Functional analysis of an activating receptor LMIR4 as a counterpart of an inhibitory receptor LMIR3

Kumi Izawa1, Jiro Kitaura1, Yoshinori Yamanishi1, Takayuki Matsuoka1, Toshihiko Oki1, Fumi Shibata1, Hidetoshi Kumagai1, Hideaki Nakajima1, Mari Maeda-Yamamoto2, Jeffrey P. Hauchins3, Victor L. J. Tybulewicz2, Toshiyuki Takai5 and Toshio Kitamura1

1Division of Cellular Therapy, Advanced Clinical Research Center, The institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
2National Institute of Vegetable and Tea Science, National Agriculture Research Organization, 2769 Kanaya, Shizuoka 428-8501, Japan
3R&D System, Inc. 614 Mckinley Place N.E. Minneapolis, MN 55413
4Division of Immune Cell Biology, National Institute for Medical Research, London, United Kingdom
5Department of Experimental Immunology, Institute for Development, Aging and Cancer, Tohoku University, 4-1 Seiryo, Sendai 980-8575, Japan

Address correspondence to: Toshio Kitamura, Division of Cellular Therapy, Advanced Clinical Research Center, The institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Tel. +81-3-5449-5759; Fax. +81-3-5449-5428; E-Mail: kitamura@ims.u-tokyo.ac.jp

Leukocyte mono-immunoglobulin (Ig)-like receptor (LMIR) belongs to a new family of paired immunoreceptors. In this study, we analyzed an activating receptor LMIR4/CLM-5 as a counterpart of an inhibitory receptor LMIR3/CLM-1. LMIR4 is expressed in myeloid cells including granulocytes, macrophages, and mast cells, while LMIR3 is more broadly expressed. The association of LMIR4 with FcRγ among immunoreceptor tyrosine-based activation motifs (ITAM)-bearing molecules was indispensable for LMIR4-mediated functions of bone marrow-derived mast cell (BMMC), but dispensable for its surface expression. Crosslinking of LMIR4 led to lyn- and syk-dependent activation of BMMC, resulting in cytokine production and degranulation, while that of LMIR3 did not. The triggering of LMIR4 and TLR4 synergistically caused robust cytokine production in accordance with enhanced activation of ERK, while the co-ligation of LMIR4 and LMIR3 dramatically abrogated cytokine production. Importantly, intraperitoneal administration of lipopolysaccharide (LPS) strikingly upregulated LMIR3 and downregulated LMIR4, while that of G-CSF upregulated both LMIR3 and LMIR4 on granulocytes. Crosslinking of LMIR4 in bone marrow granulocytes also resulted in their activation, which was enhanced by LPS. Collectively, these results suggested that the innate immune system was at least in part regulated by the qualitative and quantitative balance of the paired receptors LMIR3 and LMIR4.

The immunoglobulin (Ig)-like receptors provide positive and negative regulation of immune cells upon recognition of various ligands (1-5). We previously identified leukocyte mono-Ig-like receptors (LMIRs) from a cDNA library of bone marrow-derived mast cell (BMMC). We and others demonstrated that LMIR1/myeloid-associated Ig-like receptor-I (MAIR-I)/CMRF-35-like-molecules-8 (CLM-8) and LMIR2/MAIR-II/CLM-4/dendritic cells-derived Ig-like receptor 1 (DiIR1) were mainly expressed in myeloid cells (6-9). The human homolog of LMIR1 is CMRF35H/IRp60/CD300a (10-14). Inhibitory effects of LMIR1 on mast cells and eosinophils and activatory roles of LMIR2 on macrophages have been recently described (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. In spite of 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 motifs (ITAM)- or the related activating motifs-bearing adaptor transmembrane proteins including DAP10, DAP12, or FcRγ via a positively charged residue in the transmembrane domain, while the latter include an immunoreceptor tyrosine-based inhibitory motifs (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 (HC) IgE alone induces the activation of mast cells, leading to the secretion of preformed and newly synthesized proinflammatory mediators (22, 23). Alternatively, inhibitory receptors such as FcγRIIb, PIR-B, gp49B1 or LMIR1 are also expressed on the same cell surface, probably preventing excessive activation or decreasing the background activation levels before the stimulation (3, 6, 7, 24-26).

In the present study, we identified new members of LMIRs, LMIR3 and LMIR4, from a BMMC cDNA library. Sequence analysis showed that LMIR3 and LMIR4 were basically identical to CLM-1 and CLM-5, respectively (8). The human homolog of LMIR3 is also called the immune receptor expressed by myeloid cell-1 (IREM-1) (27). An inhibitory receptor LMIR3/CLM-1 controls osteoclast differentiation (8). On the other hand, the function of LMIR4 was not fully understood; Fujimoto et al. recently reported the expression of the LMIR4/CLM-5 transcript in myeloid cells and its association with FcRγ in transfected cells (28). In view of the high homology of LMIR3 and LMIR4 in the Ig-like domain, we have characterized LMIR4 as a counterpart of LMIR3. Based on the finding that LMIR3 and LMIR4 are mainly expressed in myeloid cells, we have utilized BMMC or granulocyte to analyze the functions. The cross-talk between LMIR4 and LMIR3 or other receptors such as Toll-like receptor 4 (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 of relative expression levels of LMIR3 and LMIR4 in granulocytes in response to lipopolysaccharide (LPS) or G-CSF might suggest the relationship between innate immunity and differentiation of granulocytes upon bacterial infection.

