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An Activating and Inhibitory Signal from an Inhibitory Receptor LMIR3/CLM-1: LMIR3 Augments Lipopolysaccharide Response through Association with FcγR in Mast Cells

Kumi Izawa,* Jiro Kitaura,* Yoshinori Yamanishi,* Takayuki Matsuoka,* Ayako Kaitani,* Masahiro Suguchi,* Mariko Takahashi,* Akie Maehara,* Yutaka Enomoto,* Toshihiko Oki,* Toshiyuki Takai,† and Toshio Kitamura2*†

Leukocyte mono-Ig-like receptor 3 (LMIR3) is an inhibitory receptor mainly expressed in myeloid cells. Coengagement of FcγRI and LMIR3 impaired cytokine production in bone marrow-derived mast cells (BMMCs) induced by FcγRI crosslinking alone. Mouse LMIR3 possesses five cytoplasmic tyrosine residues (Y241, Y276, Y289, Y303, Y325), among which Y241 and Y289 (Y241/289) or Y325 fit the consensus sequence of ITIM or immunotyrosine-based switch motif (ITSM), respectively. The inhibitory effect was abolished by the replacement of Y325 in addition to Y241/289 with phenylalanine (Y241/189/325/F) in accordance with the potential of Y241/289/325 to cooperatively recruit Src homology region 2 domain-containing phosphatase 1 (SHP)-1 or SHP-2. Intriguingly, LMIR3 crosslinking alone induced cytokine production in BMMCs expressing LMIR3 (Y241/276/289/303/325/F) mutant as well as LMIR3 (Y241/289/325/F). Moreover, communoprecipitation experiments revealed that LMIR3 associated with ITAM-containing FcγR. Analysis of FcγR-deficient BMMCs demonstrated that both Y276/303 and FcγR played a critical role in the activating function of this inhibitory receptor. Importantly, LMIR3 crosslinking enhanced cytokine production of BMMCs stimulated by LPS, while suppressing production stimulated by other TLR agonists or stem cell factor. Thus, an inhibitory receptor LMIR3 has a unique property to associate with FcγR and thereby functions as an activating receptor in concert with TLR4 stimulation. The Journal of Immunology, 2009, 183: 925–936.
unlike ITIM, ITSM binds to adaptor molecules, such as SH2-containing adaptor protein SH2 domain protein 1A (SH2D1A) and EWS-activated transcript 2 (EAT-2), as well as to Src family kinases and the p85 regulatory subunit of PI3K (22–23). Whether an ITSM transduces an activating or an inhibitory signal depends on the immune receptor and cell type. Additionally, Y276 or Y303 of LMIR3 is situated in the putative binding motif for p85 (YxxM), respectively. Notably, this receptor by associating with an ITAM-bearing adaptor. Importantly, we demonstrate the dual functions, inhibitory and activating, of mouse LMIR3 in mast cells. Examination of the contribution of each cytoplasmic tyrosine residue to the dual functions revealed that ITSM in addition to ITIM was required for the inhibitory function, while Y276 and Y303 were involved in the activating function. Surprisingly, LMIR3 mediated an activating signal mainly through its association with FcRIγ, it is unprecedented that an ITIM-bearing receptor functions as an activating receptor by associating with an ITAM-bearing adaptor. Importantly, we delineated the biological situation where LMIR3 mediated either an inhibitory or an activating function. Notably, this positive signal of LMIR3 synergizes with TLR4 stimulation.

Materials and Methods

Abs and reagents

Rat anti-LMIR3 IgG2a mAb, designated anti-LMIR3 mAb, and goat anti-LMIR3 polyclonal Ab were obtained from R&D Systems. Anti-FLAG mAb M2, FITC-conjugated anti-FLAG mAb M2, rabbit anti-FLAG polyclonal Ab, mouse anti-DNP IgE mAb (clone SPE-7; designated SPE-7 IgE) were all purchased from Sigma-Aldrich. Mouse anti-Myc mAb (9E10) was from Roche Diagnostics. Monoclonal anti-trinitrophenyl (TNP) IgE mAb (C38-2) and FITC-conjugated mouse anti-trinitrophenyl (TNP) IgE mAb (C38-2) were purchased from MBL International. Rabbit anti-phosphotyrosine polyclonal Ab, rabbit anti-PI3K p85 polyclonal Ab, rabbit anti-mouse IgE (SouthernBiotec), which recognized mouse IgE as well as anti-IgG Ab (Zymed Laboratories), recognized mouse IgG as well as anti-mouse IgG2a mAb (Upstate Biotechnology). All other phospho-specific Abs were purchased from Cell Signaling Technology. Rabbit F(ab′)2 anti-phosphotyrosine polyclonal Ab, rabbit anti-phosphotyrosine monoclonal Ab, rabbit anti-FCERIγ subunit polyclonal Ab, and rabbit anti-FcRIk polyclonal Ab were purchased from Upstate Biotechnology. All other phospho-specific Abs were purchased from Cell Signaling Technology. Rabbit F(ab′)2 anti-IgG2a Ab (SouthernBiotec), which recognized mouse IgG as well as anti-mouse IgG2a mAb (Upstate Biotechnology) were purchased from Cell Signaling Technology. Rabbit anti-phosphotyrosine polyclonal Ab, rabbit anti-PI3K p85 polyclonal Ab, rabbit anti-FCERIγ subunit polyclonal Ab, and rabbit anti-FcRIk polyclonal Ab were purchased from Upstate Biotechnology. All other phospho-specific Abs were purchased from Cell Signaling Technology. Rabbit F(ab′)2 anti-IgG2a Ab (SouthernBiotec), which recognized mouse IgG as well as anti-mouse IgG2a mAb (Upstate Biotechnology). All other phospho-specific Abs were purchased from Cell Signaling Technology. Rabbit F(ab′)2 anti-IgG2a Ab (SouthernBiotec), which recognized mouse IgG as well as anti-mouse IgG2a mAb (Upstate Biotechnology)

