The Journal of Immunology

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J Immunol 2006; 177:2051-2055; ;
doi: 10.4049/jimmunol.177.4.2051
http://www.jimmunol.org/content/177/4/2051

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Inhibition of TLR and FcR Responses in Macrophages by Triggering Receptor Expressed on Myeloid Cells (TREM)-2 and DAP12[1]

Jessica A. Hamerman, Jessica R. Jarjoura,* Mary Beth Humphrey,‡§ Mary C. Nakamura,‡§ William E. Seaman,‡§ and Lewis L. Lanier[2]*†*

***DAP12 is an ITAM-containing adapter that associates with receptors in myeloid and NK cells. DAP12-associated receptors can give activation signals leading to cytokine production; however, in some situations, DAP12 inhibits cytokine production stimulated through TLRs and FcRs. Here we show that Triggering Receptor Expressed on Myeloid cells (TREM)-2 is responsible for the DAP12-mediated inhibition in mouse macrophages. A chimeric receptor composed of the extracellular domain of TREM-2 and the cytoplasmic domain of DAP12 inhibited the TLR- and FcR-induced TNF production of DAP12-deficient macrophages, whereas a TREM-1 chimera did not. In wild-type macrophages, TREM-2 knockdown increased TLR-induced TNF production. A TREM-2 Fc fusion protein bound to macrophages, indicating that macrophages express a TREM-2 ligand. Thus, the interaction of TREM-2 and its ligand results in an inhibitory signal that can reduce the inflammatory response. The Journal of Immunology,* 2006, 177: 2051–2055.**

DAP12 is a transmembrane-anchored signaling adapter with a minimal extracellular domain and an ITAM within its cytoplasmic domain. An aspartic acid in the DAP12 transmembrane allows it to associate with receptors expressing positively charged amino acids in their transmembranes (1). A variety of receptors in myeloid and NK cells associate with and signal through DAP12 (2). DAP12 signaling is mediated by the phosphorylation of tyrosines in its ITAM. These phosphotyrosines recruit syk and/or Zap70, which initiate downstream signaling pathways. In NK cells, DAP12 signaling results in cytokine production and cytotoxicity, whereas in macrophages DAP12 can cause the production of inflammatory cytokines.

Although in some circumstances cross-linking of DAP12-associated receptors on macrophages can initiate cytokine production, under different conditions DAP12 inhibits the secretion of these same cytokines when they are induced by TLRs or FcRs (3). This dual functionality of DAP12 was revealed by the finding that TLR- or FcR-mediated activation of DAP12-deficient bone marrow-derived macrophages resulted in more cytokine secretion than did activation of wild-type macrophages. The inhibitory function of DAP12 in macrophages requires the tyrosines within the ITAM and presumably involves the syk tyrosine kinase, as syk-deficient bone marrow-derived macrophages also exhibit enhanced cytokine production in response to TLR-induced activation. Therefore, the initial steps in the signal transduction cascade for activation and inhibition of cytokine production mediated by DAP12 are similar.

Critical to understanding DAP12's inhibitory function is whether an associated receptor is required to inhibit TLR-induced cytokine production. It is possible that DAP12, regardless of the receptor with which it is associated, can give a constitutive inhibitory signal. Alternatively, a specific DAP12-paired receptor may be required. DAP12-associated receptors in myeloid cells include: MDL-1, TREM-1, TREM-2, TREM-3, TREM-5 (CD300LB), PILRβ, MAIR-II (CD300c), SIRPβ, CD200R2, CD200R3, and CD200R4 (2). We set out to identify which, if any, DAP12-associated receptors can provide inhibitory signals in macrophages.

Materials and Methods

Mice

DAP12-deficient mice (*tyrobp*$^{-/-}$) (19) were backcrossed nine generations to C57BL/6. Protocols were approved by our Institutional Animal Care and Use Committee.

Retrovirus

The HA-DAP12 construct was generated by introducing DNA encoding amino acids 28–114 of DAP12 into the pMX-pie vector (containing an IRES-GFP) encoding a CD8 leader segment and HA tag. The HA-DAP12 TM

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Received for publication April 7, 2006. Accepted for publication June 12, 2006.

