Dual assemblies of an activating immune receptor, MAIR-II, with ITAM-bearing adapters DAP12 and FcR gamma chain on peritoneal macrophages

著者	Nakahashi Chigusa, Tahara-Hanaoka Satoko, Totsuka Naoya, Okoshi Yasushi, Takai Toshiyuki, Ohkohchi Nobuhiro, Honda Shin-ichiro, Shibuya Kazuko, Shibuya Akira, 田原, 聡子
journal or publication title	Journal of immunology
volume	178
number	2
page range	765-770
year	2007-01
権利	(C) 2007 by The American Association of Immunologists
URL	http://hdl.handle.net/2241/102019

Dual assemblies of an activating immune receptor, MAIR-II, with ITAM-bearing adapters DAP12 and FcRγ chain on peritoneal macrophages

Running title: MAIR-II associates with DAP12 and FcRγ

Chigusa Nakahashi [*, †], Satoko Tahara-Hanaoka [*], Naoya Totsuka [*], Yasushi Okoshi [*], Toshiyuki Takai[‡, §], Nobuhiro Ohkochi[†], Shin-ichiro Honda [*], Kazuko Shibuya [*], Akira Shibuya[1, *]

[*]Department of Immunology, Institute of Basic Medical Sciences and Center for TARA, [†]Department of Surgery, Institute of Clinical Medicine, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Ten-nodai, Tsukuba, Ibaraki 305-8575, Japan
[‡]Department of Experimental Immunology, Institute of Development, Aging and Cancer, Tohoku University, Seiryo 4-1, Aoba-Ku, Sendai 980-8575, Japan
[§]CREST, Japan Science and technology Agency, Honcho 4-1-8, Kawaguchi, Saitama 332-0012, Japan
[1]Address Correspondence to Akira Shibuya, e-mail; ashibuya@md.tsukuba.ac.jp, FAX : (81)29-853-3410, Phone: (81)29-853-3474

This research was supported in part by the grants provided by the Ministry of Education, Science and Culture of Japan.

KeyWords: Monocytes/Macrophages, Cell Surface Molecules, Cell Activation, Inflammation, Signal Transduction

Abstract

Certain activating immune receptors expressed on myeloid cells non-covalently associate with either DAP12 or FcRγ chain, the immunoreceptor tyrosine-based activating motif (ITAM)-bearing transmembrane adapter proteins. An activating receptor, myeloid-associated immunoglobulin-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 monoclonal antibody induced secretion of significant amount of the inflammatory cytokines TNF-α and IL-6 from DAP12-/- as well as wild type peritoneal macrophages. We show that MAIR-II associates with not only DAP12 but also FcRγ chain homodimers in peritoneal macrophages. LPS enhanced the FcRγ chain expression and FcRγ 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 FcRγ chain as well as DAP12. Our findings present the first case of an activating receptor that uses either DAP12 or FcRγ chain as a signaling adapter. FcRγ chain may provide cooperation with and/or compensation for DAP12 in MAIR-II-mediated inflammatory responses by peritoneal macrophages.

Introduction

Antigen recognition by the T cell antigen receptor (TCR) and the B cell antigen receptor (BCR) initiates signals for activation, cell differentiations, and effector function via the non-covalently associating transmembrane (TM) adaptor molecules CD3γ, CD3δ, CD3ε, and ζ subunits of the TCR and α and β subunits of BCR, respectively. These adapter proteins bear immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic regions, which are tyrosine phosphorylated by src family protein tyrosine kinases (PTKs) after antigen recognition, resulting in activation of down stream signaling molecules.

Fc receptors, including the high affinity IgE receptor (FcεRI), the IgG receptors (FcγRI, III, and IV) and the receptor for IgA (FcαRI; CD89), also associate with the ITAM-bearing adapter FcRγ chain that is responsible for Fc receptor-mediated cell activation (1). FcRγ 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 (KARAP) (7), is another family member of ITAM-containing TM adapter proteins. DAP 12 is expressed in NK cells and associates with several activating NK receptors (reviewed in (8)), and is also expressed in myeloid cells. Several myeloid cell-specific receptors that associate

with DAP12 have been reported, including human and mouse TREM-1, TREM-2, MDL-1, and PILRβ, human SIRPβ, and mouse TREM-3 and CD200RLa (reviewed in (9)).