Experimental Procedures

Antibodies (Abs) and reagents - Rat anti-LMIR3 or LMIR4 (derived from CBA/J mice) IgG2a monoclonal antibody (mAb), designated anti-LMIR3 or LMIR4 (CBA) mAb, respectively, was obtained from R&D Systems (Minneapolis, MN). Anti-FLAG M2 mAb, fluorescein isothiocyanate (FITC)-conjugated anti-FLAG M2 mAb, mouse anti-dinitrophenyl (DNP) IgE mAb (clone SPE-7), designated SPE-7 IgE, were all purchased from Sigma-Aldrich (St. Louis, MO). Anti-myc mAb and R-phycocerythrin (PE)-conjugated goat anti-rat IgG2a Ab were from Roche Diagnosis (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 BD Biosciences (Palo Alto, CA). PE- or FITC-conjugated anti-CD3, c-kit, CD45R/B220, CD11b, and 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 the anti-phospho-specific Abs were purchased from Cell Signaling Technology (Beverly, MA). F(ab’)_2 fragments were prepared by digesting anti-FLAG M2 mAb or mouse IgG1 mAb with immobilized pepsin followed by removing intact mAb with protein A affinity chromatography (Pierce Biotechnology, Inc., Rockford, IL). Cytokines were obtained from R&D Systems. PP2, PP3, and piceatannol were from EMD Biosciences (San Diego, CA). All other reagents were from Sigma-Aldrich, unless otherwise stated.

Cell culture and isolation - All the hematopoietic cell lines were cultured as described elsewhere. CBA/J mice or C57BL/6 mice (Charles River Laboratories) were used at 8-10 weeks of age for isolation of tissues and cells. All procedures were approved by an institutional review committee. To generate BMMCs or fetal liver-derived mast cells (FLMCs) with 90% purity (c-kit+/FcεRI+ by flow cytometry), bone marrow cells or fetal liver cells were cultured for 4-6 weeks in RPMI 1640...
supplemented with 10% FCS and 10 ng/ml IL-3 alone for BMDCs or plus 20 ng/ml SCF for FLMCs, respectively, as described previously (22,29). The following mutant mice were used: FcγR-/-, DAP12-/-, FcγR-/-/DAP12-/-, lyn-/-, and syk+/-. Bone marrow-derived macrophage (BMMΦ), plasmacytoid dendritic cell (BMPDC), and myeloid dendritic cell (BMmDC) were generated as described (30,31). Peritoneal cells were isolated by peritoneal lavage with 8 ml of PBS. Granulocytes were obtained as reported (32,33). Briefly, the whole bone marrow prepared from mice was centrifuged and washed in PBS. After the red blood cells were hypotonically lysed with 0.2% NaCl, this solution was resuspended to isotonicity with 1.2% NaCl. After washing, the solution was delicately applied over a 62% Percoll gradient before it was centrifuged for 30 minutes at 1,500 g. The neutrophil pellet was then isolated. Greater than 90% neutrophil purity was confirmed with a cytospin preparation. For in vivo experiments, 10 ng of G-CSF or 20 ng of LPS was intraperitoneally injected into mice 12 hours before analysis of granulocytes.

**Cell stimulation** - BMDCs were sensitized with 0.5 μg/ml of anti-TNP IgE for 12h, washed twice, and stimulated with various concentrations of TNP-BSA. Alternatively, BMDCs were directly stimulated with either SPE-7 IgE, LPS, F(ab')2 anti-FLAG mAb or their combinations. For granulocytes, these cells preincubated with 20 μg/ml of either anti-LMIR4 (CBA) Ab or rat IgG2a for 1h on ice were washed before stimulation with 10 μg/ml of F(ab')2 anti-rat IgG Ab. Alternatively, granulocytes were incubated with RPMI including 10% FCS 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 GeneBank/EMBL/DDBJ database was searched with the amino acid sequence of the Ig-like domain of LMIR1. Accession No. AY457049 (LMIR3 (B6)) and AY457051 (LMIR4 (B6)) with close similarity in the extracellular domain were selected for cloning. On the basis of these sequence data, cDNA of LMIR3 and LMIR4 were isolated from a BMMC cDNA library (derived from CBA/J mice or B6/B6 mice) by PCR and confirmed by sequencing [Accession No. AB292061 (LMIR3 (CBA)) and AB292062 (LMIR4 (CBA))], as described (6).

**Plasmid constructs** - An expression plasmid encoding a FLAG epitope-tagged DAP10, DAP12, or FcγR was generated as described (6). Either LMIR3 (CBA), LMIR3 (B6), LMIR4 (CBA), or LMIR4 (B6) was ligated into pMXs-IREs-neo' (pMXs-IN) or pMXs-IREs-puro' (pMXs-IP) (34). The entire sequence excluding leader sequence of LMIR3 (B6) or LMIR4 (B6) was amplified, and the resulting fragment was ligated into a pME vector including signal sequence of SLAM (CD150) given 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-IP or IN, generating pMXs-FLAG- or Myc- LMIR3 or LMIR4-IP or IN. All constructs were verified by DNA sequencing.

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

**RT-PCR** - Expression of LMIR4 was analyzed by reverse transcriptase (RT)-PCR amplification, as described (6). Total RNAs were extracted from each cell line and CBA mouse-derived tissues and cells. A fragment of LMIR4 was amplified with primers of 5'-ctgagattgcaagcatacgc-3' and 5'-gattcctgcagttgac-3'. This set of primers does not cross-react with LMIR3 (data not shown). For normalization, a fragment of β-actin was amplified with primers of 5'-catactattggcaagaccg-3' and 5'-acgacattacaacg-3'.