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 (3–5).

Measurement of cytokines

Culture supernatants of stimulated BMCCs were measured using ELISA kits of IL-6 or TNF-α from R&D Systems as described (3–5).

Statistical analysis

Data are shown as the means ± SD, and statistical significance was determined by Student’s t test, with p < 0.05 taken as statistically significant.

Results

The inhibitory effect of LMIR3 on FcεRI-mediated cytokine production in mast cells

We generated BMCCs with 95% purity (FcεRIγ/c-kit−) and confirmed surface expression of endogenous LMIR3 in BMCCs by using anti-LMIR3 Ab (Fig. 1A). IgE-bound FcεRI in BMCCs was engaged by plate-coated F(ab′)2 anti-IgG Ab (see Materials...
and Methods), resulting in cytokine production of BMMCs in an IgE-dose dependent manner (supplemental Fig. S1A). Since LMIR3 contains ITIM in the cytoplasmic region, the role of LMIR3 as an inhibitory receptor was expected in mast cells. Indeed, coligation of anti-LMIR3 Ab-bound LMIR3 and IgE-bound FceRI in BMMCs significantly impaired the cytokine (IL-6 and TNF-α) production induced by crosslinking of IgE-bound FceRI alone (see Materials and Methods and Fig. 1, B and C). No detectable cytokine production was observed in BMMCs stimulated by LMIR3 crosslinking alone (Fig. 1, B and C). Additionally, anti-LMIR3 Ab, but not control Ab, dose-dependently suppressed IL-6 production, suggesting an LMIR3-dependent inhibitory effect on FceRI-mediated cytokine production in mast cells (Fig. 1B). Furthermore, Western blot analysis demonstrated increased tyrosine phosphorylation of LMIR3 at 2 min followed by attenuated ERK phosphorylation at 10 min after the coligation (Fig. 1D), indicating the involvement of LMIR3 cytoplasmic tyrosine residues in the inhibitory signal.

Y325 in addition to Y241 and Y289 played an important role in the inhibitory function of LMIR3 in mast cells

LMIR3 contains five cytoplasmic tyrosine residues, Y241, Y276, Y289, Y303, and Y325, which are hereafter abbreviated as Y1, Y2, Y3, Y4, and Y5, respectively (Fig. 2D). To investigate the role in the inhibitory function of each cytoplasmic tyrosine residue, we generated FLAG-tagged wild-type (WT) LMIR3 and different mutants where one or several cytoplasmic tyrosine residues were replaced with phenylalanine, as depicted in Fig. 2D and Table I. First, BMMCs with 95% purity (FceRI/FcγRI/kit) were retrovirally transduced with FLAG-tagged LMIR3(WT) (Fig. 2A, left panel). Surface expression of transduced LMIR3 in BMMCs was confirmed by using anti-FLAG Ab (Fig. 2A, right panel). We also confirmed that IgE-bound FceRI in BMMCs was engaged by plate-coated F(ab′)2 anti-mouse IgG Ab (see Materials and Methods), leading to cytokine production of BMMCs in an IgE-dose dependent manner (supplemental Fig. S1B). As with endogenous LMIR3, transduced FLAG-tagged LMIR3(WT) displayed an inhibitory effect on FceRI-mediated IL-6 production and ERK activation of BMMCs (see Materials and Methods and Fig. 2, B and 4 The online version of this article contains supplemental material. 