We have recently identified paired activating and inhibitory immunoglobulin-like receptors, designated myeloid-associated immunoglobulin-like receptor (MAIR)-I and MAIR-II, whose extracellular domains are highly conserved with each other (10). MAIR-I, also named LMIR-1 (11) or CMRF-35-like molecule (CLM)-8 (12), contains immunoreceptor tyrosine-based inhibitory motifs (ITIM) in its cytoplasmic domain, and inhibits IgE-mediated degranulation of mast cells (10, 13). On the other hand, MAIR-II, also named LMIR-II (11), CLM-4 (12) or DIgR1 (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 9 genes on a small segment of mouse chromosome 11 (our unpublished observation and (12)). MAIR family genes are most similar to the human CD300 family (CMRF), which is located on human chromosome 17 (15), syntenic region of mouse chromosome 11.

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 (Tokyo, Japan). 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 week old mice before or three days after i.p. injection of 2 ml of 4% sterile thioglycollate. 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, Bergisch Granbach, Germany) and anti-CD11b MicroBeads, respectively. Bone marrow cells were cultured in the presence of M-CSF (10 ng/ml) for 4 days and analyzed by flow cytometry, showing that more

than 80 % of cultured cells were $CD11b^+$ macrophages. RBL-2H3 transfectant stably expressing MAIR-II-IRES GFP and N-terminus Flag-tagged DAP12 and the BW5147 transfectant stably expressing N-terminus Flag-tagged FcRγ chain, as described previously (10). The 2B4 transfectant stably expressing N-terminus Flag-tagged DAP12 was provided by Hisashi Arase (Osaka University, 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 (10).

Antibodies

Control rat IgGs, anti-CD11b and anti-CD16/CD32 2.4G2 mAbs were purchased from BD Biosciences (San Jose, CA); anti-phosphotyrosine (4G10) and anti FcεRIγ (FcRγ chain) mAbs were purchased from Upstate Biotechnology (Lake Placid, NY); anti-p42/44 ERK and anti-phospho p42/44 ERK were purchased from Cell Signaling (Beverly, MA); and rabbit anti-Flag polyclonal antibody was purchased from Sigma-Aldrich. Anti-mouse DAP12 polyclonal antibody was a gift from M. Ono (Tohoku University School of Medicine, Sendai, Japan). TX52 (anti-MAIR-II) mAb was

generated in our laboratory as described previously (10). $F(ab')_2$ fragments of TX52 mAb were prepared, as described previously (10). Purity of the $F(ab')_2$ fragments were determined by SDS-PAGE. To exclude LPS contamination, antibodies were treated with polymixin B (10 ug/ml; Sigma Aldrich) before use.

Biochemistry

Cells were lysed in 1% digitonin (Calbiochem, San Diego, CA), 0.12% Triton-X (Sigma-Aldrich), 150 mM NaCl, 20 mM triethanolamine, and protease and phosphatase inhibitors. After overnight pre-clearing with 2.4G2mAb-conjugated agarose beads (ImmunoPure Immobilized Protein L, Pierce Chemical), immunoprecipitations and/or immunoblotting experiments were performed, as described (10). For tyrosine phosphorylation studies, cells were stimulated with $F(ab')_2$ fragments of control rat IgG or anti-MAIR-II (TX52), followed by crosslinking with $F(ab')_2$ fragments of rabbit anti-rat IgG (Southern Biotechnology, Birmingham, AC). For stripping and re-blotting, Restore Western Blot Stripping Buffer (Pierce Chemical) was used according to the manufacturer's instruction.

Stimulation of macrophages and cytokine measurement

Peritoneal macrophages ($5x10^5$/well) were stimulated for 24 hrs in 96-well flat-bottom plates coated with $F(ab')_2$ fragments of control rat IgG or anti-MAIR-II (TX52). The amounts of TNF-α and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA; BD Pharmingen, Franklin Lakes, NJ).

Statistics

Statistical analyses were performed by unpaired Student's *t*-test.