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[1] This work was supported by an Arthritis Investigator Award (to J.A.H.), National Institutes of Health Grants AI068129 (to L.L.L.), AR050038 (to M.C.N.), and CA87922 (to W.E.S.), Department of Veterans Affairs (to M.B.H. and M.C.N.), American Cancer Society (to M.C.N.), Rosalind Russell Arthritis Center (to M.B.H., M.C.N., and W.E.S.), and Abbott Scholar in Rheumatology (to M.B.H.). L.L.L is an American Cancer Society Research Professor.

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[3] Abbreviation used in this paper: TREM, triggering receptor expressed on myeloid cells.

0022-1767/06/$02.00

mutant changed an aspartic acid at position 52 to alanine. The TREM-1+DAP12 and TREM-2+DAP12 chimeras were generated by replacing the HA tag of the HA-DAP12 TM mutant with the extracellular domains of TREM-1 (aa 1–200) or TREM-2 (aa 1–169).

Macrophage culture, infection, and stimulation

Bone marrow-derived macrophages were infected with VSVg-pseudotyped retroviruses (3). Cells were stimulated with 0.01 μM CpG DNA ODN1826 (InvivoGen) or 10 μg zymosan (InVitroGen) for 4 h with 10 μg/ml Brefeldin A (Sigma-Aldrich). For FcR activation, 24-well dishes were coated with 2.4G2 (3), and cells were added to the wells with Brefeldin A for 4 h. Receptors were detected with biotinylated anti-TREM-1 (a gift from M. Colonna, Washington University, St. Louis, MO), anti-TREM-2 clone no.150 (5) or anti-HA mAb (Roche) and PE-labeled streptavidin (BD).

shRNA-mediated knockdown

Lentiviruses were generated by using pLentiLox 3.7. A nonspecific shRNA virus (15) was provided by H. Neumann (European Neuroscience Institute, Göttingen, Germany). The knockdown lentivirus, containing the TREM-2 sequence gaagcggaatgggagcaca, was as described (RNAi no.2, (5)). At day 3 of culture, 1×10^6 cells per well were plated in 6-well dishes. 10–15 μl of VSVg-pseudotyped, concentrated lentivirus and 8 μg/ml polybrene were added, and cells were centrifuged at 1024 $\times$ *g* for 1 h at RT. After 6 h, the medium was replaced. At day 6, cells were re-plated and on day 7 stimulated with TLR ligands.

TREM-2 Fc staining

0.5 μg of TREM-2 Fc or NKp30 Fc (R&D Systems) was precomplexed with 2.5 μg PE-labeled anti-human IgG Fc $F(ab')_2$ (Jackson ImmunoResearch Laboratories) for 30 min. 2×10^5 cells were stained for 1 h on ice. For blocking, 10 μg $F(ab')_2$ of control or anti-TREM-2 mAb (no. 78) was preincubated with TREM-2 Fc.

Results and Discussion

Receptor association is required for DAP12 inhibitory function

To understand whether DAP12 must associate with a receptor to inhibit TLR and FcR-induced TNF production in macrophages, we made a variant of DAP12 that cannot pair with receptors. A mutant DAP12, in which the aspartic acid in the transmembrane was mutated to an alanine (DAP12 TM mutant), was generated. This aspartic acid is required for receptor pairing, as it interacts with an arginine or lysine in the transmembrane of DAP12-pairing receptors (4). Unlike wild-type DAP12, which is retained in the cytoplasm and degraded in the absence of a pairing receptor, the DAP12 TM mutant is expressed on the cell surface in the absence of any partner (4). HA-DAP12 and HA-DAP12 TM mutant were introduced into DAP12-deficient bone marrow-derived macrophages with retroviruses containing an IRES-GFP for identification of transduced cells. These cells were then stimulated with CpG DNA, which signals through TLR9, with zymosan, which signals through TLR2, or by plating on wells coated with 2.4G2, a mAb that binds FcγRII (CD32) and FcγRIII (CD16). After 4 h, TNF production was assessed by intracellular staining. DAP12 reduced TNF production of DAP12-deficient macrophages to a level similar to that seen in wild-type macrophages in response to CpG DNA, zymosan, or FcR stimulation, but the DAP12 TM mutant had no effect on TNF production (Fig. 1 and data not shown). DAP12 and the TM mutant were expressed similarly on the surface of the transduced macrophages and were functional in a mouse T cell reporter system (data not shown). Therefore, DAP12 must associate with a receptor present in the macrophage to inhibit TLR- and FcR-induced TNF production.

