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Dual Assemblies of an Activating Immune Receptor, MAIR-II, with ITAM-Bearing Adapters DAP12 and FcRγ Chain on Peritoneal Macrophages

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Certain activating immune receptors expressed on myeloid cells noncovalently associate with either DAP12 or FcεRIγ (FcRγ chain), the ITAM-bearing transmembrane adapter proteins. An activating receptor, myeloid-associated Ig-like receptor (MAIR) II, is expressed on a subset of B cells and macrophages in the spleen and peritoneal cavity of mice and associates with DAP12 in these cells. However, we demonstrate here that cross-linking MAIR-II with mAb induced secretion of a significant amount of the inflammatory cytokines TNF-α and IL-6 from DAP12−/− as well as wild-type (WT) peritoneal macrophages. We show that MAIR-II associates with not only DAP12 but also FcεRI chain homodimers in peritoneal macrophages. LPS enhanced the FcεRI chain expression and FcεRI chain-dependent cell surface expression of MAIR-II and had additive effects on MAIR-II-mediated inflammatory cytokine secretion from peritoneal macrophages. The lysine residue in the transmembrane region of MAIR-II was involved in the association with FcεRI chain as well as DAP12. Our findings present the first case of an activating receptor that uses either DAP12 or FcεRI chain as a signaling adapter. The FcεRI chain may provide cooperation with and/or compensation for DAP12 in MAIR-II-mediated inflammatory responses by peritoneal macrophages. The Journal of Immunology, 2007, 178: 765–770.

Antigen recognition by the TCR and the BCR initiates signals for activation, cell differentiations, and effector function via the noncovalently associating transmembrane (TM) adapter molecules CD3γ, CD3δ, CD3ε, and ζ subunits of the TCR and α and β subunits of BCR, respectively. These adapter proteins bear ITAMs in their cytoplasmic regions, which are tyrosine phosphorylated by src family protein tyrosine kinases (PTKs) after Ag recognition, resulting in activation of downstream signaling molecules.

Fc receptors, including the high-affinity IgE receptor (FceRI), the IgG receptors (FcγRI, III, and IV), and the receptor for IgA (FceRI; CD89), also associate with the ITAM-bearing adapter FcεRIγ (FcεRI chain) that is responsible for FcεRI-mediated cell activation (1). The FcεRI chain is also an adapter that associates with several myeloid cell-specific receptors, including mouse PIR-A (2, 3) and human ILT-1 (4) and ILT-7 (5).

DAP12 (6), also named killer cell-activating receptor-associated protein (7), is another family member of ITAM-containing TM adapter proteins. DAP12 is expressed in NK cells and associates with several activating NK receptors (reviewed in Ref. 8), and is also expressed in myeloid cells. Several myeloid cell-specific receptors that associate with DAP12 have been reported, including human and mouse triggering receptor expressed on myeloid cell (TREM) 1, TREM-2, myeloid DAP12-associating lectin 1 (MDL), and paired Ig-like receptor (PILR) β, human signal regulatory protein β, and mouse TREM-3 and CD200RLα (reviewed inRef. 9).

We have recently identified paired activating and inhibitory Ig-like receptors, designated myeloid-associated Ig-like receptor (MAIR) I and MAIR IIa (MAIR-II), whose extracellular domains are highly conserved with each other (10). MAIR-I, also named LMR-1 (11) or CMRF-35-like molecule 8 (12), contains ITIMs in its cytoplasmic domain and inhibits IgE-mediated degranulation of mast cells (10, 13). In contrast, MAIR-II, also named LMR-II (11), CMRF-35-like molecule 4 (12), or DlgR1 (14), is expressed on subsets of peritoneal macrophages and B cells and associates with DAP12, which mediates activating signals, resulting in inflammatory cytokine secretion from macrophages (10). By screening a macrophage cDNA library and the mouse genome, we and another group found that MAIR-I and MAIR-II are members of a multigene family consisting of at least nine genes on a small segment of mouse chromosome 11 (our unpublished observation and Ref. 12). MAIR family genes are most similar to the human CMRF (CD300) family, which is located on human chromosome 17 (15), syntenic region of mouse chromosome 11. MAIR-I and MAIR-II were assigned to be CD300a and CD300d, respectively.