**Biochemistry** - To detect the association of LMIR4 and ITAM- or the related activating motifs-bearing molecules, COS-7 cells or 293T cells were co-transfected with two constructs of interest (pME-Myc-LMIR4 and either pMKIT-FLAG-DAP10, DAP12, or FcγR). Cells were harvested at 48 h after transfection and lysed in a lysis buffer containing 20mM Tris-HCl, PH 7.4, 137 mM NaCl, 10% glycerol and 1% Nonidet P-40 in the presence of protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Cleared supernatants of cell lysates were used for immunoprecipitation with appropriate Abs and Protein A Sepharose CL-4B (Pharmacia, North Peapack, NJ). Samples were separated by sodium
signal sequence trap based on retrovirus-mediated expression screening (SST-REX) (38) and the GeneBank/EMBL/DDBJ database (6). To find new members of LMIR, we searched the same database using the sequence of the Ig-like domain of LMIR1 and identified four homologous cDNA sequences. Among them, we cloned two cDNA (1014 and 666 nucleotides in an open-reading frame, termed LMIR3 and LMIR4, respectively) from a BMMC cDNA library derived from CBA/J mice. LMIR3 and LMIR4 proteins were 337 and 221 aa in length, respectively, and 91% identical in the Ig-like domain (Fig. 1A). Both LMIR3 and LMIR4 cDNA encoded for a type I transmembrane protein including 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 contained only a short cytoplasmic tail. Thus, LMIR3 displayed a structure typical of an inhibitory receptor. In contrast, LMIR4 had a unique property as an activating receptor. That is, LMIR4 did not possess a positively charged residue such as an arginine or lysine residue that is supposed to associate with an ITAM-bearing adaptor protein, but instead contained a negatively charged residue, glutamic acid residue, in its transmembrane domain (1,2,5,39). Sequence analysis showed that LMIR3 and LMIR4 were basically identical to CLM1 and CLM5, respectively (8). The difference of 13 aa between LMIR3 and CLM4 or 19 aa between LMIR4 and CLM5 was due to the difference between mouse strains. LMIR or CLM was derived from CBA/J or B57BL/6J, respectively (Fig. 1A). Amino acid alignment showed that the LMIR4 protein did not have apparent N-linked glycosylation sites within its extracellular domain, but had several potential O-linked glycosylation sites within a serine/threonine-rich region. Immunoprecipitation of the FLAG-tagged LMIR4 transduced into Ba/F3 cells revealed ~36 kD and ~28 kD protein, when analyzed under both reducing and non-reducing conditions. However, N-glycosyldase-F treatment did not decrease the mobility of LMIR4 (Fig. 1B and data not shown). 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 mainly expressed in myeloid cells-To
investigate the expression patterns of LMIR4, RT-PCR was performed on various tissues. High expression 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 found that expression of LMIR4 was observed in myeloid cell lines, including mast cell lines and macrophage lines (Fig. 2B), and in primary myeloid cells such as BMMΦ, BMDC, and BMMC (Fig. 2C). To confirm the surface expression level of LMIR4 on hematopoietic cells, we generated anti-LMIR3 (CBA) mAb and anti-LMIR4 (CBA) mAb. The staining of Ba/F3 cells over-expressing 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-1<sup>high</sup>Mac-1<sup>high</sup>) and monocytes/macrophages (Gr-1<sup>low</sup>Mac-1<sup>low</sup>). Granulocytes in peripheral blood highly expressed LMIR4 (Fig. 2E and data not shown), while a weak but detectable level of LMIR4 was also observed in bone marrow-derived cells (Fig. 2F and data not shown). LMIR4 expression was not detected in B cells or T cells (Figure 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 (Figure 2E-F). In summary, LMIR4 was expressed rather exclusively in myeloid cells as 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 an ITAM- and the related activating motifs-bearing adaptor protein, co-immunoprecipitation (IP) experiments were performed using COS7 cells co-transfected with a FLAG-tagged DAP10, DAP12, FcRγ or a control construct together with a Myc-tagged LMIR4. As depicted in Fig. 3A, only FcRγ was coimmunoprecipitated 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 COS7 cells dramatically increased by overexpression of FcRγ, but not DAP10 and DAP12 (Fig. 3B). These results suggested that FcRγ associated with and stabilized LMIR4, resulting in its increased expression.