FIGURE 1. Coligation of FceRI and LMIR3 in BMMCs impaired cytokine production induced by FceRI crosslinking alone. A, Surface expression levels of c-kit and IgE-bound FceRI (left panel) as well as those of endogenous LMIR3 (right panel) in BMMCs were analyzed by flow cytometry. B, IgE-bound or unbound FceRI and endogenous LMIR3 in BMMCs were coligated by various concentrations (0, 4, 20 μg/ml) of anti-LMIR3 Ab or rat IgG2a Ab as control on F(ab′)2 anti-rat IgG Ab-coated plates as described in Materials and Methods. IL-6 released into the culture supernatants was measured by ELISA. All data points correspond to the mean and the SD of three independent experiments. Rat IgG2a or anti-LMIR3 indicates rat IgG2a Ab or rat anti-LMIR3 Ab, respectively. IgE(+) or IgE(−) indicates BMMCs sensitized with IgE or not, respectively. C, IgE-bound FceRI and endogenous LMIR3 in BMMCs were coligated by 20 μg/ml anti-LMIR3 Ab, rat IgG2a Ab, or PBS on F(ab′)2 anti-rat IgG Ab-coated plates. TNF-α released into the culture supernatants was measured by ELISA. All data points correspond to the mean and the SD of three independent experiments. D, IgE-bound FceRI and endogenous LMIR3 in BMMCs were coligated by 20 μg/ml anti-LMIR3 Ab or 20 μg/ml rat IgG2a Ab as control on F(ab′)2 anti-rat IgG Ab-coated plates for 2 or 10 min. Cell lysates were subjected to immunoblotting with anti-phospho-p44/42 MAPK (pERK1/2) Ab. Equal loading was evaluated by reprobing the immunoblots with anti-ERK1/2 Ab or anti-LMIR3 Ab. Immunoprecipitates of cell lysates with anti-LMIR3 Ab were immunoblotted with anti-phosphotyrosine (pY) mAb. •, p < 0.05.

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Next, we generated different LMIR3 mutant-transduced BMMCs that showed equivalent levels of mast cell maturity and surface LMIR3 expression, although higher expression levels of LMIR3 were observed in LMIR3(YallF) mutant-transduced BMMCs (supplemental Fig. S2). To examine the inhibitory effect of each LMIR3 mutant, we measured the ratio of the amounts of IL-6 produced by each transfectant when FLAG-tagged LMIR3 mutant and IgE-bound FcεRI were coligated by using F(ab’2) anti-FLAG mAb to those only when IgE-bound FcεRI was engaged by using F(ab’2) anti-mouse IgG Ab to those only when IgE-bound FcεRI was engaged by using F(ab’2) anti-mouse IgG Ab-coated plates for 10 min. Cell lysates were subject to immunoblotting with anti-phospho-p44/42 MAPK (pERK1/2) Ab. Equal loading was evaluated by reprobing the immunoblot with anti-ERK1/2 Ab. IgE(+) or IgE(−) indicates BMMCs sensitized with IgE or not, respectively. Y1 and Y3 are located in ITIM, while Y5 is located in ITSM. TM indicates the transmembrane region.

***Figure 2.*** LMIR3 transmits an inhibitory signal in BMMCs via Y325 located in ITSM in addition to Y241/Y289 located in ITIM. A: BMMCs from mice were transduced with FLAG-tagged LMIR3. Surface expression levels of c-kit and IgE-bound FcεRI as well as those of FLAG-tagged LMIR3 were analyzed by flow cytometry. B: IgE-bound FcεRI and FLAG-tagged LMIR3 in the transduced BMMCs were coligated by using various concentrations (0, 0.1, 1, 10 μg/ml) of F(ab’2) anti-FLAG mAb or mouse IgG1 mAb as control on F(ab’2) anti-mouse IgG Ab-coated plates as described in Materials and Methods. IL-6 released into the culture supernatants was measured by ELISA. All data points correspond to the mean and the SD of three independent experiments. mIgG1 or anti-FLAG indicates F(ab’2) mouse IgG1 mAb or F(ab’2) anti-FLAG mAb. C: IgE-bound or unbound FcεRI and FLAG-tagged LMIR3 in the transduced BMMCs were coligated by 10 μg/ml F(ab’2) anti-FLAG mAb or 10 μg/ml mouse IgG1 mAb as control on F(ab’2) anti-mouse IgG Ab-coated plates for 10 min. Cell lysates were subject to immunoblotting with anti-phospho-p44/42 MAPK (pERK1/2) Ab. Equal loading was evaluated by reprobing the immunoblot with anti-ERK1/2 Ab. IgE(+) or IgE(−) indicates BMMCs sensitized with IgE or not, respectively. D: Structure of FLAG-tagged LMIR3(WT) containing cytoplasmic five tyrosine residues, Y241, Y276, Y289, Y303, and Y325, abbreviated as Y1, Y2, Y3, Y4, and Y5, respectively. Y1 and Y3 are located in ITIM, while Y5 is located in ITSM. TM indicates the transmembrane region. E: The ratio of the amounts of IL-6 produced by each transfectant when FLAG-tagged LMIR3 mutant and IgE-bound FcεRI were coligated by using F(ab’2) anti-FLAG mAb to those only when IgE-bound FcεRI was engaged by using F(ab’2), mouse IgG1 mAb. BMMCs were transduced with FLAG-tagged LMIR3(WT), different mutants, or mock. Data are representative of three independent experiments. All data points correspond to the mean and the SD.
Y4 are not important for the inhibitory function of LMIR3. On the other hand, LMIR3(Y1F), (Y3F), or (Y5F) mutant-mediated inhibition was only 45–60% when compared with LMIR3(WT)-mediated inhibition. In the LMIR3(Y1/3F) mutant where both Y1 and Y3 located in ITIM were replaced with phenylalanine, the inhibition was 30%. Coligation of FcεRI and LMIR3(Y1/3/5F) mutant, where Y5 located in ITSM in addition to Y1/3 was replaced with phenylalanine, did not result in the inhibition of IL-6 production at all. Collectively, these results indicated that the inhibitory effect of LMIR3 on FcεRI-mediated cytokine production was dependent on both ITIM and ITSM in the cytoplasmic region.