RESULTS

MAIR-II mediates pro-inflammatory cytokine production from DAP12-deficient peritoneal macrophages

We previously demonstrated that MAIR-II was co-immunoprecipitated 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 co-immunoprecipitated with DAP12 in both $B220^+$ B cells and $CD11b^+$ macrophages purified from spleen (**Fig. 1**). Further experiments showed similar results in $B220^+$ B cells and $CD11b^+$ macrophages purified from peritoneal exudative cells (PEC) (**Fig. 1**), 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 from WT mice produced TNF-α when stimulated with $F(ab')_2$ fragments of anti-MAIR-II mAb, *DAP12-/-* macrophages did not respond to the antibody stimulation for TNF-α secretion (**Fig. 2**), indicating that DAP12 is required for MAIR-II-mediated activating signal in splenic macrophages. Unexpectedly, however, cross-

linking MAIR-II produced significant amount of both TNF-α and IL-6 from *DAP12-/-* as well as WT peritoneal macrophages (**Fig. 2**). These results suggest that another MAIR-II-associating adapter molecule, in addition to DAP12, may mediate activating signals specifically in peritoneal macrophages.

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

Because B cells and macrophages express FcRγ chain as well as DAP12 (10), we investigated whether MAIR-II also associates with FcRγ chain in these cells from the spleen and PEC. We observed co-immunoprecipitation of MAIR-II with FcRγ chain in lysates of peritoneal macrophages and bone marrow-derived cultured macrophages (**Fig. 1**). Moreover, it was significantly enhanced after culture of peritoneal macrophages in the presence of LPS (**Fig. 1**), 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 (**Fig. 1**).

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

The signaling pathways downstream of DAP12 and FcRγ chain have well been characterized; both adapter proteins activate a common pathway, including stimulation of Src family PTKs, which phosphorylate ITAMs on tandem tyrosine residues, thereby leading to the recruitment and activation of PTKs of Syk family and subsequent downstream signal transduction (9, 19). ERK is a tyrosine phosphorylated signaling molecule downstream of both DAP12 and FcRγ chain. To determine whether either DAP12, 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 $F(ab')_2$ fragments of 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 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 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 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 amount of MAIR-II on the cell surface, culture in the presence of LPS upregulated 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 interferon (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 (data not shown).

We next examined whether LPS had any effect on MAIR-II-mediated signaling in peritoneal macrophages as a result of enhancement of cell surface expression of MAIR-II. To address this question, peritoneal macrophages were cultured in the presence of LPS and then stimulated with $F(ab')_2$ fragments of anti-MAIR-II mAb. Stimulation with LPS alone significantly induced TNF-α secretion from WT, *DAP12-/-*, *FcRγ-/-*, and DKO 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 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 FcRγ chain (21), with a MAIR-II-IRES-GFP construct together with Flag-tagged DAP12. Experiments were performed to examine whether both DAP12 and FcRγ chain were co-immunoprecipitated with MAIR-II by using anti-Flag or anti-FcRγ chain mAbs. As demonstrated in **Fig. 5,** MAIR-II was co-immunoprecipitated with either homodimers of DAP12 or 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 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 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 with WT *MAIR-II,* mutant *MAIR-II* also did not induce cell surface expression of FcRγ chain. Moreover, FcRγ chain as well as DAP12 were co-immunoprecipitated with WT, but not mutant, MAIR-II (**Fig. 6C**). Together, these results indicated that the lysine residue in the TM region of MAIR-II was involved in the association with FcRγ chain as well as DAP12.

Discussion

We have presented here the first case of an activating receptor that uses either DAP12 or FcRγ chain as the signaling adapter. At present, it is unclear whether any other activating receptors on myeloid or lymphoid cells have a similar property. Although NKG2D non-covalently associates with DAP10 in $CD8^+$ T cells, a mouse NKG2D isoform pairs with either DAP10 or DAP12 in activated mouse NK cells (22, 23). Unlike DAP12, DAP10 has a YxxM motif in the cytoplasmic domain, which recruits the p85 subunit of the phosphatidylinositol 3-kinase (PI 3-kinase), thereby providing a different function from DAP12. On the contrary, DAP12 and FcRγ chain seem to initiate the same signal transduction pathways by recruiting Syk and/or ZAP70 (19). Because LPS enhanced FcRγ chain expression and FcRγ chain-dependent cell surface expression of MAIR-II on peritoneal macrophages, FcRγ chain may provide cooperation with and/or compensation for DAP12 in MAIR-II-mediated inflammatory responses mediated by peritoneal macrophages.