**Crosslinking of LMIR4, but not LMIR3, induces the activation of BMMCs-To** obtain strong activation of primary cells stimulated by LMIR4 crosslinking, we generated FLAG-tagged LMIR4-transduced BMMCs. When stimulated with F(ab')<sub>2</sub>anti-FLAG mAb, but not control mAb, LMIR4 transfectants showed increased tyrosine phosphorylation in total cellular proteins as revealed by anti-phosphotyrosine blot (Fig. 3C). Moreover, the phosphorylation of Akt and MAPK such as ERK, JNK, and p38 was recognized by using phospho-specific Abs (Fig. 3D). These data indicated that aggregation of LMIR4 induced activation of mast cells. As activated mast cells have the potential to produce various chemical mediators, we next examined whether BMMC stimulated by LMIR4 crosslinking produced cytokines. In response to F(ab')<sub>2</sub>anti-FLAG mAb, the LMIR4 transfectants, but not LMIR3 or control transfectants, produced a large amount of IL-6 and TNFα almost comparable to the levels by IgE stimulation (Fig. 3E and data not shown). As recently reported, mast cells treated with highly cytokinergic (HC) IgE survive by an autocrine mechanism under the IL-3-depleted conditions (22,40,41). Then we explored whether the triggering of LMIR4 exerts the same survival effect. As shown in Fig. 3F, LMIR4 stimulation displayed an anti-apoptotic effect on mast cells, although HC IgE was more effective. We also measured the released histamine, indicative of degranulation in mast cells. Crosslinking of LMIR4 induced 15% release of histamine, a relatively low level compared with the stimulation by SPE-7 IgE, but significant (Fig. 3G). In summary, the aggregation of LMIR4 activated...
mast cells, resulting in secretion of newly synthesized and preformed chemical mediators.

Crosslinking of LMIR4 induced cytokine production of BMMC through lyn and syk kinase
—To investigate the role of two major tyrosine kinases, lyn and syk, in mast cell functions caused by LMIR4 aggregation, pharmacological experiments were conducted (29,42-44). Pretreatment with piceatannol, a syk kinase inhibitor or PP2, a src family kinase inhibitor, but not PP3, a control analogue of PP2, dramatically reduced cytokine production of LMIR4-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 the mast cell functions, depending on the intensity of FcRII aggregation (45-49). To clarify the role of lyn in LMIR4 signaling pathways, LMIR4 was transduced into wild type or lyn-deficient mast cells (48). Comparable expression levels of c-kit, and FcRI, and transduced-LMIR4 were confirmed by flow cytometry (Fig. 4B). The stimulation of IgE plus 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, LMIR4-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 stimulation mode we used (Fig. 4C). Next, we performed similar experiments using syk-deficient FLMCs with their expression levels of FcRI and c-kit comparable to those of wild-type FLMCs (Fig. 4D). As shown in Fig. 4E, IL-6 production by LMIR4 crosslinking was completely abolished in syk-deficient FLMCs in accordance with negligible activation of ERK (Fig. 5D), although that by PMA stimulation as control was comparable between wild type- and syk-deficient FLMCs (29). Collectively, these data indicated that both lyn and syk kinase were required for positive signals downstream of LMIR4.

The role of FcRγ in the function of mast cells stimulated by LMIR4 crosslinking—To further explore the effect of FcRγ on LMIR4 expression on the cell surface, FcRγ- or DAP12-deficient BMMCs were used. As the genetic background strain of these mice is B57BL/6 and anti-LMIR4 (CBA) mAb does not detect LMIR4 (B6), FLAG-tagged LMIR4 was retrovirally transduced into BMMCs derived from FcRγ-/-, DAP12-/-, or FcRγ-/-/DAP12-/- mice (50-52). We first confirmed that surface expression levels of c-kit were comparable among these transfectants and those of FcRγ in FcRγ-deficient mast cells were not detectable, as reported (Fig. 5A, upper panel) (48,49). Flow cytometry analysis using anti-FLAG mAb revealed that surface expression of LMIR4 in FcRγ-deficient BMMCs were still detectable but significantly lower than that in wild type or DAP12-deficient BMMCs (Fig. 5A, lower panel). This result confirmed the finding in Fig 3, while it suggested that FcRγ was not essential for surface expression of LMIR4. Next, we attempted to clarify the functional role of FcRγ in LMIR4 signaling. To this end, cytokine production of BMMCs derived from wild type or FcRγ-deficient mice was measured in response to LMIR4 triggering. Strikingly, FcRγ-deficient mast cells induced no detectable production of IL-6 in accordance with no activation of ERK (Fig. 5B-C). The same tendency was observed in HC IgE stimulation; FcRγ also shared FcRγ essential to the signaling downstream of FcRγ, while control stimulation by PMA 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 LMIR4 and is essential for downstream functions although it is dispensable for surface expression.

Cooperation of LMIR4 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 crosslinking of TREM1 (4,17). Therefore, we examined the effects of LMIR4 aggregation on the functions downstream of other receptors. As shown in Fig. 6A, crosslinking of LMIR4 synergistically enhanced the cytokine production of BMMCs stimulated by LPS through TLR4 (upper panel), and IgE plus antigen through FcRI (lower panel). In particular, the strong synergy between LMIR4- and TLR4- signaling was noteworthy, which was in accordance with 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 FLAG-tagged LMIR4-transduced BMMCs displaying comparable expression levels of FcRI 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, crosslinking of LMIR4 alone resulted in the production of a large amount of IL-6, while co-ligation of LMIR3 and LMIR4 by F(ab′)2 anti-FLAG mAb dramatically abrogated it (Fig. 6E). This result supported the notion that LMIR4 or LMIR3 functioned as an activating or as an inhibitory receptor, respectively.