The family of TM adapter proteins described above are all characterized by a small extracellular region, an aspartic acid (D), a negatively charged amino acid, in the TM domain, and a cytoplasmic domain that contains one or more ITAM (16). Receptors that
associate with these TM adapters have a positively charged amino acid, such as lysine (K) or arginine (R), in their TM region and possess short cytoplasmic regions without any signaling motif. Certain activating immune receptors expressed on lymphoid and myeloid cells selectively associate either with DAP12 or FcRγ; however, there is not prior evidence that a receptor can pair with both of these adapter proteins. We demonstrate here that MAIR-II physically and functionally associates with not only DAP12 but also FcRγ chain in peritoneal macrophages.

Materials and Methods

Mice

C57BL/6J mice were purchased from Clea. DAP12-deficient and FcRγ chain-deficient mice were previously described (17, 18). These mutant mice were backcrossed to C57BL/6J mice for nine generations. All experiments were performed according to the guidelines of the animal ethics committee of the University of Tsukuba Animal Research Center.

Cells and transfectant

Mouse peritoneal macrophages were collected by peritoneal lavage with 5 ml of PBS from 8- to 12-wk-old mice before or 3 days after i.p. injection of 2 ml of 4% sterile thioglycolate. B cells and macrophages from the spleen and peritoneal cavity were purified by using a MACS selection system with biotin-conjugated anti-B220 mAb and streptavidin-coated MicroBeads (Miltenyi Biotec) and anti-CD11b MicroBeads, respectively. The purities of B220+ B cells and CD11b+ macrophages were >95% and 88%, respectively, as determined by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry.

Antibodies

Control rat IgGs, anti-CD11b, and anti-CD16/CD32 2.4G2 mAbs were purchased from BD Biosciences; anti-phosphotyrosine (4G10) and anti FcγR (FcγR chain) mAbs were purchased from Upstate Biotechnology; anti-p42/44 ERK and anti-phospho-p42/44 ERK were purchased from Cell Signaling; and rabbit anti-Flag polyclonal Ab was a gift from M. Ono (Osaka University, Osaka, Japan). These BW5147 and 2B4 transfectants were further transfected with WT or mutated MAIR-II, which contained a codon for A195 (GCG) instead of K195 (AAG), as described elsewhere (10).

Biochemistry

Cells were lysed in 1% digitonin (Calbiochem), 0.12% Triton X-100 (Sigma-Aldrich), 150 mM NaCl, 20 mM triethanolamine, protease inhibitors (1 mM PMSF and 10 U/ml aprotinin), and phosphatase inhibitors (1 mM EGTA, 10 mM NaF, 1 mM Na4P2O7, 0.1 mM β-glycerophosphate, and 1 mM Na3VO4). After overnight preclearing with 2.4G2 mAb-conjugated agarose beads (ImmunoPure Immobilized Protein L; Pierce), immunoprecipitations and/or immunoblotting experiments were performed as described previously (10). For tyrosine phosphorylation studies, cells were stimulated with F(ab′)2 of control rat IgG or anti-MAIR-II (TX52), followed by cross-linking with F(ab′)2 of rabbit anti-rat IgG (Southern Biotechnology Associates). For stripping and reblotting, Restore Western Blot Stripping Buffer (Pierce) was used according to the manufacturer’s instruction.

Stimulation of macrophages and cytokine measurement

Peritoneal macrophages (5 × 106/well) were stimulated for 24 h in 96-well flat-bottom plates coated with F(ab′)2 of control rat IgG or anti-MAIR-II (TX52). The amounts of TNF-α and IL-6 were measured by ELISA (BD Pharmingen).

Statistics

Statistical analyses were performed using the unpaired Student’s t test.