LMIR3 associated with SHP-1 and SHP-2 via phosphorylated Y241, Y276, Y289, and Y303, while associating with the p85 subunit of PI3K via phosphorylated Y276

We next explored which molecules LMIR3 associated with through the phosphorylation of its cytoplasmic tyrosine residues. When FLAG-tagged LMIR3(WT)-transduced Ba/F3 cells were stimulated by sodium pervanadate, Western blot analysis displayed a mobility shift of tyrosine-phosphorylated LMIR3 (Fig. 3A, left panel). Generally, ITIM can associate with phosphatases such as SHP-1, SHP-2, or SHP, while ITSM can bind not only to phosphatases but also to the p85 regulatory subunit of PI3K or to other adaptor molecules, depending on receptor type and cellular context (22, 23). Moreover, Y276 or Y303 fits the putative binding motif for p85 or Grb2, respectively. Therefore, we performed coimmunoprecipitation experiments using LMIR3(WT)-transduced Ba/F3 cells stimulated by sodium pervanadate. Immunoprecipitates of lysates with anti-FLAG Ab were subjected to probing with anti-SHP-1, SHP-2, or p85, or Grb2 Ab, confirming that Grb2 associated with phosphorylated LMIR3 (Fig. 3A, middle panel). Because Grb2 was not easy to discern from the nonspecific Ig L chain due to the similar mobilities in the Western blot, immunoprecipitates of lysates with anti-Grb2 Ab were also probed with anti-LMIR3 Ab, confirming that Grb2 associated with phosphorylated LMIR3 (Fig. 3A, right panel). Moreover, similar results that tyrosine-phosphorylated LMIR3 associated with SHP-1, SHP-2, or p85 were also obtained by coimmunoprecipitation experiments using LMIR3(WT)-transduced BMMCs (supplemental Fig. S3). To further explore the contribution of each tyrosine residue to the association of LMIR3 with SHP-1, SHP-2, or p85, different LMIR3 mutant-transduced Ba/F3 cells were generated. Equivalent expression levels of surface LMIR3 were confirmed among different transfectants by using anti-FLAG Ab (supplemental Fig. 4). We also performed coimmunoprecipitation experiments on Ba/F3 transfectants expressing the mutants. The association of LMIR3 with SHP-1, SHP-2, or p85 as well as tyrosine phosphorylation and concomitant mobility shift of LMIR3 upon sodium pervanadate stimulation was abolished by the replacement of all five tyrosine residues with phenylalanine in the cytoplasmic LMIR3 (Fig. 3B, left panel). This confirmed that tyrosine phosphorylation of LMIR3 was necessary for the association of LMIR3 with SHP-1, SHP-2, or p85. As shown in Fig. 3B, only LMIR3(Y2F) mutant did not coimmunoprecipitate p85 among LMIR3 mutants where a single tyrosine residue was replaced with phenylalanine (left panel). In parallel, only LMIR3(Y2Y) mutant coimmunoprecipitated p85 among LMIR3 mutants where four of five tyrosine residues were replaced with phenylalanine, at levels comparable to LMIR3(WT) (Fig. 3B, right panel). Thus, Y276 was indispensable for the association of LMIR3 with p85. On the other hand, the association of LMIR3 with SHP-1 or SHP-2 was slightly reduced in LMIR3(Y1F), (Y3F), or (Y5F) mutants (Fig. 3B, right panel), while LMIR3(Y1), (Y3), or (Y5) mutant did not coimmunoprecipitate SHP-1 or SHP-2 at all (Fig. 3B, right panel). These results suggested that the combination of Y1, Y3, or Y5 is required for the association. Analysis of LMIR3(Y1/3F), (Y1/5F), (Y3/5F), (Y1/3/5F), (1/3Y), (1/ 5Y), (3/5Y), or (1/3/5Y) mutant demonstrated that Y1/3/5 (> Y1/ 5 > Y3/5 > Y1/3 in order) played a critical role in the association of LMIR3 with SHP-1 or SHP-2 (Fig. 3C), which was in accordance with the finding that Y1, Y3, and Y5 are required for the maximum inhibitory effect of LMIR3 on FcεRI-mediated cytokine production (Fig. 2E).