Modulation of expression levels of LMIR3 and LMIR4 on granulocytes by in vivo and in vitro administration of LPS or G-CSF—Some activating or inhibitory receptors were reported to increase their surface expression levels on immune cells under inflammatory conditions. For example, TLT2 expression on monocytes was upregulated by in vivo administration of LPS (53). As both LMIR3 and LMIR4 are expressed predominantly in myeloid cells, the possibility arose that expression levels of these receptors were regulated by inflammation. To test this, LPS was administrated into the peritoneal cavity of CBA/J mice. As a result, the granulocytes (Gr-1high) included in bone marrow, peripheral blood, or peritoneal cavity showed an increased mean fluorescence intensity (MFI) for LMIR3 and decreased MFI for LMIR4 (Fig. 7A). On the other hand, intraperitoneal injection of G-CSF increased 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 hours with either LPS or G-CSF. Similar to in vivo findings, G-CSF increased the expression of both LMIR3 and LMIR4, while LPS increased the expression of LMIR3 and decreased that of LMIR4, respectively (Fig. 7C). Finally, the effect of LMIR4 engagement on granulocytes was examined. To obtain mature granulocytes (CD11bhighGr-1high) with high expression levels of LMIR4, bone marrow granulocytes were purified from CBA/J mice given an intraperitoneal injection of G-CSF. When these granulocytes were stimulated by LMIR4 engagement with or without LPS, the adhesion of these cells to plastic plates was examined. Although LMIR4 crosslinking alone induced only a weak adhesion, additional LPS stimulation accelerated the adhesive property of granulocytes triggered by LMIR4 (Fig. 7D). When these granulocytes are stimulated by LMIR4 engagement, cytokine production of IL-6, but not TNFα was observed. In addition, LPS-induced production of IL-6 and TNFα in granulocytes was significantly enhanced by LMIR4 engagement (Fig. 7E). Collectively, the activation of granulocytes was induced by engagement of endogenous LMIR4, which was enhanced by LPS. Thus, synergistic activation by LMIR4 and TLR4 engagement was recognized in granulocytes as well as in mast cells.

DISCUSSION

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

Although alignment of the aa sequence of LMIR4 displayed that its transmembrane domain contained a negatively charged residue instead of a positively charged amino acid, our results (Fig. 3, Fig. 4, Fig. 5) strongly suggested that FcRγ was indispensable for the function of LMIR4 by selective binding to and stabilization of LMIR4. Indeed, we generated LMIR4 mutants where glutamic acid residue in transmembrane was mutated to lysine with a negative charge or 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 including knockout mice are now under way.

In response to LMIR4 engagement, high levels of cytokine production were observed in wild type mast cells, but it was dampened in FcRγ-, lyn-, and syk-deficient mast cells (Fig. 4 and Fig. 5). Together with previous findings on the signaling pathways downstream of ITAM-bearing molecules, we assume that the triggering of LMIR4 induces the phosphorylation of ITAM in FcRγ by lyn, the recruitment of syk to the phosphorylated ITAM, and the activation of syk by lyn (5,21,23). In summary, all the events induced by crosslinking 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. 6A-B and Fig. 7D-E, LMIR4 signaling synergistically enhanced TLR4 signaling in mast cells and granulocytes. These data suggested 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 Figure 6E, co-ligation abrogated the cytokine production of mast cells induced by the crosslinking 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 on mast cells should influence the activation events induced by the aggregation of LMIR4 with the same ligand. Importantly, the expression levels of both LMIR3 and LMIR4 increased on granulocytes under the pre-inflammatory conditions where G-CSF was mobilized in face of bacterial infection, while that of LMIR3 or LMIR4 dramatically increased or 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 change upon bacterial infection. Accordingly, it is tempting to assume that upregulation of LMIR3 in myeloid cells under the inflammatory situations suppresses the excessive activation induced by the aggregation of LMIR4 with the same ligand. Thus, both the qualitative and the 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 identification of the ligands for LMIR3 and LMIR4 is indispensable for complete understanding of the functions, it has been unsuccessful despite of extensive trial using expression cloning or biochemical approaches. Fine-tuning of LMIR3, LMIR4, and their ligands might open a new therapeutic strategy in the regulation of allergy and innate immunity.

REFERENCES


FOOTNOTES

We gratefully thank Tadashi Yamamoto and Tohru Tezuka for kindly providing lyn-/- mice and thank Takashi Saito and Sho Yamasaki for kindly providing fetal liver cells of syk-/- mice and thank Hisashi Arase for providing pME18S expression vector containing a mouse CD150 leader segment vector. We are grateful to Dr. Dovie Wylie for her excellent language assistance. We thank Mihoko Shibuya and Kaori Ema for technical assistance. This work was supported by the Ministry of Education, Science, Technology, Sports and Culture and the Ministry of Health and Welfare, Japan.

The abbreviations used are: BMMC, bone marrow-derived mast cell; BMMPΔ; bone marrow-derived macrophage; BMmDC, bone marrow-derived myeloid dendritic cell; BMpDC, bone marrow-derived plasmacytoid dendritic cell, CLM, CMRF-35-like molecules; Dlgr, dendritic cells-derived Ig-like receptor; DNP, dinitrophenyl; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-regulated kinase; FcεRI, high-affinity IgE receptor; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; FLMC, fetal liver-derived mast cell; G-CSF, granulocyte-colony stimulating factor; HC, highly cytokinergic; Ig, Immunoglobulin; IL, interleukin; IREM, immune receptor expressed by myeloid cell; IRP, inhibitory receptor protein; ITAM, immunoreceptor tyrosine-based activation motifs; ITIM, immunoreceptor tyrosine-based inhibitory motifs; JNK, c-Jun N-terminal kinases; LMR, leukocyte mono-immunoglobulin-like receptor; LPS, lipopolysaccharide; mAb, monoclonal antibody; MAIR, myeloid-associated Ig-like receptor; MAPK,
mitogen-activated protein kinase; MFI, mean fluorescence intensity; PCR, polymerase chain reaction; PE, phycoerythrin; PIR, paired immunoglobulin (Ig)-like receptor; PMA, phorbol-12-myristate, 13-acetate; PVDF, polyvinylidene difluoride; RT, reverse transcriptase; SCF, stem cell factor; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; TNP, trinitrophenyl; TLR, Toll-like receptor