Results

MAIR-II mediates proinflammatory cytokine production from DAP12-deficient peritoneal macrophages

We previously demonstrated that MAIR-II was coimmunoprecipitated with DAP12, but not FcRγ chain, in primary spleen cells (10). Because MAIR-II was expressed on subsets of B cells and macrophages in spleen, we first examined whether MAIR-II associated with DAP12 either in B cells, macrophages or both. We demonstrate that MAIR-II was coimmunoprecipitated with DAP12 in both B220+ B cells and CD11b+ macrophages purified from spleen (Fig. 1A). Additional experiments showed similar results in B220+ B cells and CD11b+ macrophages purified from peritoneal exudate cells (PEC) (Fig. 1A), suggesting that DAP12 is a physiological partner of MAIR-II in B cells and macrophages in the spleen and peritoneal cavity. In fact, whereas splenic macrophages...
FastcR toneal macrophages. In contrast, association of MAIR-II with DAP12 and PEC derived from WT and macrophages in the presence of LPS (Fig. 1), suggesting that not A plate-coated F(ab')2 of anti-MAIR-II or isotype-matched control Ig for 68 and 24 h, respectively. Culture supernatants were harvested, and TNF-α and IL-6 concentrations were measured by ELISA. Data are the mean ± SD and are representative of three independent experiments.

MAIR-II associates with FcRγ chain as well as DAP12 in peritoneal macrophages

We then performed immunoblotting analyses of B cells and macrophages from the spleen and PEC, demonstrating that both cell types express FcRγ chain as well as DAP12 (Fig. 1B), although macrophages expressed the adaptors much more than B cells, consistent with our previous report (10). These results led us to investigate whether MAIR-II also associates with FcRγ chain in these cells from the spleen and PEC. We observed coimmunoprecipitation of MAIR-II with FcRγ chain in lysates of peritoneal macrophages and bone marrow-derived cultured macrophages (Fig. 1A). Moreover, it was enhanced after culture of peritoneal macrophages in the presence of LPS (Fig. 1A), suggesting that not only DAP12 but also FcRγ chain associates with MAIR-II in peritoneal macrophages. In contrast, association of MAIR-II with FcRγ chain was not detected in splenic B cells and macrophages and peritoneal B cells, even after culture in the presence of LPS, although these cells expressed the FcRγ chain as well as DAP12 (Fig. 1B).

MAIR-II mediates activating signals through both DAP12 and the FcRγ chain in peritoneal macrophages

The signaling pathways downstream of DAP12 and the FcRγ chain have well been characterized; both adapter proteins activate a common pathway, including stimulation of the Src family PTKs, which phosphorylate ITAMs on tandem tyrosine residues, thereby leading to the recruitment and activation of PTKs of the Syk family and subsequent downstream signal transduction (9, 19). ERK is a tyrosine-phosphorylated signaling molecule downstream of both DAP12 and the FcRγ chain. To determine whether either DAP12, the FcRγ chain, or both mediate signals after cross-linking MAIR-II, we stimulated peritoneal macrophages derived from WT, DAP12−/−, FcRγ−/−, and double-deficient mice (DKO) with F(ab')2 of the anti-MAIR-II mAb. Immunoblotting studies demonstrated that ERK 1/2 were tyrosine phosphorylated after stimulation with anti-MAIR-II in peritoneal macrophages from WT, DAP12−/−, and FcRγ−/−, but not DKO, mice (Fig. 3A). Consistent with these signaling events, cross-linking MAIR-II also induced TNF-α and IL-6 productions from WT, DAP12−/−, and FcRγ−/− peritoneal macrophages, but not from DKO mice (Fig. 3B). Taken together, these results suggest that both DAP12 and the FcRγ chain are responsible for MAIR-II-mediated activating signals in peritoneal macrophages.

LPS enhances FcRγ chain-dependent cell surface expression of MAIR-II in peritoneal macrophages

We demonstrated that LPS enhanced the association of MAIR-II with the FcRγ chain, but not DAP12 (Fig. 1), suggesting that LPS up-regulates FcRγ chain expression, accelerating the complex formation and cell surface expression of MAIR-II and the FcRγ chain. In fact, we found that overnight culture with LPS induced up-regulation of FcRγ chain expression in WT peritoneal macrophages (Fig. 4A). In contrast, DAP12 expression was not affected by LPS in peritoneal macrophages (Fig. 4A), although it was enhanced after culture in the presence of LPS in spleen B cells (10). Moreover, whereas peritoneal macrophages derived from WT, DAP12−/−, FcRγ−/−, and DKO mice expressed comparable amounts of MAIR-II on the cell surface, to culture in the presence

FIGURE 2. MAIR-II mediates cytokine production from DAP12−/− as well as WT peritoneal macrophages. Macrophages (Mφ) from the spleen and PEC derived from WT and DAP12−/− mice were stimulated with plate-coated F(ab')2 of anti-MAIR-II or isotype-matched control Ig for 68 and 24 h, respectively. Culture supernatants were harvested and TNF-α and IL-6 concentrations were measured by ELISA. Data are the mean ± SD and are representative of three independent experiments.