Crosslinking of LMIR3(Y241/276/289/303/325/F) mutant as well as LMIR3(Y241/289/325/35/F) mutant resulted in IL-6 production in the transduced BMMCs

The potential of LMIR3 to associate with p85 or Grb2 via its tyrosine phosphorylation prompted us to postulate that LMIR3 could transmit an activating signal, at least in LMIR3 mutants that lost the inhibitory function. In fact, cytokine production of the transduced BMMCs was strongly induced by crosslinking of LMIR3(Y1/3/5F) mutant that had lost the inhibitory function (Fig. 4A). Consistently, engagement of LMIR3(Y1/3F) mutant that partially lost the inhibitory function resulted in lower but significant levels of IL-6 production compared with that of LMIR3(Y1/3/5F) mutant (Fig. 4A). Moreover, Western blot analysis demonstrated that crosslinking of LMIR3(Y1/3/5F) in the transduced BMMCs induced the tyrosine phosphorylation of LMIR3 and the association of LMIR3 with p85, suggesting the importance of Y2 and Y4 in the activating role of LMIR3 (Fig. 4C). In contrast, cytokine production was not significantly induced by crosslinking of LMIR3(WT) or LMIR3(Y1F), (Y2F), (Y3F), (Y4F), (Y5F), (Y2/4F), or (Y2/4/5F) mutants (Fig. 4A). Collectively, these results are consistent with the finding recently reported by Alvarez-Errico et al. on IREM-1/human LMIR3 that both Y236 and Y263 in IREM-1 played an important part in IREM-1-mediated activating signal (25). Furthermore, similar experiments were conducted on LMIR1, another ITIM-containing receptor among the LMIR family. In BMMCs transduced with LMIR1 WT or LMIR1(Y258/270F) mutant in which the inhibitory function was disrupted by the replacement

Table I. LMIR3 WT or different mutants

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Residues at 241, 276, 289, 303, 325</th>
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<tbody>
<tr>
<td>WT</td>
<td>Y241, Y276, Y289, Y303, Y325</td>
</tr>
<tr>
<td>Y1F</td>
<td>F241, Y276, Y289, Y303, Y325</td>
</tr>
<tr>
<td>Y2F</td>
<td>Y241, F276, Y289, Y303, Y325</td>
</tr>
<tr>
<td>Y3F</td>
<td>Y241, Y276, F289, Y303, Y325</td>
</tr>
<tr>
<td>Y4F</td>
<td>Y241, Y276, Y289, F303, Y325</td>
</tr>
<tr>
<td>Y5F</td>
<td>Y241, Y276, Y289, Y303, F325</td>
</tr>
<tr>
<td>Y1/3F</td>
<td>F241, Y276, F289, Y303, Y325</td>
</tr>
<tr>
<td>Y1/3/5F</td>
<td>Y276, Y289, Y303, Y325</td>
</tr>
<tr>
<td>Y2/3/4/5F or 1/3Y</td>
<td>Y241, Y276, Y289, F303, F325</td>
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<td>Y241, Y276, F289, Y303, Y325</td>
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<tr>
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<td>Y1/2/3/4/5F or Y5F</td>
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*LMIR3 cytoplasmic tyrosine residues, Y241, Y276, Y289, Y303, and Y325 are abbreviated as Y1, Y2, Y3, Y4, and Y5, respectively. In LMIR3 mutants, single or several tyrosine residues (Y) were replaced with phenylalanine (F).
of both Y258 and Y270 situated in ITIM with phenylalanine (21), comparable surface expression levels of LMIR1 as well as mast cell maturity were confirmed as well (supplemental Fig. 2). When either transfectant was stimulated by LMIR1 crosslinking, we found no detectable levels of cytokine production (Fig. 4A), indicating the specificity of LMIR3-mediated activation events. However, unlike the results for IREM-1, crosslinking of LMIR3(YallF) mutant that did not contain phosphorylatable tyrosine residues led to the production of a significant level of IL-6 in the transduced BMMCs. These results let us postulate the potential of LMIR3 to transmit an activating signal independent of its cytoplasmic tyrosine residues.

LMIR3 associated with FcRγ, but not DAP10 or DAP12, in BMMCs

To clarify the mechanism of LMIR3-mediated activating signal independent of LMIR3 tyrosine phosphorylation (Fig. 4A), we attempted to test the possibility that LMIR3 associated with FcRγ, DAP10, or DAP12, although it was unlikely that ITIM-containing inhibitory receptors associated with ITAM- or the related activating motif-bearing adaptor molecules. Surprisingly, coimmunoprecipitation experiments using COS-7 cells cotransduced with LMIR3(WT) and either Myc-tagged FcRγ, DAP10, DAP12, or mock clearly demonstrated that LMIR3 associated with FcRγ, but not DAP10 or DAP12 (Fig. 4B, left panel). Similar coimmunoprecipitation experiments also revealed that not only LMIR3(WT) but also LMIR3(Y1/3/5F) or LMIR3(YallF) mutant associated with FcRγ (Fig. 4B, right panel), suggesting that the association of LMIR3 with FcRγ was independent of tyrosine residues of LMIR3. Additionally, in either WT or FcRγ-deficient BMMCs transduced with LMIR3(WT), (Y1/3/5F) mutant, or (YallF) mutant, we confirmed the association of LMIR3, irrespective of WT or mutants, with endogenous FcRγ in mast cells (Fig. 4D). Collectively, an inhibitory receptor LMIR3 associated with FcRγ in mast cells.