FIGURE LEGENDS

Fig. 1. Molecular and biochemical characteristics of LMIR4. A, amino acid sequences of LMIR4 derived from the different strains, CBA/J mouse and C57BL/6 mouse. Ig-like domains are indicated. The cysteines potentially involved in generating the interchain disulfide bridge of the Ig-like domains are circled. The putative leader sequence is indicated in gray background. The predicted transmembrane domain is underlined; the negative charged glutamate residue is boxed. Alignment of the Ig-like domains of LMIR3 derived from either CBA/J mouse or C57BL/6J mouse was also indicated. Identical amino acids are denoted by asterisks. B, Ba/F3 cells expressing the FLAG-tagged LMIR3 or LMIR4 were lysed and proteins were immunoprecipitated with polyclonal anti-FLAG Ab. Immunoprecipitates were resolved by SDS-PAGE under reduced conditions and blotted with anti-FLAG mAb. Molecular weight markers are indicated. C, flow cytometric analysis demonstrated that LMIR3 or LMIR4 on Ba/F3 cells expressing the FLAG-tagged LMIR3 or LMIR4, respectively, were stained by FITC-conjugated anti-FLAG mAb.

Fig. 2. Expression of LMIR4. A-C, RT-PCR analysis in various mouse tissues, hematopoietic cell lines, and bone marrow-derived cells, using specific primers for LMIR4. β-actin was amplified as a control. 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-F, single-cell suspensions were prepared from peripheral blood, bone marrow, splenocytes, and peritoneal cells from CBA/J mice. The cells gated in the population (FSC<sup>high</sup>SSC<sup>high</sup>) were stained with anti-LMIR3 (CBA) Ab, anti-LMIR4 (CBA) mAb, or control rat IgG2a, followed by staining with PE-conjugated anti-rat IgG2a mAb. For double staining, FITC-conjugated mAbs indicated were used. The peripheral blood cells gated in the population (FSC<sup>high</sup>SSC<sup>high</sup>) and the splenocytes gated in the population (FSC<sup>low</sup>SSC<sup>low</sup>) were analyzed. Bone marrow-derived myeloid or plasmacytoid dendritic cells were generated from CBA/J mice. The cells were stained as described.

Fig. 3. LMIR4 functions as an activating receptor. A, COS7 cells were transiently co-transfected with either FLAG-tagged DAP10, DAP12, FcRγ or mock construct and Myc-tagged LMIR4 construct. Lysates of these transfecants were immunoprecipitated with anti-myc mAb. The precipitates were probed with anti-FLAG mAb or anti-myc mAb. The same series of lysates was also immunoprecipitated with anti-FLAG polyclonal Ab, and the precipitates were probed with anti-FLAG mAb. B, surface expression of LMIR4 on these transfecants was analyzed using anti-myc mAb using flow cytometry. Mean fluorescent intensity (MFI) of LMIR4 expression is presented. One representative of three independent experiments is shown. C, BMMCs were infected with LMIR4-expressing retroviruses. Phosphotyrosine blot analysis of lysates from the transduced BMMCs stimulated with F(ab')<sub>2</sub> mouse IgG1 or anti-FLAG mAb for indicated time. D, the amount of phosphorylated ERK1/2, JNK, p38 or Akt was examined by western blot analysis using either anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) Ab, anti-phospho-JNK (Thr183/Tyr185) Ab, anti-phospho-p38 (Thr180/Tyr182) Ab, or anti-phospho-Akt (Ser473) Ab, respectively. Equal loading was evaluated by reprobing immunoblots with antibodies specific for ERK1/2, JNK, p38, or Akt. E, G, either mock, LMIR3-, or LMIR4-transduced BMMCs were generated. Crosslinking of LMIR4, but not LMIR3, of BMMCs using F(ab')<sub>2</sub> anti-FLAG mAb induced IL-6 production or histamine release. IL-6 (E) or histamine (G) secreted into culture media was measured. All data points correspond to the mean and the SD of four independent experiments. F, BMMCs were deprived of IL-3 in the presence of either F(ab')<sub>2</sub> anti-FLAG mAb, F(ab')<sub>2</sub> mouse IgG1 or SPE-7 IgE for indicated periods before staining with PE-conjugated annexin V. The percent of apoptotic cells was analyzed by flow cytometry. All data points
correspond to the mean and the SD of four independent experiments. Asterisks (P<0.05) indicate statistically differences.

Fig. 4. Cytokine production of BMMCs by the aggregation of LMIR4 is dependent on lyn and syk. A, IL-6 production of FLAG-tagged LMIR4-transduced BMMCs stimulated by F(ab’2), anti-FLAG mAb was measured. Cells were preincubated for 30 min with the indicated concentration of PP2, PP3, or piceatanol. B, BMMCs derived from wild type or lyn/-/- mice were infected with LMIR4-expressing retroviruses. The surface expression levels of c-kit and IgE-bound FcεRI as well as those of LMIR4 were analyzed by flow cytometry. C, IL-6 production of these transfectants stimulated by F(ab’)2 mouse IgG1 or anti-FLAG mAb (right panel) or stimulated by anti-TNP IgE plus TNP-BSA (left panel) was measured. D, FLMCs derived from wild type or syk/-/- mice were infected with LMIR4-expressing retroviruses. The surface expression levels of c-kit and IgE-bound FcεRI as well as those of LMIR4 were analyzed by flow cytometry. E, IL-6 production of these transfectants stimulated by F(ab’)2 mouse IgG1, anti-FLAG mAb or PMA. All data points correspond to the mean and the SD of four independent experiments.