Several activating receptors, including CD16, NKp46, and NKp30, associate with heterodimers of FcRγ and ζ, as well as the homodimer of these adapter proteins

(24), 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 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 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 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 the N-terminal segment of the TM region and non-covalently 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 FcRγ chain, it cannot be concluded that the molecular and structural characteristics of the assemblies of MAIR-II with FcRγ chain are similar to that 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 (25).

We have demonstrated that, although MAIR-II associated with either DAP12 or 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 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.

Acknowledgement

We thank Lewis Lanier for critical reading of the manuscript and Yurika Soeda for secretarial assistance.

References

1. Nimmerjahn, F., and J. V. Ravetch. 2006. Fcgamma receptors: old friends and new family members. *Immunity 24:19-28*.
2. Kubagawa, H., P. D. Burrows, and M. D. Cooper. 1997. A novel pair of immunoglobulin-like receptors expressed by B cells and myeloid cells. *Proc Natl Acad Sci U S A 94:5261-5266*.
3. Hayami, K., D. Fukuta, Y. Nishikawa, Y. Yamashita, M. Inui, Y. Ohyama, M. Hikida, H. Ohmori, and T. Takai. 1997. Molecular cloning of a novel murine cell-surface glycoprotein homologous to killer cell inhibitory receptors. *J Biol Chem 272:7320-7327*.
4. Nakajima, H., J. Samaridis, L. Angman, and M. Colonna. 1999. Human myeloid cells express an activating ILT receptor (ILT1) that associates with Fc receptor gamma-chain. *J Immunol 162:5-8*.
5. Cao, W., D. B. Rosen, T. Ito, L. Bover, M. Bao, G. Watanabe, Z. Yao, L. Zhang, L. L. Lanier, and Y. J. Liu. 2006. Plasmacytoid dendritic cell-specific receptor ILT7-Fc{varepsilon}RI{gamma} inhibits Toll-like receptor-induced interferon production. *J Exp Med*.
6. Lanier, L. L., B. C. Corliss, J. Wu, C. Leong, and J. H. Phillips. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature 391:703-707*.
7. Tomasello, E., L. Olcese, F. Vely, C. Geourgeon, M. Blery, A. Moqrich, D. Gautheret, M. Djabali, M. G. Mattei, and E. Vivier. 1998. Gene structure, expression pattern, and biological activity of mouse killer cell activating receptor-associated protein (KARAP)/DAP-12. *J Biol Chem 273:34115-34119*.
8. Lanier, L. L. 2005. NK cell recognition. *Annu Rev Immunol 23:225-274*.
9. Lanier, L. L., and A. B. Bakker. 2000. The ITAM-bearing transmembrane adaptor DAP12 in lymphoid and myeloid cell function. *Immunol Today 21:611-614*.
10. Yotsumoto, K., Y. Okoshi, K. Shibuya, S. Yamazaki, S. Tahara-Hanaoka, S. Honda, M. Osawa, A. Kuroiwa, Y. Matsuda, D. G. Tenen, A. Iwama, H. Nakauchi, and A. Shibuya. 2003. Paired activating and inhibitory

immunoglobulin-like receptors, MAIR-I and MAIR-II, regulate mast cell and macrophage activation. *J Exp Med 198:223-233*.