FcRγ played a critical role in LMIR3-mediated cytokine production in LMIR3(Y241/289/325F) or (Y241/276/303/325F) mutant-transduced BMMCs

As shown in Fig. 5A, equivalent surface expression levels of c-kit with no detectable FceRI were observed in FcRγ-deficient
BMMCs transduced with LMIR3(WT), (Y1/3/5F) mutant, (YallF) mutant, or mock (26). When surface expression levels of LMIR3(WT) or mutants were compared between WT and FcR/H9253-deficient BMMCs, no significant difference was observed, suggesting that FcR/H9253 was dispensable for efficient surface expression of LMIR3 in BMMCs despite its association with LMIR3 (Fig. 5A). To clarify the role of FcR/H9253 in the activating function of LMIR3, we measured the amounts of IL-6 released from LMIR3(Y1/3/5F)-transduced WT or FcR/H9253-deficient BMMCs stimulated by LMIR3 crosslinking. Notably, the deficiency of FcR/H9253 severely, but not completely, impaired IL-6 production of the transduced BMMCs stimulated by LMIR3 crosslinking. As expected, the deficiency of FcR/H9253 completely abolished IgE-dependent IL-6 production, although PMA stimulation led to comparable amounts of IL-6 production in both transfectants (Fig. 5B). Collectively, these results suggested that FcRy played a predominant role in cytokine production of BMMCs stimulated by crosslinking of LMIR3(Y1/3/5F) or (YallF) mutant.

Crosslinking of LMIR3 enhanced cytokine production of BMMCs triggered by TLR4 agonist, while it suppressed that stimulated by other TLR agonists or SCF

Since the potential of LMIR3 to transmit an activating signal in mast cells had been demonstrated, we next attempted to find the physiological situation where LMIR3 functions as an activating receptor. Intriguingly, crosslinking of LMIR3 dramatically enhanced cytokine production of LMIR3(WT)-transduced BMMCs...
FIGURE 5. Cytokine production of the transduced BMMCs induced by engagement of LMIR3(Y241/289/325F) or LMIR3(Y241/276/289/303/325F) is severely or completely, respectively, impaired by FcRγ deficiency. A, WT or FcRγ-deficient BMMCs were transduced with FLAG-tagged LMIR3(WT), (Y1/3/5F) mutant, (YallF) mutant, or mock. Surface expression levels of c-kit and IgE-bound FcεRI as well as those of FLAG-tagged LMIR3 were analyzed by flow cytometry. B and D, WT or FcRγ-deficient BMMCs transduced with LMIR3(Y1/3/5F) (B) or LMIR3(YallF) (D) were stimulated by F(ab')2 anti-FLAG mAb or F(ab')2 mouse IgG1 as control (left panel), SPE-7 IgE (middle panel), or PMA (right panel). IL-6 released into the culture supernatants was measured by ELISA. All data points correspond to the mean and the SD of four independent experiments. C, WT or FcRγ-deficient BMMCs transduced with LMIR3(Y1/3/5F) were stimulated by F(ab')2 anti-FLAG mAb for the indicated times. Cell lysates were subject to immunoblotting with anti-phospho-p44/42 MAPK (pERK1/2) Ab (left panel) or anti-phospho-Akt Ab (right panel). Equal loading was evaluated by reprobing the immunoblots with anti-ERK1/2 Ab (left panel) or anti-Akt Ab (right panel). One representative of three independent experiments is shown.
stimulated by LPS, a TLR4 ligand (Fig. 6A). To delineate the contribution of FcRγ and/or LMIR3 cytoplasmic tyrosine residues to the LMIR3-mediated enhancement of cytokine production of mast cells stimulated by LPS, we utilized either LMIR3(WT) or LMIR3(YallF)-transduced WT or FcRγ-deficient BMMCs. We then measured the ratio of the amount of cytokine production in BMMCs stimulated by LPS plus LMIR3 crosslinking to that in BMMCs stimulated by LPS alone, finding the ratios to be 4.7 in LMIR3(WT)-transduced WT BMMCs, 2.2 in LMIR3(YallF)-transduced WT BMMCs, or 1.1 in LMIR3(YallF)-transduced FcRγ-deficient BMMCs. Taken together, these results indicated that both FcRγ and LMIR3 cytoplasmic tyrosine residues played an important role in LMIR3-mediated enhancement of cytokine production of BMMCs stimulated by LPS (Fig. 6B). In contrast, LMIR3 crosslinking impaired cytokine production of LMIR3-transduced BMMC stimulated by zymosan, poly(I:C), or CpG-ODN, which are ligands for TLR2, TLR3, or TLR9, respectively, or SCF (Fig. 6C). Finally, and most importantly, endogenous LMIR3 crosslinking induced significant levels of enhancement of cytokine production in BMMCs stimulated by LPS (Fig. 6D). In summary, LMIR3 functions as an activating receptor in BMMCs stimulated by TLR4 ligand, while LMIR3 functions as an inhibitory receptor in BMMCs stimulated by other TLR agonists or SCF.