Fig. 5. FcRγ is essential to the activation of mast cells stimulated by crosslinking of LMIR4 but is dispensable for surface expression on mast cells. A, BMMC generated from DAP12/-/-, FcRγ/-/-, DAP12/-/-/FcRγ/-/-, or wild type mice were transfected with LMIR4-expressing retroviruses. The surface expression levels of c-kit and IgE-bound FcεRI as well as LMIR4 were analyzed by flow cytometry. B, IL-6 production of these transfectants stimulated by F(ab’)2 mouse IgG1 or anti-FLAG mAb was measured. All data points correspond to the mean and the SD of four independent experiments. C-D, the amount of phosphorylated ERK1/2 in lysates either from wild type- or FcRγ-deficient BMMCs (C) or from wild type- or syk-deficient FLMCs (D) was examined by western blot analysis using anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) Ab. Equal loading was evaluated by reprobing immunoblots with Abs specific for ERK1/2.

Fig. 6. Cross-talk of the signaling downstream of LMIR4 and other receptors. A, either 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 depicted in Methods. IL-6 production was measured. B, LMIR4-transduced BMMCs were stimulated with either F(ab’)2 anti-FLAG mAb, LPS, or F(ab’)2 anti-FLAG mAb plus LPS for indicated time. The amount of phosphorylated ERK1/2, JNK, p38 or Akt was examined by western blot analysis, as described in Fig. 3D. C, either mock, FLAG-tagged LMIR3-, FLAG-tagged LMIR4-, or both-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 of these transfectants were confirmed by immunoprecipitation followed by immunoblot as described. E, IL-6 production of these transfectants stimulated either by F(ab’)2 mouse IgG1 or anti-FLAG mAb (left panel) or by PMA (right panel) was measured. All data points correspond to the mean and the SD of three independent experiments.

Fig. 7. Neutrophils upregulated LMIR3 and downregulated LMIR4 in response to LPS stimulation, while these upregulated both LMIR3 and LMIR4 in response to G-CSF. A, CBA/J mice were intraperitoneally injected with LPS (20 ng). After 12 h, cells were isolated from the peritoneal cavity, peripheral blood, or bone marrow and then stained for Gr-1, LMIR3, and LMIR4. The percent of neutrophils (Gr-1high) in the population (FSChighSSC) and the MFI of LMIR3 or LMIR4 on neutrophils (Gr-1high) was measured by flow cytometry. Data are representative of experiments done three times independently. B, similar experiments were done using G-CSF (10 ng) instead of LPS in an intraperitoneal injection. Data are representative of experiments done three times independently. C, granulocytes were isolated from CBA/J mice. Expression levels of LMIR3 or LMIR4 were measured by flow cytometry after incubation with 1000 ng/ml LPS or 100 ng/ml G-CSF for 24h. Data are representative of experiments done four times independently. D-E, mature granulocytes were stimulated with either anti-LMIR4(CBA) Ab or rat IgG2a plus F(ab’2) anti-rat IgG Ab for 1h before adhesion assay (E) or measuring cytokine production (F) of
TNFα (left panel) and IL-6 (right panel).
Figure 1

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMIR4 (CBA)</td>
<td>MWQSALLLF FLPGCCTAQN</td>
<td>PVTGEEVSQ 150</td>
</tr>
<tr>
<td>LMIR4 (B6)</td>
<td>***********</td>
<td>***********</td>
</tr>
<tr>
<td>LMIR3 (CBA)</td>
<td>***********</td>
<td>***********</td>
</tr>
<tr>
<td>LMIR3 (B6)</td>
<td>***********</td>
<td>***********</td>
</tr>
</tbody>
</table>

B

Vector  LMIR3  LMIR4

(kDa)

50 -

37 -

25 -

C

Vector  LMIR3  LMIR4

anti-FLAG

0

20

40

60

80

100

0

20

40

60

80

100
Figure 2

A

<table>
<thead>
<tr>
<th>Brain</th>
<th>Thyroid</th>
<th>Thymus</th>
<th>Heart</th>
<th>Liver</th>
<th>Trachea</th>
<th>Kidney</th>
<th>Stomach</th>
<th>Small intestine</th>
<th>Colon</th>
<th>Stomach</th>
<th>Colon</th>
<th>Spleen</th>
<th>Stomach</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LMIR4

β-actin

B

<table>
<thead>
<tr>
<th>Myeloid</th>
<th>Mφ</th>
<th>Myeloid</th>
<th>Mast cell</th>
<th>Megakaryocyte</th>
<th>B lineage</th>
<th>T lineage</th>
<th>Stroma cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDC-P1</td>
<td>J77.4-1</td>
<td>RAW 264.7</td>
<td>L-G</td>
<td>32D.cl1</td>
<td>P815</td>
<td>L9267</td>
<td>BA/F3</td>
</tr>
<tr>
<td>Myeloid</td>
<td>Mφ</td>
<td>Myeloid</td>
<td>Mast cell</td>
<td>Megakaryocyte</td>
<td>B lineage</td>
<td>T lineage</td>
<td>Stroma cell</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>J77.4-1</td>
<td>RAW 264.7</td>
<td>L-G</td>
<td>32D.cl1</td>
<td>P815</td>
<td>L9267</td>
<td>BA/F3</td>
</tr>
</tbody>
</table>