FIGURE 3. Both DAP12 and the FcRγ chain are involved in MAIR-II-mediated signaling in peritoneal macrophages. Peritoneal macrophages from the mice indicated were stimulated or not with plate-coated F(ab')2 of anti-MAIR-II or isotype-matched control Ig for 5 min (A) or for 24 h (B). The lysates were immunoblotted with anti-phospho-p42/44 ERK or anti-p42/44 ERK (A). Culture supernatants were harvested and TNF-α and IL-6 concentrations were measured by ELISA (B). Data are the mean ± SD and are representative of five independent experiments.
of LPS up-regulated cell surface expression of MAIR-II in WT and DAP12−/−, but not FcRγ−/− and DKO, peritoneal macrophages (Fig. 4B). Together, these results suggested that LPS enhanced FcRγ chain-dependent cell surface expression of MAIR-II. In contrast with LPS, both a Th1 cytokine IFN-γ and a Th2 cytokine IL-4, which are thought to induce classical and alternative activation of macrophages, respectively (20), did not have any effects on cell surface expression of MAIR-II on peritoneal macrophages. Cross-linking MAIR-II further increased TNF-α secretion from WT and DAP12−/−, but not from FcRγ−/− or DKO, peritoneal macrophages that had been stimulated with LPS (Fig. 4C), indicating that LPS-induced, FcRγ chain-dependent MAIR-II expression could amplify inflammatory responses by peritoneal macrophages.

**MAIR-II associates with DAP12 or the FcRγ chain homodimers**

To determine whether DAP12 and FcRγ can form heterodimers, we transfected a rat basophilic leukemia, RBL-2H3, which does not express MAIR-II and DAP12, but does the FcRγ chain (21), with a MAIR-II-ires-GFP construct along with Flag-tagged DAP12. Experiments were performed to examine whether both DAP12 and the FcRγ chain were coimmunoprecipitated with MAIR-II by using anti-Flag or anti-FcRγ chain mAbs. As demonstrated in Fig. 5, MAIR-II was coimmunoprecipitated with either homodimers of DAP12 or the FcRγ chain. Heterodimers between DAP12 and FcRγ were not observed.

The lysine residue in the TM region of MAIR-II is involved in association with both DAP12 and the FcRγ chain

Because MAIR-II contains a lysine TM region, but not an arginine in the TM region, a question was raised whether the lysine residue is involved in the association with the FcRγ chain as well as DAP12. We generated WT and mutant MAIR-II cDNA, which encoded MAIR-II containing an alanine instead of the lysine in the TM region. These cDNA were introduced into the 2B4 and BW5147 mouse T cell transfectants stably expressing Flag-tagged DAP12 and Flag-tagged FcRγ chain, respectively. As demonstrated in Fig. 6A, whereas introducing WT MAIR-II into Flag-tagged DAP12-expressing 2B4 induced cell surface expression of DAP12, introduction of the mutant MAIR-II did not. Similarly, in contrast to WT MAIR-II, mutant MAIR-II also did not induce cell surface expression of the FcRγ chain. Moreover, the FcRγ chain as well as DAP12 were coimmunoprecipitated with WT, but not mutant MAIR-II (Fig. 6B). Of note, mutant MAIR-II was expressed higher than WT MAIR-II on the cell surface of both 2B4 and BW5147 transfectants (Fig. 6A). This difference might be dependent on the retrovirus transduction efficiency because the total amount of mutant MAIR-II was higher than that of WT MAIR-II in each transfectant (Fig. 6B). Together, these results indicated that the lysine residue in the TM region of MAIR-II
In our study, it was interesting that TNF-α secretion through DAP12 was inhibited by TLR signaling from macrophages through DAP12 (25, 26). In recently, triggering receptor expressed on myeloid cells (TREM-2) when they are induced by TLR under different conditions. Very and/or ZAP70 (19). Because LPS enhanced FcR chain expression and FcR chain-dependent cell surface expression of MAIR-II on peritoneal macrophages, the FcR chain may provide cooperation with and/or compensation for DAP12 in MAIR-II-mediated inflammatory responses by peritoneal macrophages. This may be reasonable, because peritoneal macrophages are required to be activated more quickly and sufficiently for innate immunity than spleen macrophages or B cells that are involved in adaptive immunity or link between innate and adaptive immunity.