**Discussion**

The biological role of paired immune receptors remains incompletely understood, but recent advances postulate their main functions in innate immunity. Paired activating receptors respond rapidly and effectively to foreign pathogens such as bacteria or viruses, while paired inhibitory receptors suppress the steady-state response to self proteins or excessive inflammation under pathological conditions. Generally, the former associate with ITAM or
ITAM-related activating motif-bearing adaptor molecules to transmit an activating signal, while the latter contain ITIM in the cytoplasmic region to transmit an inhibitory signal (1, 2, 27, 28). On the other hand, growing evidence has recently established the concept of dual functionality of an immune receptor that is related to the concept of inhibitory ITAM or activating ITIM (29–34). In fact, some ITAM-containing receptors can deliver an inhibitory signal in a cell type and stimulation-dependent manner, as exemplified by the inhibitory effect of triggering receptor expressed on myeloid cells 2 (TREM-2) on LPS response (35, 36) or Fc signal in a cell type and stimulation-dependent manner, as exemplified by the inhibitory effect of triggering receptor expressed on platelet-endothelial cell adhesion molecule-1 (PECAM-1) can promote endothelial cell migration (38). Additionally, ITSM-containing receptors, such as SLAM family immunoreceptors, also exhibit dual functionality (23). For instance, 2B4 (CD244)-mediated NK cell activity was promoted or suppressed by the association of its ITSM with SH2D1A or EAT-2, respectively (39).

In the present study, we delineated the dual function of LMIR3 in mast cells. As expected from the structural characteristic that LMIR3 contains two ITIMs in the cytoplasmic region, LMIR3 exerted an inhibitory effect on FceRI-mediated cytokine production in mast cells. However, analysis of different LMIR3 mutants demonstrated that the maximum inhibitory function as well as the maximum association of LMIR3 with SHP-1 or SHP-2 required an ITSM (including Y5) in addition to two ITIMs (including Y1 and Y3) (Fig. 2E). Remarkably, both Y1 and Y5 (Y1/5) played a predominant role (Y1/5 > Y3/5 > Y1/3) in the association of LMIR3 with SHP-1 or SHP-2, while a single tyrosine residue (Y1, Y3, or Y5) had a minimum role. These results strongly suggested an indispensable role of ITSM and the differential roles of two ITIMs in the inhibitory function of LMIR3. However, adaptor molecules with positive functions to associate with LMIR3 via its ITSM might be able to interfere with the inhibitory function of LMIR3. Additionally, how SHP-1 and SHP-2 differentially contribute to LMIR3-mediated inhibition in mast cells remains to be resolved.

On the other hand, the potential of ITIM-containing LMIR3 to function as an activating receptor was illustrated by experimental results that in mast cells, crosslinking of LMIR3(Y1/3/5F) mutant lacking the inhibitory function induced high amounts of cytokine production (Fig. 4A) as well as the activation of ERK and Akt (Fig. 4C) and the association of p85 with phosphorylated LMIR3(Y1/3/5F) (Fig. 4D). In support of this, LMIR3 associated with p85 exclusively via phosphorylated Y2 (Fig. 3B). Therefore, it is anticipated that P13K will be important to the LMIR3-mediated activating pathway. Additionally, the association of LMIR3 with Grb2 was also recognized (Fig. 3A), but LMIR3 tyrosine residues responsible for the binding could not be identified (data not shown) notwithstanding the fact that Y4 is located in the binding motif for Grb2. This might be either because Grb2 is not easy to discriminate from Ig L chain in coimmunoprecipitation experiments or because LMIR3 associates with Grb2 but not necessarily via Y4. Further examination is necessary, but the association of LMIR3 with Grb2 should lead to the activation of Ras/ERK in the LMIR3-mediated activating pathway. Indeed, these results do not always conflict with recent reports on IREM-1/human LMIR3, but there exists the striking difference between mouse and human LMIR3. Unlike IREM-1, crosslinking of LMIR3(YallF), in which all cytoplasmic tyrosine residues were replaced with phenylalanine, still induced significant levels of cytokine production in mast cells. We then asked if there exists an LMIR3-mediated activating signaling pathway independent of LMIR3 cytoplasmic tyrosine residues, and concluded that LMIR3 associates with ITAM-bearing FcγRI, thereby transmitting an activating signal in mast cells independently of tyrosines. Since LMIR3 contains no charged residues in the transmembrane domain, the association of LMIR3 with FcγRI appears not to be through pairwise interaction of basic and acidic residues. Related to this, surface expression levels of LMIR3, unlike typical activating receptors associating with FcγRI, were not affected by FcγRI deficiency. Simultaneously, we also found that cytokine production induced by triggering LMIR3(Y1/3/5F) or LMIR3(YallF) mutant, with the former being at much higher levels than the latter, was severely or completely, respectively, suppressed by FcγRI deficiency. This suggested that FcγRI played a predominant role as compared with Y2/4 of LMIR3 in LMIR3-mediated cytokine production. With the exception that killer cell Ig-like receptor (KIR)2DL4 contains ITIM in the cytoplasmic domain and a basic transmembrane residue through which it associates with FcγRI, LMIR3 is unusual in that an ITIM-containing receptor associates with an ITAM-bearing adaptor. Intriguingly, KIR2DL4 functions only as an activating receptor despite its bearing ITIM, whereas LMIR3 can function as either an inhibitory receptor via ITIM and ITSM or an activating receptor via Y276/303 and association with FcγRI. Additionally, several differences exist between mouse and human LMIR3. 1) IREM-1 associated with p85 via Y236 and Y263, both of which fit the binding motif for p85, while mouse LMIR3 did so only via Y2 (Fig. 3B). 2) Membrane-proximal ITIM played a predominant role in the inhibitory function of IREM-1, whereas ITSM did so in the case of LMIR3 (Figs. 2E and 3). 3) IREM-1 associated with SHP-1, but not SHP-2, while LMIR3 associated with both SHP-1 and SHP-2 (Fig. 3). 4) Coligation of IREM-1 and FceRI in RBL cells induced an inhibition of the release of β-hexosaminidase, a marker of degranulation, while coligation of mouse LMIR3 and FceRI in BMMCs led to the impairment of cytokine production, but not significant degranulation (Fig. 1 and data not shown). The difference might be explained by the different experimental systems including the quality of Ab used. On the other hand, because degranulation requires stronger FceRI aggregation than cytokine production in BMMCs, a stronger inhibitory signal may be necessary for the inhibition of degranulation as compared with cytokine production (13, 41, 42). Accordingly, it is possible that mouse LMIR3 has relatively weaker inhibitory activities in comparison to human LMIR3, while having stronger activating activities via FcγRI in mast cells. From this point of view, the potential of mouse, but not human, LMIR3 to associate with SHP-2 might influence the magnitude of the inhibitory signal.

