Functional Analysis of Activating Receptor LMIR4 as a Counterpart of Inhibitory Receptor LMIR3*

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The leukocyte mono-Ig-like receptor (LMIR) belongs to a new family of paired immunoreceptors. In this study, we analyzed activating receptor LMIR4/CLM-5 as a counterpart of inhibitory receptor LMIR3/CLM-1. LMIR4 is expressed in myeloid cells, including granulocytes, macrophages, and mast cells, whereas LMIR3 is more broadly expressed. The association of LMIR4 with Fc receptor-γ among immunoreceptor tyrosine-based activation motif-bearing molecules was indispensable for LMIR4-mediated functions of bone marrow-derived mast cells, but dispensable for its surface expression. Cross-linking of LMIR4 led to Lyn- and Syk-dependent activation of bone marrow-derived mast cells, resulting in cytokine production and degranulation, whereas that of LMIR3 did not. The triggering of LMIR4 and TLR4 synergistically caused robust cytokine production in accordance with enhanced activation of ERK, whereas the co-ligation of LMIR4 and LMIR3 dramatically abrogated cytokine production. Notably, intraperitoneal administration of lipopolysaccharide strikingly up-regulated LMIR3 and down-regulated LMIR4, whereas that of granulocyte colony-stimulating factor up-regulated both LMIR3 and LMIR4 in granulocytes. Cross-linking of LMIR4 in bone marrow granulocytes also resulted in their activation, which was enhanced by lipopolysaccharide. Collectively, these results suggest that the innate immune system is at least in part regulated by the qualitative and quantitative balance of the paired receptors LMIR3 and LMIR4.

The Ig-like receptors provide positive and negative regulation of immune cells upon recognition of various ligands (1–5). We identified previously leukocyte mono-Ig-like receptors (LMIRs) from a cDNA library of bone marrow-derived mast cells (BMMCs). We (6) and others (7–9) demonstrated that LMIR1/MAIR1 (myeloid-associated Ig-like receptor I)/CLM-8 (CMRF-35-like molecules-8) and LMIR2/MAIRII/CLM-4/ DlgR1 (dendritic cell-derived Ig-like receptor 1) are expressed mainly in myeloid cells. The human homolog of LMIR1 is CMRF-35H/IRp60 (inhibitory receptor protein of 60 kDa)/ CD300a (10–14). The inhibitory effects of LMIR1 on mast cells and eosinophils and the activatory roles of LMIR2 in macrophages have been described recently (6, 7, 11). In addition to LMIRs, a variety of Ig-like paired receptors are expressed by myeloid cells (2, 15–17), but the biological significance of a paired receptor remains incompletely understood. Despite the similarity in the extracellular Ig-like domains, a striking structural difference between activating and inhibitory receptors exists in the transmembrane and cytoplasmic regions. In general, the former associate with an immunoreceptor tyrosine-based activation motif (ITAM)– or the related activating motif-bearing adaptor transmembrane protein, including DAP10, DAP12, or Fc receptor–γ (FcRγ), via a positively charged residue in the transmembrane domain, whereas the latter include an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain (1, 5, 18, 19).

Cells of the myeloid lineage such as granulocytes and mast cells are the major component of the innate immune response. Mast cells are implicated in a wide variety of inflammatory processes through a high affinity IgE receptor (FcεRI) or other immune receptors (20, 21). Aggregation of FcεRI with IgE plus antigen or highly cytokinergic IgE alone induces the activation of mast cells, leading to the secretion of preformed and newly synthesized pro-inflammatory mediators (22, 23). Alternatively, inhibitory receptors such as FcRγIb, paired Ig-like receptor B, gp49B1, and LMIR1 are also expressed on the same cell surface, probably preventing excessive activation or decreasing the background activation levels before stimulation (3, 6, 7, 24–26).

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‡ These abbreviations used are: LMIRs, leukocyte mono-Ig-like receptors; BMMCs, bone marrow-derived mast cells; ITAM, immunoreceptor tyrosine-based activation motif; FcRγ, Fc receptor–γ; ITIM, immunoreceptor tyrosine-based inhibitory motif; FcεRI, high affinity IgE receptor I; TLR, Toll-like receptor; LPS, lipopolysaccharide; G-CSF, granulocyte colony-stimulating factor; mAbs, monoclonal antibodies; FITC, fluorescein isothiocyanate; PE, R-phycocerythin; Ab, antibody; TNP, trinitrophenyl; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; FLMCs, fetal liver-derived mast cells; IL, interleukin; RT, reverse transcription; MAPKs, mitogen-activated protein kinases.