11. Kumagai, H., T. Oki, K. Tamitsu, S. Z. Feng, M. Ono, H. Nakajima, Y. C. Bao, Y. Kawakami, K. Nagayoshi, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, T. Kawakami, and T. Kitamura. 2003. Identification and characterization of a new pair of immunoglobulin-like receptors LMIR1 and 2 derived from murine bone marrow-derived mast cells. *Biochem Biophys Res Commun 307:719-729*.
12. Chung, D. H., M. B. Humphrey, M. C. Nakamura, D. G. Ginzinger, W. E. Seaman, and M. R. Daws. 2003. CMRF-35-like molecule-1, a novel mouse myeloid receptor, can inhibit osteoclast formation. *J Immunol 171:6541-6548*.
13. Okoshi, Y., S. Tahara-Hanaoka, C. Nakahashi, S. Honda, A. Miyamoto, H. Kojima, T. Nagasawa, K. Shibuya, and A. Shibuya. 2005. Requirement of the tyrosines at residues 258 and 270 of MAIR-I in inhibitory effect on degranulation from basophilic leukemia RBL-2H3. *Int Immunol 17:65-72*.
14. Luo, K., W. Zhang, L. Sui, N. Li, M. Zhang, X. Ma, L. Zhang, and X. Cao. 2001. DIgR1, a novel membrane receptor of the immunoglobulin gene superfamily, is preferentially expressed by antigen-presenting cells. *Biochem Biophys Res Commun 287:35-41*.
15. Clark, G. J., B. Cooper, S. Fitzpatrick, B. J. Green, and D. N. Hart. 2001. The gene encoding the immunoregulatory signaling molecule CMRF-35A localized to human chromosome 17 in close proximity to other members of the CMRF-35 family. *Tissue Antigens 57:415-423*.
16. Feng, J., D. Garrity, M. E. Call, H. Moffett, and K. W. Wucherpfennig. 2005. Convergence on a distinctive assembly mechanism by unrelated families of activating immune receptors. *Immunity 22:427-438*.
17. Kaifu, T., J. Nakahara, M. Inui, K. Mishima, T. Momiyama, M. Kaji, A. Sugahara, H. Koito, A. Ujike-Asai, A. Nakamura, K. Kanazawa, K. Tan-Takeuchi, K. Iwasaki, W. M. Yokoyama, A. Kudo, M. Fujiwara, H. Asou, and T. Takai. 2003. Osteopetrosis and thalamic hypomyelinosis with synaptic degeneration in DAP12-deficient mice. *J Clin Invest 111:323-332*.
18. Takai, T., M. Li, D. Sylvestre, R. Clynes, and J. V. Ravetch. 1994. FcR gamma chain deletion results in pleiotrophic effector cell defects. *Cell 76:519-529*.

19. Vivier, E., J. A. Nunes, and F. Vely. 2004. Natural killer cell signaling pathways. *Science 306:1517-1519*.
20. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol 3:23-35*.
21. Tomasello, E., C. Cant, H. J. Buhring, F. Vely, P. Andre, M. Seiffert, A. Ullrich, and E. Vivier. 2000. Association of signal-regulatory proteins beta with KARAP/DAP-12. *Eur J Immunol 30:2147-2156*.
22. Diefenbach, A., E. Tomasello, M. Lucas, A. M. Jamieson, J. K. Hsia, E. Vivier, and D. H. Raulet. 2002. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat Immunol 3:1142-1149*.
23. Gilfillan, S., E. L. Ho, M. Cella, W. M. Yokoyama, and M. Colonna. 2002. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat Immunol 3:1150-1155*.
24. Lanier, L. L. 2001. On guard--activating NK cell receptors. *Nat Immunol 2:23-27*.
25. Bakema, J. E., S. de Haij, C. F. den Hartog-Jager, J. Bakker, G. Vidarsson, M. van Egmond, J. G. van de Winkel, and J. H. Leusen. 2006. Signaling through mutants of the IgA Receptor CD89 and consequences for Fc receptor gamma-chain interaction. *J Immunol 176:3603-3610*.

Figure Legends

Figure 1. MAIR-II associates not only with DAP12 but also with FcRγ chain in peritoneal and bone marrow-derived cultured macrophages.

B cells and macrophages purified from spleen and peritoneal exudative cells (PEC) or bone marrow-derived cultured macrophages were cultured overnight in the presence or absence of LPS (100 ng/ml). Cells were lysed in 1% digitonin buffer, immunoprecipitated with control Ig or anti-MAIR-II, and immunoblotted with anti-DAP12, anti-FcRγ chain, or anti-MAIR-II. Data are representative of three independent experiments.

