.

Notably, despite the high homology of the transmembrane domains, several differences exist between LMIR7 and LMIR4. LMIR7-FcRγ interaction is weaker than LMIR4-FcRγ interaction; surface expression levels of LMIR7 are higher than those of LMIR4 (Fig. 6). Our analysis of chimera receptors delineated the relevant molecular mechanism.

The transmembrane domain of LMIR7 played a role in maintaining its surface expression at high levels, and probably no charged residue in the transmembrane domain in the absence of FcRγ. Interestingly, the extracellular juxtamembrane region of LMIR7 had a negative effect on its surface expression levels, whereas that of LMIR4 had a positive effect. We also reasoned that FcRγ expression did not further increase the surface expression of LMIR7, presumably because of the

**FIGURE 6. Both the extracellular juxtamembrane region and the transmembrane domain of LMIR4 are indispensable for stronger association of LMIR4-FcRγ as compared with LMIR7-FcRγ.** A, alignment of amino acid sequences of the extracellular juxtamembrane and transmembrane domains in LMIR4, LMIR7, and chimera receptors (LMIR7-M1, LMIR7-M2, LMIR7-M3, LMIR4-M1, and LMIR4-M2) are shown using SMART (simple modular architecture research tool) software. Different amino acid residues between LMIR4, LMIR7, or mouse IgG1 as control followed by PE-conjugated anti-mouse IgG goat F(ab')2 Antibody (anti-FcRγ/H11006). Cells were stained with FITC-conjugated anti-Myc mAb. The results of staining (left panel) are shown as a filled histogram (Ba/F3 cells without FcRγ) or bold line histogram (Ba/F3 cells expressing FcRγ). The geometric mean fluorescence intensities (Geom. MFI) of Myc-tagged receptors were measured by flow cytometry (right panel). All data points correspond to the mean ± S.D. of three independent experiments. One representative of three independent experiments is shown. B, Ba/F3 cells were transiently co-transfected with (a Myc-tagged LMIR4, LMIR4-M1, LMIR4-M2, or mock) plus (FcRγ or alone). Cells were stained with FITC-conjugated anti-Myc mAb. Total cell lysates of these transfectants were also immunoblotted with anti-FcRγ Ab. Total cell lysates of these transfecants were also immunoblotted with anti-FcRγ Ab. Total cell lysates of these transfecants were also immunoblotted with anti-Myc mAb. A representative of three independent experiments is shown.
weak interaction of LMIR7 with FcRy. In addition, the weak but significant levels of LMIR7-FcRy interaction might be explained by a leucine zipper-like interaction between the transmembrane region of LMIR7 and FcRy. As reported (25), a leucine zipper-like interaction formed by FcRy residues (Leu-14 and Leu-21) and FcαRI residues (Leu-217, Leu-220, and Leu-224) as well as an Arg/Asp charge interaction formed by the FcRy residue (Asp-11) and the FcαRI residue (Arg-209) contribute to the tight interaction of both receptors. Interestingly, both LMIR7 and LMIR4 maintain the leucine zipper-like sequences in the transmembrane: LMIR7 residues Leu-202, Leu-205, and Leu-209; and LMIR4 residues Leu-190, Leu-193, and Leu-197. Therefore, it is possible that the leucine zipper-like sequences in the transmembrane of LMIR7 play a role in the LMIR7-FcRy interaction. In addition, the transmembrane domain of LMIR7 probably contributes to the LMIR7-FcRy interaction by maintaining surface LMIR7 high levels. Alternatively, an unknown adaptor molecule might intervene between LMIR7 and FcRy. Further examination will be necessary to completely understand the precise mechanism of how LMIR7 interacts with FcRy.

On the other hand, we also speculated that some other mechanism was involved in the strong interaction of LMIR4 with FcRy. Indeed, we clearly demonstrated by analysis done on chimera receptors that the extracellular juxtamembrane region (amino acid sequence SPRHTR) of LMIR4 played a critical role in the up-regulation of surface LMIR4 by its interaction with FcRy (Fig. 6). Because there is variability in terms of the expression levels of the chimeric or parent receptors, it is difficult to assess the relative importance of a transmembrane domain versus an extracellular juxtamembrane region. However, our results suggested that the transmembrane domain of LMIR4 plays a major role in the high affinity interaction of LMIR4 with FcRy (Fig. 6E). Although the cytoplasmic region, as well as the transmembrane domain, of GPVI is reportedly required for its interaction with FcRy (23), to our knowledge the present work is the first demonstration that an extracellular juxtamembrane region of an activating receptor plays an important part in the tight interaction with FcRy. One possibility is that the extracellular juxtamembrane region of LMIR4 interacts with the short extracellular domain of FcRy. Alternatively, the extracellular juxtamembrane region of LMIR4 might be folded in three-dimensional structure and thereby kept in contact with the transmembrane domain of FcRy. According to a recent report (24), three polar positions formed by one basic T cell receptor (TCR) α and two ζζ basic aspartic acid transmembrane residues are critical for ζζ dimerization and assembly with T cell receptor. In most cases, this theory is probably valid for activating receptors coupled with FcRy, considering the high degree of sequence homology between ζ and FcRy. However, in FcRy-coupled receptors without a positively charged residue in the transmembrane, the notion presented in this study highlights the novel molecular mechanism of the interaction between activating receptors and FcRy.

Whereas LMIR4 is expressed predominantly in neutrophils (4), real-time PCR and flow cytometric analysis demonstrated that LMIR7 is highly expressed in mast cells and monocytes/macrophages. Notably, high expression levels of LMIR7 were also observed in an immature subtype of dendritic cell (CD11c<sup>+</sup>) in PB but not in myeloid or plasmacytoid dendritic cells. Because an inhibitory LMIR3 is broadly expressed in myeloid cells (2, 4), it is likely that LMIR3 pairs with LMIR7 in mast cells and monocytes/macrophages or with LMIR4 in neutrophils. Intriguingly, we showed previously that LMIR3 transmits an inhibitory signal while it interacts with FcRy and thereby transmits an activating signal in concert with an LPS/TLR4 signal (4). In addition, we demonstrated that like the LMIR4 signal, the LMIR7 signal synergizes with the LPS signal. Therefore, if LMIR3/4/7, with a highly conserved Ig-like domain, had a similar or the same ligand, the LMIR3 signal might inhibit the LMIR7-mediated activating signal or, conversely, cooperate with the LMIR7 signal to enhance the TLR4 signal in vivo. In any case, it is possible that LMIR7 or LMIR4 modulates the innate immune responses in a cell type-dependent manner. Complete understanding of the in vivo functions of LMIR requires both analysis of each of the knock-out mice and identification of the ligands of each LMIR.

In conclusion, LMIR7 is an activating receptor found among the LMIR family, which transmits an activating signal by interacting with FcRy. LMIR7 shares similarities with LMIR4 as a counterpart of LMIR3, whereas LMIR7 is regulated differently from LMIR4 in regard to distribution, expression, and function, suggesting a nonredundant role for both activating receptors in other cell types.

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