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In this study, we identified new members of the LMIR family (LMIR3 and LMIR4) from a BMMC cDNA library. Sequence analysis showed that LMIR3 and LMIR4 are basically identical to CLM-1 and CLM-5, respectively (8). The human homolog of LMIR3 is also called IREM-1 (immune receptor expressed by myeloid cell-l) (27). Inhibitory receptor LMIR3/CLM-1 controls osteoclast differentiation (8). On the other hand, the function of LMIR4 was not fully understood; Fujimoto et al. (28) recently reported the expression of the LMIR4/CLM-5 transcript in myeloid cells and its association with FcRγ in transfected cells. In view of the high homology of LMIR3 and LMIR4 in the Ig-like domain, we have characterized LMIR4 as a counterpart of LMIR3. On the basis of the finding that LMIR3 and LMIR4 are expressed mainly in myeloid cells, we have utilized BMMCs or granulocytes to analyze the functions. The cross-talk between LMIR4 and LMIR3 or other receptors such as TLR4 or FcεRI leads us to postulate that LMIR4 is involved in a wide array of immune responses, including innate immunity and allergy. Moreover, the change in the relative expression levels of LMIR3 and LMIR4 in granulocytes in response to lipopolysaccharide (LPS) or granulocyte colony-stimulating factor (G-CSF) might suggest the relationship between innate immunity and differentiation of granulocytes upon bacterial infection.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Rat anti-LMIR3 and anti-LMIR4 (derived from CBA/J mice) IgG2a monoclonal antibodies (mAbs), designated anti-LMIR3 and anti-LMIR4 (CBA) mAbs, respectively, was obtained from R&D Systems (Minneapolis, MN). Anti-FLAG mAb M2, fluorescein isothiocyanate (FITC)-conjugated anti-FLAG mAb M2, and mouse anti-dinitrophenyl IgE mAb (clone SPE-7; designated SPE-7 IgE) were all purchased from Sigma. Anti-Myc mAb and R-phycocerythrin (PE)-conjugated goat anti-rat IgG2a antibody (Ab) were from Roche Diagnostics (Mannheim, Germany) and Southern Biotech (Birmingham, AL), respectively. Mouse anti-trinitrophenyl (TNP) IgE mAb (C-38) and FITC-conjugated anti-mouse IgE mAb were from BioSource. PE- or FITC-conjugated anti-CD3, anti-c-Kit, anti-CD45R/B220, anti-CD11b, and anti-Gr-1 Abs were from eBioscience (San Diego, CA). Anti-ERK, anti-p38, anti-JNK, and anti-Akt Abs were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All phospho-specific Abs were purchased from Cell Signaling Biotechnology (Beverly, MA). F(ab’2)2 fragments were prepared by digesting anti-FLAG mAb M2 or mouse IgG1 mAb with immobilized pepsin, followed by removing intact mAb by protein A affinity chromatography (Pierce). Cytokines were obtained from R&D Systems. PP2, PP3, and piceatannol were from EMD Biosciences, Inc. (San Diego, CA). All other reagents were from Sigma unless stated otherwise.

**Cell Culture and Isolation**—All hematopoietic cell lines were cultured as described (6). CBA/J or C57BL/6 (B6) mice (Charles River Laboratories, Inc.) were used at 8–10 weeks of age for isolation of tissues and cells. To generate BMMCs or fetal liver-derived mast cells (FLMCs) with 90% purity (c-Kit+/FceRI+ by flow cytometry), BMMCs or FLMCs were cultured for 4–6 weeks in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 ng/ml IL-3 ( interleukin-3) alone or with 20 ng/ml stem cell factor, respectively, as described previously (22, 29). The following mutant mice were used: FcRγ−/−, Dap12−/−, FcγRI−/−, Lyn−/−, and syk+−/−. Bone marrow-derived macrophages, plasmacytoid dendritic cells, and myeloid dendritic cells were generated as described (30, 31). Peritoneal cells were isolated by peritoneal lavage with 8 ml of phosphate-buffered saline. Granulocytes were obtained as described previously (32, 33). Briefly, the whole bone marrow prepared from mice was centrifuged and washed with phosphate-buffered saline. After the red blood cells were hypotonically lysed with 0.2% NaCl, this solution was returned to isotonicity with 1.2% NaCl. After washing, the solution was delicately applied over a 62% Percoll gradient before it was centrifuged for 30 min at 1500 × g. The neutrophil pellet was then isolated. >90% neutrophil purity was confirmed with a Cytospin preparation. For in vivo experiments, 10 ng of G-CSF or 20 ng of LPS was intra-peritoneally injected into mice 12 h before analysis of granulocytes.