Figure 2. MAIR-II mediates cytokine production from *DAP12-/-* as well as WT peritoneal macrophages.

Macrophages from the spleen and PEC derived from WT and *DAP12-/-* mice were stimulated with plate-coated $F(ab')_2$ fragments of anti-MAIR-II or isotype-matched control Ig for 68 h and 24 h, respectively. Culture supernatants were harvested, and

TNF-α and IL-6 concentrations were measured by ELISA. Data are representative of three independent experiments.

Figure 3. Both DAP12 and FcRγ chain are involved in MAIR-II-mediated signaling in peritoneal macrophages

Peritoneal macrophages from mice indicated were stimulated or not with plate-coated F(ab)'$_2$ fragments 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 representative of five independent experiments.

Figure 4. LPS has additive effects on MAIR-II-mediated inflammatory cytokine secretion from peritoneal macrophages

(A) 3 x 10^6 peritoneal macrophages from WT mice were stimulated or not with LPS (100 ng/ml) for 48 hrs, lysed, and immunoblotted with anti-DAP12 or anti-FcRγ chain (left). Relative density of each signal was measured by densitometry (right). (B) PEC

from mice indicated were cultured in the presence or absence of LPS (100 ng/ml) for 48 hrs and then stained with Alexa 647-conjugated F(ab)'$_2$ fragments of anti-MAIR-II (open histogram) or isotype-matched control Ig (shaded histogram), together with anti-CD11b and anti-F4/80. CD11b$^+$ and F4/80$^+$ cells were analyzed by flow cytometry. (C) Peritoneal macrophages from mice indicated were cultured overnight in the presence of LPS (100 ng/ml) and then stimulated with plate-coated F(ab)'$_2$ fragments of anti-MAIR-II or isotype-matched control Ig for 24 hrs. Culture supernatants were harvested and TNF-α and IL-6 concentrations were measured by ELISA. Data are representative of four to five experiments.

Figure 5. MAIR-II associates with the FcRγ chain or DAP12 homodimers

RBL-2H3 that constitutively expresses FcRγ chain was transfected with a MAIR-II-IRES-GFP construct together with Flag-tagged *DAP12* in a retrovirus vector. Two days after infection, MAIR-II expressing cells were sorted, based on GFP expression, lysed and immunoprecipitated with control Ig, anti-Flag, or anti-FcRγ chain, and immunoblotted with anti-MAIR-II, anti-Flag, or anti-FcRγ chain.

Figure 6. The lysine residue of MAIR-II is involved in association of FcRγ chain as well as DAP12 homodimers

2B4 and BW5147 transfectants stably expressing Flag-tagged DAP12 and Flag-tagged FcRγ chain, respectively, were transfected with WT or mutated (K195A) MAIR-II in a retrovirus vector. Two days after infection, cells were stained with Alexa 647-conjugated anti-MAIR-II and biotin-conjugated anti-Flag mAbs, followed by FITC-conjugated streptavidin, and analyzed by flow cytometry (**A**). These cells were also lysed, immunoprecipitated with anti-MAIR-II, and immunoblotted with anti-Flag or anti-MAIR-II (**B**).

Figure 1

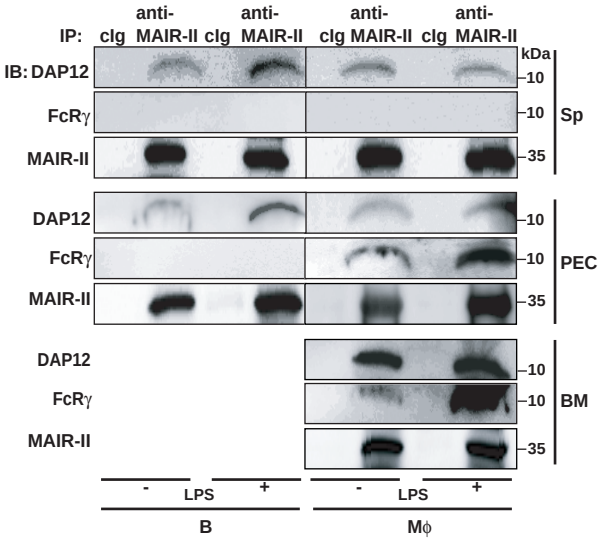

IP:
cIg
anti-MAIR-II
cIg
anti-MAIR-II
cIg
anti-MAIR-II
cIg
anti-MAIR-II
kDa
IB: DAP12
FcRγ
MAIR-II
10
10
35
Sp
DAP12
FcRγ
MAIR-II
10
10
35
PEC
DAP12
FcRγ
MAIR-II
10
10
35
BM
-
LPS
+
-
LPS
+
B
Mϕ