Lanier and colleagues (24) demonstrated that, although cross-linking of DAP12-associated receptors on macrophages can initiate cytokine production in some circumstances, these same cytokine secretions were increased from DAP12−/− macrophages when they are induced by TLR under different conditions. Very recently, triggering receptor expressed on myeloid cells (TREM-2) has been found to mediate inhibition of cytokine secretion induced by TLR signaling from macrophages through DAP12 (25, 26). In our study, it was interesting that TNF-α secretion induced by LPS seemed to be decreased, rather than increased, from FcRγ−/− peritoneal macrophages upon cross-linking with MAIR-II (Fig. 4C), suggesting a possibility that MAIR-II also inhibited LPS-mediated TNF-α secretion through DAP12. Further analysis is required to determine this possibility.

Several activating receptors, including CD16, NKG2D, and NKp30, associate with heterodimers of FcRγ and ζ, as well as the homodimer of these adapter proteins (27), suggesting that FcRγ and ζ are redundant. We have demonstrated that MAIR-II associated with either the homodimers of DAP12 or FcRγ, but not the heterodimers of DAP12 and FcRγ. These results were reasonable, because, unlike FcRγ and ζ, DAP12 and the FcRγ chain have the extracellular cysteines and the TM aspartic acid residues at quite different positions from each other, which may prevent the heterodimer formation by these two adapter proteins. Specifically, DAP12 has two cysteine residues per chain in the membrane-proximal extracellular region, which may be involved in dimer formation, whereas the FcRγ chain has one cysteine per chain in the TM domain (16). Moreover, the negatively charged residue aspartic acid, which is involved in physical association with activating receptors containing a positively charged amino acid residue in the TM domain, is located close to the center of the TM region of DAP12, but it is in the N-terminal segment of the TM domain of the FcRγ chain (16). DAP12, which has an aspartic acid close to the center of the TM region, preferentially associates with immune receptors, including NK receptors and activating myeloid receptors, which bear a charged amino acid residue (usually lysine) at the center, rather than at the N-terminal segment, of the TM domain (16). In contrast, FcRγ chain has an aspartic acid in the N-terminal segment of the TM region and noncovalently associates with immune receptors, including several Fc receptors, and others, which bear a charged amino acid residue (often arginine) also at the N-terminal segment of the TM domain. Although the lysine residue in the TM region of MAIR-II is involved in the association of MAIR-II with both DAP12 and the FcRγ chain, it cannot be concluded that the molecular and structural characteristics of the assemblies of MAIR-II with the FcRγ chain are similar to those with DAP12, because not only a charged amino acid but also the orientation of receptors toward TM adaptors play an important role in association of receptors with adaptors (28).

We have demonstrated that, although MAIR-II associated with either DAP12 or the FcRγ chain on peritoneal and bone marrow-derived cultured macrophages, it did with DAP12 alone on macrophages from the spleen. Macrophages are widely distributed in tissues and have marked phenotypic heterogeneity, which are likely to result from developmental signals encountered within individual tissue sites. In addition, each macrophage also acquires new functional capacities in response to stimuli encountered in the tissue microenvironment. These heterogeneity of tissue macrophages might lead to the functional difference also in the adapter usage of MAIR-II. In contrast, B cells from the peritoneal cavity as well as the spleen expressed MAIR-II that associated with DAP12 alone, probably because B cells may have little phenotypic heterogeneity between tissues. Future studies are required to clarify the molecular basis for the assembly of both adapters by MAIR-II.

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**Disclosures**

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