**Cell Stimulation**—BMMCs were sensitized with 0.5 μg/ml anti-TNP IgE for 12 h, washed twice, and stimulated with various concentrations of TNP-conjugated bovine serum albumin. Alternatively, BMMCs were directly stimulated with SPE-7 IgE, LPS, F(ab’2)2 anti-FLAG mAb, or their combinations. For granulocytes, these cells were preincubated with 20 μg/ml anti-LMIR4 (CBA) Ab or rat IgG2a for 1 h on ice were washed before stimulation with 10 μg/ml F(ab’2)2 anti-rat IgG2a Ab. Alternatively, granulocytes were incubated with RPMI 1640 medium including 10% fetal calf serum in the presence of 1000 ng/ml LPS or 100 ng/ml G-CSF for 24 h before analysis.

**Cloning of LMIR3 and LMIR4**—The DDBJ/EBI/GenBank™/Data Bank was searched using the amino acid sequence of the Ig-like domain of LMIR1. Accession numbers AY457049 (LMIR3 (B6)) and AY457051 (LMIR4 (B6)), which were derived from B6 mice, with close similarity in the extracellular domain were selected for cloning. On the basis of these sequence data, the cDNAs of LMIR3 and LMIR4 were isolated from a BMMC cDNA library (derived from CBA/J or C57BL/6 mice) by PCR and confirmed by sequencing (accession numbers AB292061 (LMIR3 (CBA)) and AB292062 (LMIR4 (CBA))) as described (6).

**Plasmid Constructs**—An expression plasmid encoding FLAG epitope-tagged DAP10, DAP12, or FcRγ was generated as described (6). LMIR3 (CBA), LMIR3 (B6), LMIR4 (CBA), or LMIR4 (B6) was ligated into pMXs-IREs-neor or pMXs-IREs-puro’ (34). The entire sequence excluding the leader sequence of LMIR3 (B6) or LMIR4 (B6) was amplified, and the resulting fragment was ligated into a pME vector including the signal sequence of SLAM (CD150; provided by Dr. H. Arase, Osaka University) (35). The resulting SLAM signal sequence-FLAG- or -Myc-LMIR3 (B6) or -LMIR4 (B6) fragment was subcloned into pMXs-IREs-puro’ or pMXs-IREs-neo’, generating pMXs-FLAG- or -Myc-LMIR3– or -LMIR4–IREs-puro’ or -IREs-neo’. All constructs were verified by DNA sequencing.

**Transfection and Infection**—Retroviral transfection was as described previously (6, 34). Briefly, retroviruses were generated by transient transfection of PLAT-E packaging cells (36) with FuGENE 6 (Roche Diagnostics). Cells were infected with...
retroviruses in the presence of 10 μg/ml Polybrene. Selection with G418 or puromycin was started 48 h after infection.

Reverse Transcription (RT)-PCR—The expression of LMIR4 was analyzed by RT-PCR amplification as described (6). Total RNAs were extracted from each cell line and CBA/J mouse-derived tissues and cells. A fragment of LMIR4 was amplified with primers 5′-ctgagattgcaagcatacacg-3′ and 5′-gattcctgcagt-gacctcc-3′. This set of primers does not cross-react with LMIR3 (data not shown). For normalization, a fragment of actin was amplified with primers 5′-catcactattggcaacgagc-3′ and 5′-acgcagctcagtaacagtcc-3′. For Biochemistry—To detect the association of LMIR4 and ITAM- or the related activating motif-bearing molecules, COS-7 or 293T cells were cotransfected with two constructs of interest (pME-Myc-LMIR4 and pMKIT-FLAG-DAP10, -DAP12, or -FcR). Cells were harvested at 48 h after transfection and lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 10% glycerol, and 1% Nonidet P-40 in the presence of protease and phosphatase inhibitor mixtures (Sigma). Cleared supernatants of cell lysates were used for immunoprecipitation with appropriate Abs and protein A-Sepharose CL-4B (GE Healthcare). Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membranes were blocked and incubated with anti-FLAG mAb, followed by horseradish peroxidase-conjugated anti-mouse Ig (Sigma). To detect phosphorylation of several proteins, stimulated cells were lysed in lysis buffer, and lysates were subject to protein assay using a Bio-Rad protein assay kit. An equal amount of total lysate was separated by SDS-PAGE. The membrane was incubated with anti-phosphotyrosine mAb or phospho-specific Abs and then incubated with the appropriate horseradish peroxidase-conjugated secondary Abs (Sigma). Proteins were detected by enhanced chemiluminescence (Amersham Biosciences) as described (6, 37).

Flow Cytometry—Briefly, cells were incubated with the appropriate antibodies for 60 min on ice after blocking the Fc receptor. When necessary, the samples were incubated with secondary antibodies for an additional 60 min on ice. To monitor apoptosis, cells were incubated with 1 μg/ml PE-labeled annexin V (BD Biosciences) at room temperature for 20 min in the dark. Flow cytometric analysis of the stained cells was performed with a FACSCalibur (BD Biosciences) equipped with CellQuest software and Flowjo software (TreeStar Inc.) as described (6, 22, 37).