LMIR4

β-actin

C

<table>
<thead>
<tr>
<th>BMDC</th>
<th>BMpDC</th>
<th>Peritoneal Mφ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LMIR4

β-actin

D

<table>
<thead>
<tr>
<th>Vector</th>
<th>LMIR3 (CBA)</th>
<th>LMIR3 (B6)</th>
<th>LMIR4 (CBA)</th>
<th>LMIR4 (B6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isotype control

anti-LMIR3 (CBA) mAb

anti-LMIR4 (CBA) mAb

E

<table>
<thead>
<tr>
<th>Peripheral blood</th>
<th>Bone marrow</th>
<th>Peritoneal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isotype control

anti-LMIR3 (CBA) mAb

anti-LMIR4 (CBA) mAb

F

<table>
<thead>
<tr>
<th>BMmDC</th>
<th>BMpDC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isotype control

anti-LMIR3 (CBA) mAb

anti-LMIR4 (CBA) mAb
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>Vector</th>
<th>DAP10</th>
<th>FcRy</th>
<th>LMIR4</th>
<th>Vector</th>
<th>DAP10</th>
<th>FcRy</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP:</td>
<td>anti-myc</td>
<td>anti-FLAG</td>
<td></td>
<td>anti-FLAG</td>
<td>anti-myc</td>
<td>anti-FLAG</td>
<td></td>
</tr>
<tr>
<td>IB:</td>
<td>anti-FLAG</td>
<td>anti-FLAG</td>
<td></td>
<td>anti-FLAG</td>
<td>anti-FLAG</td>
<td>anti-FLAG</td>
<td></td>
</tr>
</tbody>
</table>

B

C

mIgG1 anti-FLAG

(kDa)

IB: anti-pY

D

mIgG1 anti-FLAG

IB: anti-pY

anti-pERK1/2

anti-ERK

anti-pJNK

anti-JNK

anti-p38

anti-38

anti-pAKT

anti-AKT

E

F

G

IL-8 (ng/ml)

%Apoptosis

%Histamine Release

Vector | LMIR3 | LMIR4

Vector | LMIR3 | LMIR4

Vector | LMIR3 | LMIR4

Vector | LMIR3 | LMIR4

Vector | LMIR3 | LMIR4

Vector | LMIR3 | LMIR4

Vector | LMIR3 | LMIR4

Vector | LMIR3 | LMIR4

Vector | LMIR3 | LMIR4

Vector | LMIR3 | LMIR4
Figure 4

A

IL-6 (ng/ml)

PBS  0  10  50  0  10 (µg/ml)

piceatanol  PP2  PP3

anti-FLAG (10 µg/ml)

B

WT /LMIR4  Lyn -/- /LMIR4

FceRI

c-kit

WT /LMIR4  Lyn -/- /LMIR4

LMIR4 (FLAG)

C

IL-6 (ng/ml)

PBS  mlgG1  anti-FLAG (10 µg/ml)

PBS  TNP-BSA (100 ng/ml)

D

WT /LMIR4  Syk -/- /LMIR4

FceRI

c-kit

WT /LMIR4  Syk -/- /LMIR4

LMIR4 (FLAG)

E

IL-6 (ng/ml)

PBS  mlgG1  anti-FLAG  PMA (100nM)
Figure 5

A

WT  FcRγ-/-/  DAP12-/-/  FcRγ/DAP12 -/-

c-kit  FcεRI  LMIR4 (FLAG)

B

WT /LMIR4  FcRγ-/- /LMIR4

IL-6 (ng/ml)

PBS  mlgG1  10 µg/ml anti-FLAG  SPE7 IgE  100 nM PMA

C  D

IB:  IB:

WT  (-)  5'  15'  30'  (-)  5'  15'  30'

FcRγ -/-

(-)  5'  15'  30'

(-)  5'  15'  30'

anti-pERK1/2

anti-ERK

anti-pERK1/2

anti-ERK
Figure 7

A

Bone marrow
Peripheral blood
Peritoneal cells

LMIR3
6% 79.6% 38%
317 421 769

LMIR4
39.9% 93.4% 0%
750 578 769

Gr-1

B

MFI of LMIR3
MFI of LMIR4

bone marrow
Peripheral blood
bone marrow
Peripheral blood

G-CSF (-)
G-CSF (+)

C

MFI of LMIR3
MFI of LMIR4

G-CSF (-)
G-CSF (+)

D

% adhesion

LPS 0 ng/ml
LPS 1000 ng/ml
PMA 100 nM

Rat IgG2a
anti-LMIR4

E

TNF-α (pg/ml)

IL-6 (pg/ml)

LPS 0 ng/ml
LPS 1000 ng/ml

Rat IgG2a
anti-LMIR4

* indicates statistical significance.
Functional analysis of an activating receptor LMIR4 as a counterpart of an inhibitory receptor LMIR3


J. Biol. Chem. published online April 16, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M701100200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2007/04/16/jbc.M701100200.citation.full.html#ref-list-1