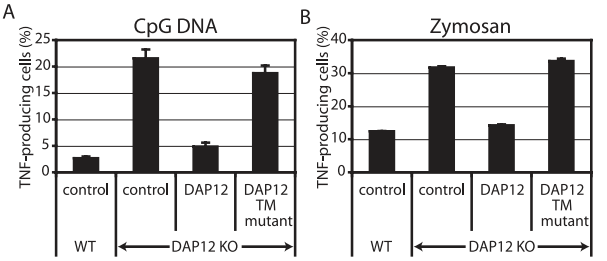

FIGURE 1. DAP12 TM mutant does not inhibit TNF production by DAP12-deficient macrophages. Wild-type macrophages were transduced with a control retrovirus and DAP12-deficient (DAP12 KO) macrophages were transduced with a control retrovirus or retroviruses (containing an IRES-GFP) encoding HA-DAP12 or HA-DAP12 TM mutant. Cells were stimulated with CpG DNA (*A*) or zymosan (*B*). Transduced cells were identified as green fluorescing cells and the percentages of TNF-producing green cells are shown. Error bars represent SD of triplicate samples. Data are representative of 4 (CpG DNA) or 6 (zymosan) independent experiments.

TREM-2 inhibits TLR- and FcR-induced TNF production

To determine which receptor is required for inhibition, we generated receptor-DAP12 chimeras for two candidate receptors, TREM-1 and TREM-2. These chimeras contained the extracellular domains of TREM-1 or TREM-2 fused to the DAP12 TM mutation to ensure that they would not associate with endogenous receptors. DAP12-deficient macrophages transduced with these chimeras were activated with CpG DNA, zymosan and anti-FcγRII/III mAb, and TNF production was assessed. For TLR and FcR activation, the TREM-2+DAP12 chimera inhibited the TNF production in DAP12-deficient macrophages (Fig. 2A-C). By contrast, the TREM-1+DAP12 chimera did not reduce cytokine production. Two other receptor-DAP12 chimeras, MAIR-II+DAP12 and PILRβ+DAP12, failed to inhibit TNF production of the DAP12-deficient macrophages (data not shown). Both MAIR-II and PILRβ are expressed on the surface of bone marrow-derived macrophages as determined by flow cytometry (data not shown).

The TREM-1 chimera was expressed very highly on the surface of transduced macrophages, whereas the untransduced macrophages did not express TREM-1 (Fig. 2*D* and data not shown). In comparison, endogenous TREM-2 was expressed on untransduced macrophages, and its surface expression was only modestly increased in macrophages transduced with the TREM-2 chimera. This suggests either that the chimera was not expressed well or that TREM-2 interacts with its ligand on the macrophages. We favor the latter interpretation, as we saw a profound functional effect of the chimera. TREM-2 may be internalized or retained intracellularly after interacting with a ligand in macrophages. This fits with a model where the macrophage expresses both the receptor and ligand required for inhibition of cytokine production (3). The amount of TREM-2 was only slightly lower in DAP12-deficient than in wild-type macrophages (Fig. 2*D*) (5). When the TREM-1+DAP12 or TREM-2+DAP12 chimeras were transduced into a mouse T cell line expressing an NFAT-lacZ reporter, both were efficiently expressed and cross-linking of the chimeras with mAbs resulted in NFAT activation, confirming that both chimeras were functional (data not shown). As TREM-2 has been shown to bind bacteria and fungi (6), the ability of the TREM-2+DAP12 chimera to inhibit FcR activation demonstrates that the inhibition of CpG and zymosan-induced TNF production