Measurement of Histamine and Cytokines and Adhesion Assays—Histamine released from stimulated cells during a 50-min incubation period was measured as described (22). After 12 h of stimulation, supernatants were measured using enzyme-linked immunosorbent assay kits from R&D Systems. For adhesion assays, BMMCs or granulocytes were incubated on 96-well plate with or without the indicated stimulation 1 h before evaluation of adherent cells using CellTiter-Glo and a MicroLumat Plus luminometer as described (37).

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.
Functional Analysis of Lmir4

(A) Expression of Lmir4 in various tissues and cell lines.

(B) Flow cytometry analysis of cell lines expressing Lmir4.

(C) Western blot analysis of Lmir4 expression.

(D) Flow cytometry analysis of peripheral blood, bone marrow, and peritoneal cells.

(E) Flow cytometry analysis of Lmir4 expression in peripheral blood and spleen.

(F) Flow cytometry analysis of BMmDC and BMpDC.
RESULTS

Cloning of LMIR4—We previously cloned paired immunoreceptors, LMIR1 and LMIR2, using a signal sequence trap based on retrovirus-mediated expression screening (38) and the DDBJ/EBI/GenBankTM Data Bank (6). To find new members of the LMIR family, we searched the same data base using the sequence of the Ig-like domain of LMIR1 and identified four homologous cDNA sequences. Among them, we cloned two cDNAs (1014 and 666 nucleotides in an open reading frame, termed LMIR3 and LMIR4, respectively) from a BMMC cDNA library derived from CBA/J mice. The LMIR3 and LMIR4 proteins are 337 and 221 amino acids in length, respectively, and 91% identical in the Ig-like domain (Fig. 1A). Both LMIR3 and LMIR4 cDNAs encode for a type I transmembrane protein containing a signal peptide, a single variable-type Ig-like domain, and a transmembrane domain, with the former harboring a cytoplasmic region containing ITIM. The latter contains only a short cytoplasmic tail. Thus, LMIR3 displays a structure typical of an inhibitory receptor. In contrast, LMIR4 has a unique property as an activating receptor, i.e. LMIR4 does not possess a positively charged residue such as an arginine or lysine that is supposed to associate with an ITAM-bearing adaptor protein, but instead contains a negatively charged residue, glutamic acid, in its transmembrane domain (1, 2, 5, 39). Sequence analysis showed that LMIR3 and LMIR4 are basically identical to CLM-1 and CLM-5, respectively (8). The difference of 13 amino acids between LMIR3 and CLM-1 or 19 amino acids between LMIR4 and CLM-5 is due to the difference between mouse strains. LMIR and CLM are derived from CBA/J and C57BL/6, respectively (Fig. 1A). Amino acid alignment showed that the LMIR4 protein does not have apparent N-linked glycosylation sites within its extracellular domain, but has several potential O-linked glycosylation sites within a serine/threonine-rich region. Immunoprecipitation of FLAG-tagged LMIR4 transduced into Ba/F3 cells revealed ~36- and ~28-kDa proteins when analyzed under both reducing and nonreducing conditions. However, N-glycosidase F treatment did not decrease the mobility of LMIR4 (Fig. 1B) (data not shown). The surface expression levels of transduced LMIR4 on Ba/F3 cells were confirmed by flow cytometry (Fig. 1C). Collectively, LMIR4 is a monomeric, O-linked glycoprotein.

LMIR4 Is Expressed Mainly in Myeloid Cells—To investigate the expression patterns of LMIR4, RT-PCR was performed on various tissues. A high expression level of LMIR4 was observed in bone marrow cells. Interestingly, LMIR4 was also highly expressed in the trachea and lung (Fig. 2A). To further delineate the expression pattern in hematopoietic cells, RT-PCR analysis was applied to a variety of cell lines and primary cells. We observed the expression of LMIR4 in myeloid cell lines, including mast cell lines and macrophage lines (Fig. 2B), and in primary myeloid cells such as bone marrow-derived macrophages, bone marrow-derived dendritic cells, and BMMCs (Fig. 2C). To confirm the surface expression level of LMIR4 on hematopoietic cells, we generated anti-LMIR3 (CBA) and anti-LMIR4 (CBA) mAbs. The staining of Ba/F3 cells overexpressing LMIR3 (CBA), LMIR4 (CBA), LMIR3 (B6), or LMIR4 (B6) demonstrated the sensitivity and specificity of these Abs. Anti-LMIR3 (CBA) mAb detected LMIR3 and LMIR4 (B6), but not LMIR4 (CBA). Anti-LMIR4 (CBA) mAb detected LMIR4 (CBA), but not LMIR4 (B6) and LMIR3 (Fig. 2D). All Abs did not cross-react with LMIR1 or LMIR2 (data not shown). Only in the analysis of the cells derived from CBA/J mice was the surface expression of LMIR3 and LMIR4 specifically detected by these mAbs. Flow cytometric analysis revealed that LMIR4 was expressed in granulocytes (Gr-1highMac-1high) and monocytes/macrophages (Gr-1lowMac-1low). Granulocytes in peripheral blood highly expressed LMIR4 (Fig. 2E) (data not shown), whereas a weak but detectable level of LMIR4 was also observed in bone marrow-derived cells (Fig. 2F) (data not shown). LMIR4 expression was not detected in B- or T-cells (Fig. 2E), in accordance with the result of LMIR4 mRNA expression patterns. In contrast, LMIR3 was more broadly expressed in hematopoietic cells other than T-cells (Fig. 2, E and F). In summary, LMIR4 was expressed rather exclusively in myeloid cells compared with LMIR3.