Figure 2

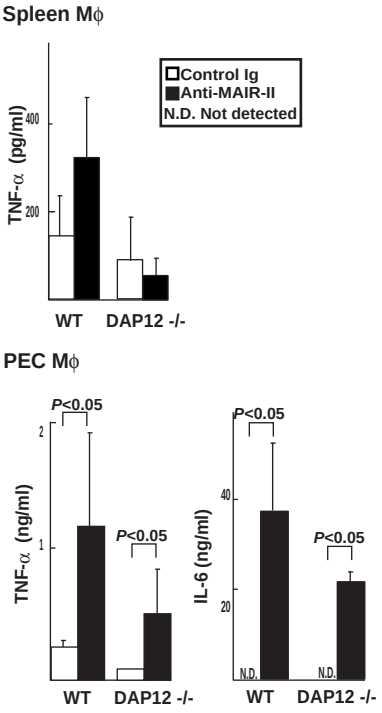

Spleen Mϕ
Control Ig
Anti-MAIR-II
N.D. Not detected
TNF-α (pg/ml)
400
200
WT
DAP12 -/-
PEC Mϕ
P<0.05
P<0.05
TNF-α (ng/ml)
2
1
WT
DAP12 -/-
P<0.05
P<0.05
IL-6 (ng/ml)
40
20
N.D.
N.D.
WT
DAP12 -/-

Figure 3

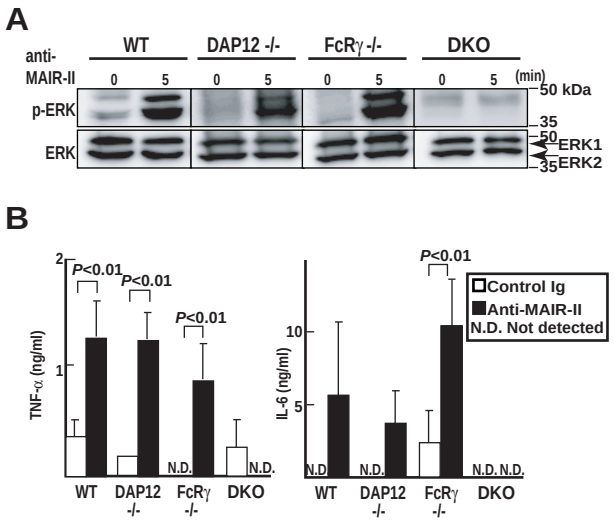

A
anti-
MAIR-II
WT
DAP12 -/-
FcRγ -/-
DKO
0
5
(min)
50 kDa
p-ERK
35
ERK
50
ERK1
35
ERK2
B
TNF-α (ng/ml)
P<0.01
P<0.01
P<0.01
N.D.
N.D.
WT
DAP12 -/-
FcRγ -/-
DKO
IL-6 (ng/ml)
P<0.01
Control Ig
Anti-MAIR-II
N.D. Not detected
N.D.
N.D.
N.D. N.D.