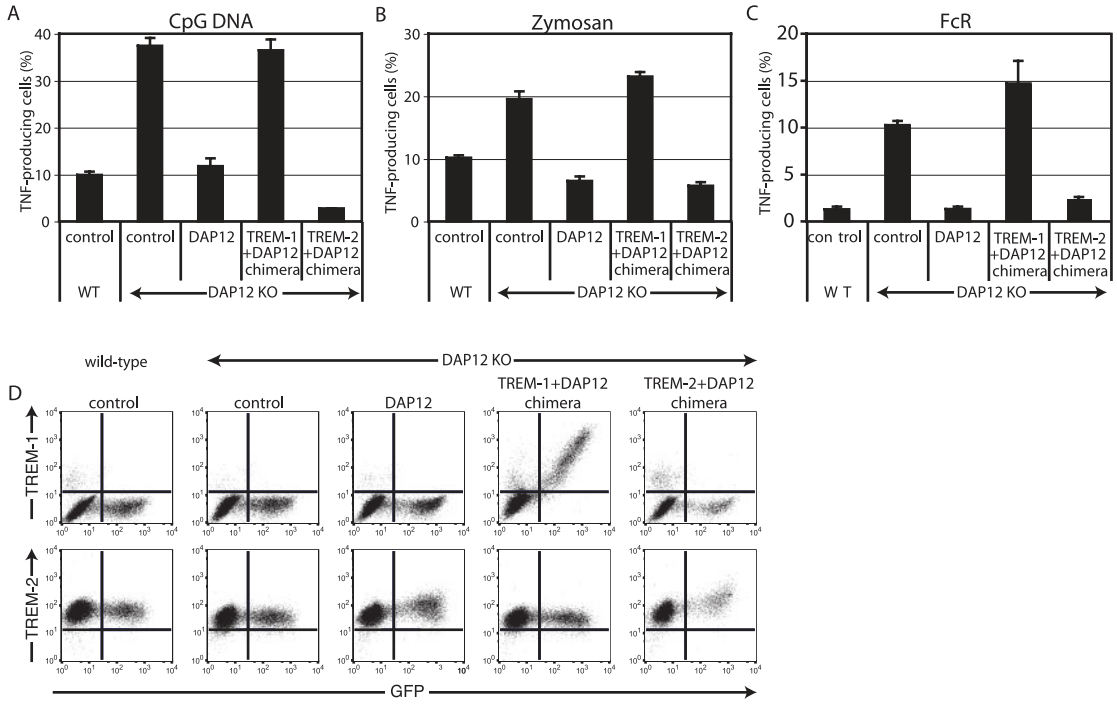

FIGURE 2. TREM-2 inhibits TNF production. Wild-type macrophages were transduced with a control retrovirus and DAP12-deficient macrophages were transduced with a control retrovirus or retroviruses encoding TREM-1+DAP12 or TREM-2+DAP12 chimera. Cells were treated with CpG DNA (*A*), zymosan (*B*) or anti-FcγRII/III mAb (*C*) and analyzed (see Fig. 1). (*D*) Expression of TREM-1 and TREM-2 in the transduced (GFP-positive) and nontransduced (GFP-negative) cells. Data are representative of four (CpG DNA), six (Zymosan), or three (FcR) independent experiments.

was not due to potential interactions between these stimuli and TREM-2.

To confirm that inhibition through the TREM-2 chimera is ITAM-dependent, we generated an ITAM-mutant TREM-2+DAP12 chimera. The ITAM mutant did not inhibit TNF production by DAP12-deficient macrophages in response to CpG DNA or zymosan (Fig. 3, *A* and *B*). In comparison with the TREM-2+DAP12 chimera, the ITAM mutant was expressed more highly on the transduced macrophages (Fig. 3*C*). A functional ITAM reduces the expression of the TREM-2+DAP12 chimeric receptor, supporting a model of ligand-induced internalization or intracellular retention of TREM-2.