LMIR4 Selectively Associates with FcRγ, but Not DAP10 or DAP12—LMIR4 is atypical as an activating receptor in that it has a negatively charged residue, glutamic acid, in the transmembrane domain. To clarify whether LMIR4 can associate with ITAM- and the related activating motif-bearing adaptor proteins, co-immunoprecipitation experiments were performed using COS-7 cells cotransfected with FLAG-tagged DAP10, DAP12, or FcRγ or a control construct together with Myc-tagged LMIR4. As depicted in Fig. 3A, only FcRγ was co-immunoprecipitated with LMIR4. The expression level of LMIR4 was significantly elevated in the presence of FcRγ. At the same time, the surface expression level of LMIR4 on COS-7 cells dramatically increased by overexpression of FcRγ, but not DAP10 and DAP12 (Fig. 3B). These results suggest that FcRγ associates with and stabilizes LMIR4, resulting in its increased expression.

Cross-linking of LMIR4, but Not LMIR3, Induces the Activation of BMMCs—To obtain strong activation of primary cells stimulated by LMIR4 cross-linking, we generated FLAG-tagged LMIR4-transduced BMMCs. When stimulated with F(ab’)2 anti-FLAG mAb, but not control mAb, LMIR4 transfectants showed increased tyrosine phosphorylation in total cellular proteins as revealed by anti-phosphotyrosine blotting (Fig. 3C). Moreover, the phosphorylation of Akt and MAPKs such as ERK, JNK, and p38 was recognized using phospho-specific Abs.

FIGURE 2. Expression of LMIR4. A–C, various mouse tissues, hematopoietic cell lines, and bone marrow (BM)-derived cells were analyzed by RT-PCR using specific primers for LMIR4. β-Actin was amplified as a control, BMMΦ, bone marrow-derived macrophages. D, LMIR4 (CBA), LMIR4 (B6), LMIR3 (CBA), and LMIR3 (B6) were transduced into Ba/F3 cells. The sensitivity and specificity of anti-LMIR3 (CBA) mAb or anti-LMIR4 (CBA) mAb were confirmed by flow cytometry. E and F, single cell suspensions were prepared from peripheral blood, bone marrow, splenocytes, and peritoneal cells from CBA/J mice. The cells grafted in the population forward scatter (FSC)high side scatter (SSC)high were stained with anti-LMIR3 (CBA) mAb, anti-LMIR4 (CBA) mAb, or control rat IgG2a, followed by staining with PE-conjugated anti-rat IgG2a mAb. For double staining, the FITC-conjugated mAbs indicated were used. The peripheral blood cells grafted in the population (FSC)high(SSC)high) and the splenocytes grafted in the population (FSC)low(SSC)low) were analyzed. Bone marrow-derived myeloid (BMMΦd) and plasmacytoid (BMPDC) dendritic cells were generated from CBA/J mice. The cells were stained as described above.
Functional Analysis of LMIR4

A

|       | Vector | DAP12 | LMR4 | DAP10 | FcRy |
|-------|--------|-------|------|-------|------|
| IP:   | anti-myc |       |      |       |      |
| IB:   | anti-FLAG |      |      |       |      |
| IP:   | anti-FLAG |      |      |       |      |
| IB:   | anti-FLAG |      |      |       |      |
| IP:   | anti-myc |       |      |       |      |
| IB:   | anti-myc |      |      |       |      |

B

E

C

mlgG1 anti-FLAG

G

D

IB: (-)