Figure 4

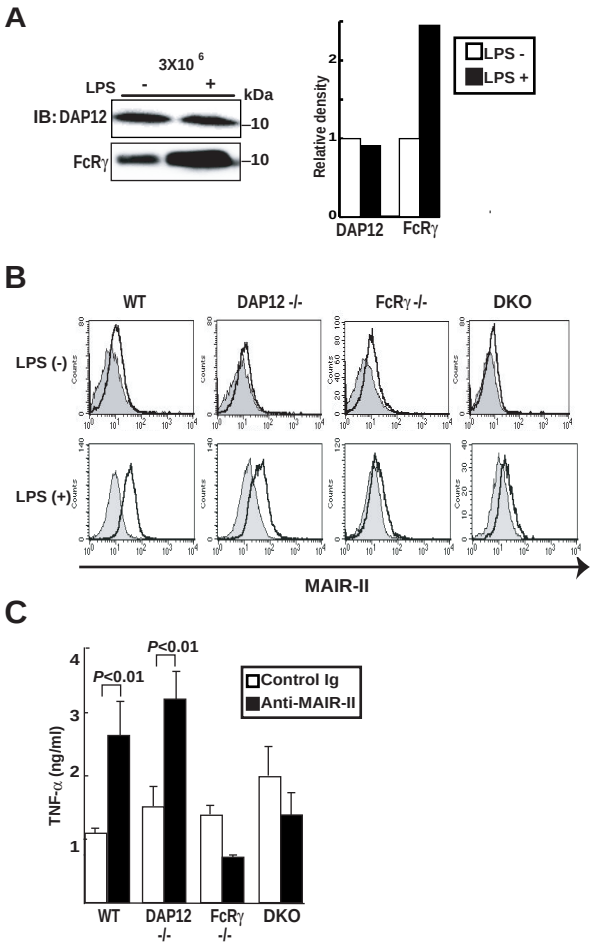

A
3X10 6
LPS - +
kDa
IB: DAP12
10
FcRγ
10
Relative density
LPS -
LPS +
DAP12
FcRγ
B
WT
DAP12 -/-
FcRγ -/-
DKO
LPS (-)
LPS (+)
MAIR-II
C
P<0.01
P<0.01
Control Ig
Anti-MAIR-II
TNF-α (ng/ml)
WT
DAP12 -/-
FcRγ -/-
DKO

Figure 5

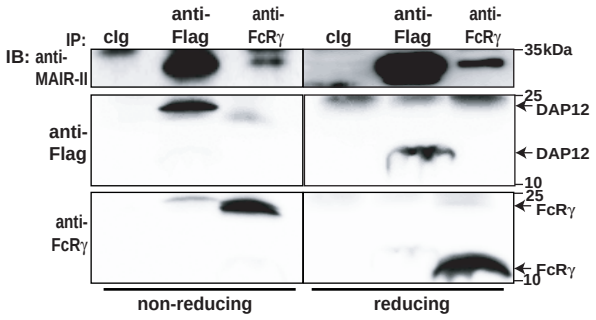
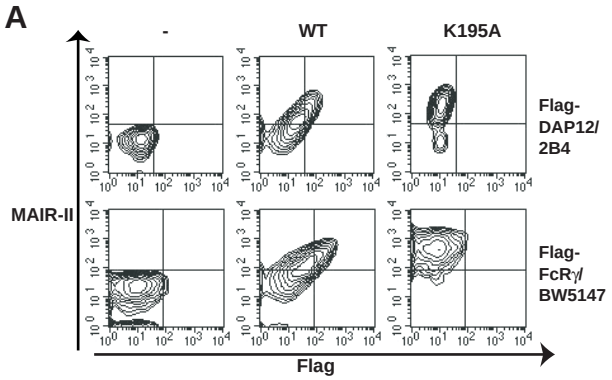
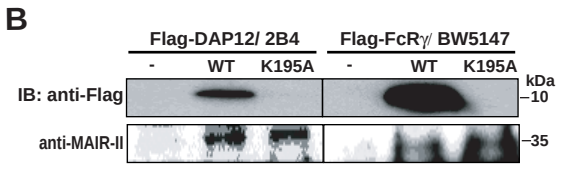

IP:
cIg
anti-Flag
anti-FcRγ
cIg
anti-Flag
anti-FcRγ
IB: anti-MAIR-II
35kDa
anti-Flag
25
DAP12
DAP12
10
anti-FcRγ
25
FcRγ
FcRγ
10
non-reducing
reducing

Figure 6


A
-
WT
K195A
Flag-
DAP12/
2B4
MAIR-II
Flag-
FcRγ/
BW5147
Flag


B
Flag-DAP12/ 2B4
Flag-FcRγ/ BW5147
-
WT
K195A
-
WT
K195A
IB: anti-Flag
anti-MAIR-II
kDa
10
35