Most importantly, we found physiological conditions under which LMIR3 functions as an activating receptor in mast cells. As clearly demonstrated in Fig. 6, cytokine production of mast cells stimulated by LPS was profoundly enhanced by LMIR3 engagement, where the activating effect of LMIR3 on TLR4 signaling was dependent on FcγRI and LMIR3 cytoplasmic tyrosine residues.

Considering that TLR4 expressed in mast cells plays a critical protective role in a model of acute septic peritonitis (43–45), LMIR3 signaling may either protect against enterobacterial infection or, conversely, cause prolonged excessive inflammation by enhancing the TLR4 signal. On the other hand, cytokine production of mast cells stimulated by SCF or other TLR agonists such as zymosan, poly(I:C), or CpG-ODN was impaired by LMIR3 crosslinking, although these stimuli did not affect surface expression levels of LMIR3 in mast cells (Fig. 6 and data not shown).

Interestingly, these inhibitory events did not require coengagement of LMIR3 and TLRs or c-kit, whereas the inhibition of FceRI signaling required coengagement of LMIR3 and FceRI. In fact, the inhibition was not significantly observed when FceRI and LMIR3 were engaged by anti-TNP IgE plus TNP-BSA and anti-LMIR3.
Ab, respectively; that is, FceRI and LMIR3 were separately engaged (data not shown). Collectively, the quality and quantity of stimuli may determine the function of LMIR3 as well as the phosphorylation levels of each LMIR3 tyrosine residue. The generation of Ab specific for each phosphorylated tyrosine of LMIR3 will give a clue to the stimulation-dependent LMIR3 function. To clarify the precise mechanism under which LMIR3 functions as an activating or inhibitory receptor is an important issue. Whether LMIR3 associates with TLR, where LMIR3- and TLR-mediated signaling pathways are combined, and how the quality and quantity of stimuli modulate the function of LMIR3 are all questions that remain to be resolved. In any case, LMIR3 can positively or negatively regulate TLR responses in mast cells, strongly suggesting the involvement of LMIR3 in the modulation of innate immunity. In view of the regulation of an activating and inhibitory signal in the immune system, the dual functionality of LMIR3 and its relevant mechanism presented here is unique and intriguing. Based on the counterbalance theory for evolution and function of paired receptors (46), we could hypothesize an in vivo function of LMIR3 as follows: if any pathogen utilized an inhibitory receptor LMIR3 to down-regulate responses against itself, another activating LMIR might have evolved to interact with it and thereby act as a counterbalance. Simultaneously, LMIR3 also might have evolutionally acquired the activating function to enhance TLR4 signal. Under normal conditions, LMIR3 constitutively inhibits weak signals in the steady-state from cytokine/chemokine or self-Ag through binding to an unknown endogenous ligand. Consequently, appropriate myeloid cell differentiation, distribution, and activation will be maintained, and the occurrence of autoimmune diseases will be avoided. Although both identification of a ligand for LMIR3 and analysis of LMIR3-deficient mice are indispensable to fully understand the function of LMIR3, this study will lead to the delineation of a novel aspect of immune regulation.

In conclusion, this study provides the first demonstration that an inhibitory receptor LMIR3 associates with FeRy and thereby enhances LPS response in mast cells. Dual functions of LMIR3 are expected to play an important part in maintaining homeostasis and in responding to emergencies in immunity.

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Disclosures

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References