E

IB: anti-pY

F

IB: anti-FLAG

G

IB: anti-FLAG

H

IB: anti-FLAG

I

IB: anti-FLAG

J

IB: anti-FLAG

K

IB: anti-FLAG

L

IB: anti-FLAG

M

IB: anti-FLAG

N

IB: anti-FLAG

O

IB: anti-FLAG

P

IB: anti-FLAG

Q

IB: anti-FLAG

R

IB: anti-FLAG

S

IB: anti-FLAG

T

IB: anti-FLAG

U

IB: anti-FLAG

V

IB: anti-FLAG

W

IB: anti-FLAG

X

IB: anti-FLAG

Y

IB: anti-FLAG

Z
These data indicate that aggregation of LMR4 induced activation of mast cells. As activated mast cells have the potential to produce various chemical mediators, we next examined whether BMMCs stimulated by LMR4 cross-linking produce cytokines. In response to F(ab)2 ant-FLAG mAb, the LMR4 transfectants, but not LMR3 or control transfectants, produced a large amount of IL-6 and tumor necrosis factor-α almost comparable with the levels obtained upon IgE stimulation (Fig. 3E) (data not shown). As reported recently, mast cells treated with highly cytokinergic IgE survive by an autocrine mechanism under IL-3-depleted conditions (22, 40, 41). We next explored whether the triggering of LMR4 exerts the same survival effect. As shown in Fig. 3F, LMR4 stimulation displayed an anti-apoptotic effect on mast cells, although highly cytokinergic IgE was more effective. We also measured the released histamine, indicative of degranulation in mast cells. Cross-linking of LMR4 induced a 15% release of histamine, a relatively low but significant level compared with the stimulation by SPE-7 IgE (Fig. 3G). In summary, the aggregation of LMR4 activates mast cells, resulting in secretion of newly synthesized and preformed chemical mediators.

Cross-linking of LMR4 Induces Cytokine Production of BMMCs through Lyn and Syk Kinases—To investigate the role of two major tyrosine kinases, Lyn and Syk, in mast cell functions caused by LMR4 aggregation, pharmacological experiments were conducted (29, 42–44). Pretreatment with picatannol (a Syk kinase inhibitor) or PP2 (a control analog of PP2), dramatically reduced cytokine production of LMR4-stimulated BMMCs (Fig. 4A). Lyn is a Src family kinase found in abundance in mast cells and can be a positive or negative regulator of mast cell functions, depending on the intensity of FcεRI aggregation (45–49). To clarify the role of Lyn in LMR4 signaling pathways, LMR4 was transduced into wild-type or Lyn-deficient mast cells (48). Comparable expression levels of c-Kit and FceRI and transduced LMR4 were confirmed by flow cytometry (Fig. 4B). Stimulation by IgE plus a high dose of antigen caused higher levels of IL-6 production in Lyn-deficient mast cells than in wild-type mast cells, as reported previously (22, 47). In contrast, LMR4-induced IL-6 production was observed only in wild-type mast cells, but not in Lyn-deficient mast cells, at least in the range of the stimulation mode we used (Fig. 4C). Next, we performed similar experiments using Syk-deficient FLMCs with expression levels of FceRI and c-Kit comparable with those of wild-type FLMCs (Fig. 4D). As shown in Fig. 4E, IL-6 production by LMR4 cross-linking was completely abolished in Syk-deficient FLMCs in accordance with negligible activation of ERK (Fig. 5D), although that by phorbol 12-myristate 13-acetate stimulation as a control was comparable between wild-type and Syk-deficient FLMCs (29). Collectively, these data indicate that both Lyn and Syk kinases are required for positive signals downstream of LMR4.

Role of FcRy in the Function of Mast Cells Stimulated by LMR4 Cross-linking—To further explore the effect of FcRy on LMR4 expression on the cell surface, FcRγ- or DAP12-deficient BMMCs were used. As the genetic background strain of these mice is B57BL/6 and as anti-LMR4 (CBA) mAb does not detect LMR4 (B6), FLAG-tagged LMR4 was retrovirally transduced into BMMCs derived from FcRγ+/−, Dap12+/−, or FcRγ−/−/Dap12−/− mice (50–52). We first confirmed that the surface expression levels of c-Kit were comparable among these transfectants and that those of FceRI in FcRγ-deficient mast cells were not detectable (Fig. 5A, upper panels), as reported (48, 49). Flow cytometric analysis using anti-FLAG mAb revealed that the surface expression of LMR4 in FcRγ-deficient BMMCs was still detectable but significantly lower than that in wild-type or DAP12-deficient BMMCs (Fig. 5A, lower panels). This result confirmed the finding in Fig. 3, whereas it suggested that FcRγ was not essential for the surface expression of LMR4. Next, we attempted to clarify the functional role of FcRy in LMR4 signaling. To this end, cytokine production of BMMCs derived from wild-type or FcRγ-deficient mice was measured in response to LMR4 triggering. Strikingly, FcRγ-deficient mast cells induced no detectable production of IL-6 in accordance with no activation of ERK (Fig. 5, B and C). The same tendency was observed upon highly cytokinergic IgE stimulation; FceRI also shared FcRγ essential to the signaling downstream of FceRI, whereas control stimulation by phorbol 12-myristate 13-acetate induced comparable amounts of cytokine production in the wild-type and FcRγ-deficient mast cells (Fig. 5B). We conclude that FcRγ selectively associates with and stabilizes LMR4 and is essential for downstream functions, although it is dispensable for surface expression.

Cooperation of LMR4 Signaling with Others—Activating receptors adjust the functions of immune cells by cooperating with other receptors. For example, cytokine production of macrophages stimulated by LPS is enhanced by cross-linking of TREM1 (4, 17). Therefore, we examined the effects of LMR4 aggregation on the functions downstream of other receptors. As shown in Fig. 6A, cross-linking of LMR4 synergistically enhanced the cytokine production of BMMCs stimulated by LPS through TLR4 (upper panel) and by IgE plus antigen...
through FceRI (lower panel). In particular, the strong synergy between LMIR4 and TLR4 signaling was noteworthy and was in accordance with the strong enhancement of ERK and Akt activation in BMMCs triggered by both LMIR4 and TLR4 (Fig. 6B).

To evaluate the effect of the co-ligation of LMIR3 and LMIR4 on mast cell functions, we generated FLAG-tagged LMIR3- and/or LMIR4-transduced BMMCs displaying comparable expression levels of FceRI and c-Kit (Fig. 6C). The expression levels of LMIR3 and/or LMIR4 of these transfectants were confirmed by Western blot analysis (Fig. 6D). As expected, cross-linking of LMIR4 alone resulted in the production of a large amount of IL-6, whereas co-ligation of LMIR3 and LMIR4 by F(ab')2 anti-FLAG mAb dramatically abrogated it (Fig. 6E). This result supports the notion that LMIR4 and LMIR3 function as activating and inhibitory receptors, respectively.

Modulation of the Expression Levels of LMIR3 and LMIR4 in Granulocytes by in Vivo and in Vitro Administration of LPS or G-CSF—Some activating and inhibitory receptors have been reported to increase their surface expression levels on immune cells under inflammatory conditions. For example, TLT2 expression on monocytes is up-regulated by in vivo administration of LPS (53). As both LMIR3 and LMIR4 are expressed predominantly in myeloid cells, the possibility arose that the expression levels of these receptors are regulated by inflammation. To test this, LPS was administrated into the peritoneal cavities of CBA/J mice. As a result, the granulocytes (Gr-1high) included in bone marrow, peripheral blood, or the peritoneal cavity showed an increased mean fluorescence intensity for LMIR3 and a decreased mean fluorescence intensity for LMIR4 (Fig. 7A). On the other hand, intraperitoneal injection of G-CSF increased the expression of both LMIR3 and LMIR4 in particular in bone marrow granulocytes, but not in peripheral blood granulocytes, indicating higher expression levels of both LMIR3 and LMIR4 in mature granulocytes (Fig. 7B). To confirm the in vivo results, granulocytes purified from bone marrow cells were incubated for 24 h with either LPS or G-CSF.
but not tumor necrosis factor-α, was observed. In addition, LPS-induced production of IL-6 and tumor necrosis factor-α in granulocytes was significantly enhanced by LMIR4 engagement (Fig. 7E). Collectively, the activation of granulocytes is induced by engagement of endogenous LMIR4, which is enhanced by LPS. Thus, synergistic activation by LMIR4 and TLR4 engagement is recognized in granulocytes as well as in mast cells.

**DISCUSSION**

In this study, we identified LMIR3 and LMIR4 from a CBA/J mouse-derived BMMC cDNA library as new members of the LMIR family. We demonstrated a diversity of LMIR/CLM molecules among mouse strains as well as a similarity in the Ig-like domain among the members of the LMIR family, possibly giving a clue to the identification of ligands for LMIRs. Staining hematopoietic cells derived from CBA/J mice with specific mAbs revealed that LMIR4 is expressed mainly in myeloid cells, whereas LMIR3 is more broadly expressed, suggesting the existence of other activating receptors of the same family in other cells and tissues. In fact, there are a couple of activating receptors related to LMIR4. Interestingly, the expression levels of LMIR4 were higher in granulocytes of peripheral blood than in those of bone marrow and were elevated after administration of G-CSF, suggesting that mature or activated granulocytes express more LMIR4. The relationship of LMIR4 expression to the differentiation of myeloid cells remains to be determined. Our results implicate LMIR4 in innate immunity because of the following reasons. First, the expression of LMIR4 is observed in myeloid cells participating in innate immunity as well as in trachea and lung exposed to incoming invaders. Second, LMIR4 ligation strongly enhances LPS-induced cytokine production of mast cells and granulocytes. In addition, LPS stimulation leads to up-regulation of LMIR3, but to down-regulation of LMIR4.

Although alignment of the amino acid sequence of LMIR4 shows that its transmembrane domain contains a negatively charged residue instead of a positively charged amino acid, our results strongly suggest that FcRγ is indispensable for the function of LMIR4 by selective binding to and stabilization of
 Indeed, we generated LMIR4 mutants in which the glutamic acid in the transmembrane domain was mutated to lysine with a negative charge or to glutamine with a neutral charge, but the expression levels or functions of LMIR4 in mast cells did not significantly alter whatever the residue was (data not shown). To fully understand the mechanism of LMIR4 functions, further studies using knock-out mice are now under way.

In response to LMIR4 engagement, high level cytokine production was observed in wild-type mast cells, but it was dampened in FcRγ-, Lyn-, and Syk-deficient mast cells (Figs. 4 and 5). On the basis of this and previous findings on the signaling pathways downstream of ITAM-bearing molecules (5, 21, 23), we assume that the triggering of LMIR4 induces the phosphorylation of ITAM in FcRγ by Lyn, the recruitment of Syk to phosphorylated ITAM, and the activation of Syk by Lyn. In summary, all the events induced by cross-linking of LMIR4 are positively regulated by FcRγ and two tyrosine kinases, Lyn and Syk.

The cross-talk between ITAM-bearing receptors and TLRs is noteworthy in terms of innate immunity (54, 55). As clearly demonstrated in Fig. 6 (A and B) and Fig. 7 (D and E), LMIR4 signaling synergistically enhanced TLR4 signaling in mast cells and granulocytes. These data suggest that the aggregation of LMIR4 by its unknown ligands would positively regulate various signaling pathways, affecting the inflammatory responses of myeloid cells. Taking into consideration that LMIR3 and LMIR4 share high homology in the Ig-like domain, these receptors may share the ligands. Based on this hypothesis, the effects of the co-ligation of LMIR3 and LMIR4 on mast cells may mimic the physiological situations. As clearly demonstrated in Fig. 6E, co-ligation abrogated the cytokine production of mast cells induced by the cross-linking of LMIR4 alone. This could be because the inhibitory effect of the ITIM-bearing receptor LMIR3 was exerted through the phosphatases or because the number and size of aggregated LMIR4 were decreased by the co-ligation of both receptors. In any case, the increase or decrease of LMIR3 expression levels in mast cells should influence the activation events induced by the aggregation of LMIR4 with the same ligand. Notably, the expression levels of both LMIR3 and LMIR4 increased in granulocytes under the pre-inflamatory conditions, where G-CSF was mobilized upon

**FIGURE 6. Cross-talk of the signaling downstream of LMIR4 and other receptors.** A, mock- or LMIR4-transduced BMMCs were stimulated by F(ab’)2 anti-FLAG mAb and either LPS (upper panel) or IgE plus antigen (lower panel) as described under “Experimental Procedures.” IL-6 production was measured. PBS, phosphate-buffered saline; BSA, bovine serum albumin. B, LMIR4-transduced BMMCs were stimulated with F(ab’2), anti-FLAG mAb, LPS, or F(ab’2), anti-FLAG mAb plus LPS for the indicated times. The amount of phosphorylated (p) ERK1/2, JNK, p38, or Akt was examined by Western blot analysis as described in the legend to Fig. 3D. IB, immunoblot. C, mock-transduced, FLAG-tagged LMIR3-transduced, FLAG-tagged LMIR4-transduced, or FLAG-tagged LMIR3- and LMIR4-transduced BMMCs were generated. The surface expression levels of c-Kit and IgE-bound FcεRI were examined by flow cytometry. D, the expression levels of LMIR3 and LMIR4 in these transfectants were confirmed by immunoprecipitation followed by immunoblotting as described in the legend to Fig. 1B. E, the IL-6 production of these transfectants stimulated by F(ab’)2, mouse (m) IgG1 or anti-FLAG mAb (left panel) or by phorbol 12-myristate 13-acetate (PMA) (right panel) was measured. All data points correspond to the mean ± S.D. of three independent experiments.
bacterial infection, whereas those of LMIR3 and LMIR4 dramatically increased and decreased, respectively, under the inflammatory conditions induced by exposure to LPS. These findings indicate that the ratio of LMIR3 and LMIR4 expression on the cell surface changes upon bacterial infection. Accordingly, it is tempting to assume that up-regulation of LMIR3 in myeloid cells under
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the inflammatory situations suppresses the excessive activation induced by the aggregation of LMIR4 with the same ligand. Thus, both the qualitative and quantitative balance of the paired receptors LMIR3 and LMIR4 might regulate the inflammatory response of immune cells, suggesting a significant role for these paired receptors under pathophysiological situations (1, 4, 5, 56). Although the identification of the ligands for LMIR3 and LMIR4 is indispensable for complete understanding of the functions, it has been unsuccessful despite an extensive trial using expression cloning or biochemical approaches. Fine-tuning of LMIR3, LMIR4, and their ligands might provide a new therapeutic strategy in the regulation of allergy and innate immunity.

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