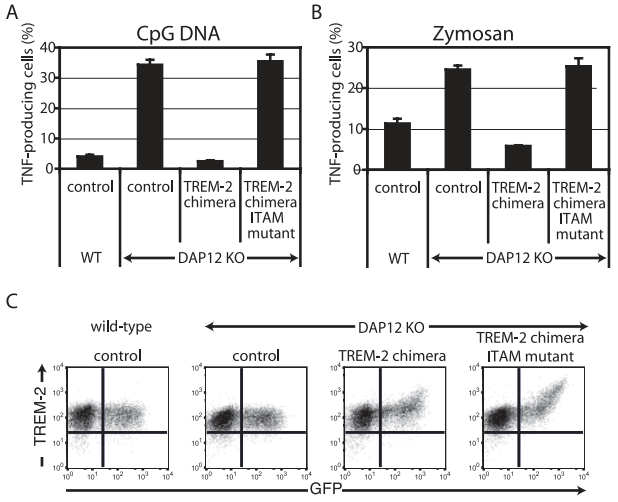

FIGURE 3. ITAM tyrosines are required for TREM-2-mediated inhibition. Wild-type macrophages were transduced with a control retrovirus and DAP12-deficient macrophages were transduced with a control retrovirus or a retrovirus encoding a TREM-2+DAP12 chimera or a TREM-2+DAP12 ITAM mutant chimera. Cells were treated with CpG DNA (*A*) or zymosan (*B*) and analyzed (see Fig. 1). *C*, Expression of TREM-2 on transduced and nontransduced macrophages. Data are representative of three independent experiments.

We also tested whether TREM-2 inhibits TLR-responses in wild-type macrophages. TREM-2 expression was reduced by using a shRNA targeting TREM-2 (5). Four days after transduction, the peak effect of knockdown was achieved and surface expression of TREM-2 was reduced 40–60% (Fig. 4*A*). This partial loss of TREM-2 was sufficient to double the number of TNF-producing cells in response to CpG DNA or zymosan (Fig. 4, *B* and *C*). There was no effect of expressing a nonspecific shRNA on TNF production in comparison with an empty vector control (data not shown). Together, the inhibition of TNF production in DAP12-deficient macrophages by the TREM-2+DAP12 chimera and the increase in TNF production in wild-type macrophages by TREM-2 knockdown provide strong evidence that TREM-2 is a DAP12-associated receptor responsible for inhibitory signaling.

Macrophages express a TREM-2 ligand

We hypothesized that the inhibitory effect of TREM-2 is caused by ligands on macrophages. Macrophages were stained with a TREM-2 Fc fusion protein complex. Macrophages stained with the TREM-2 Fc, but not with an irrelevant fusion protein (Fig. 5*A*). $F(ab')_2$ fragments of a TREM-2 mAb blocked the binding of the TREM-2 Fc (Fig. 5*B*). Freshly isolated resident peritoneal macrophages also stained with mAb to TREM-2 and with the TREM-2 Fc reagent (data not shown).

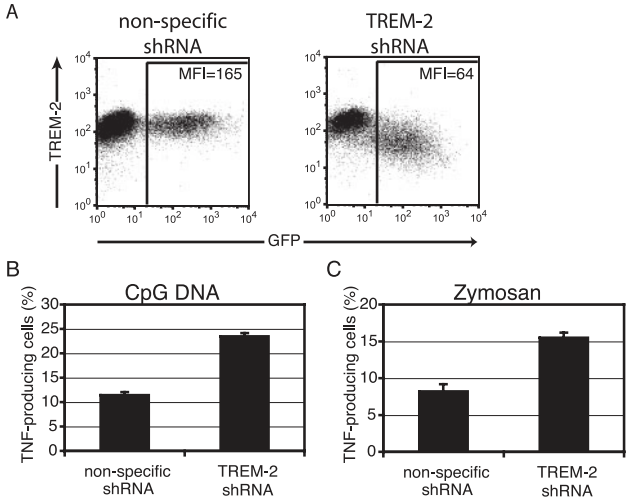

FIGURE 4. TREM-2 knockdown increases TLR-induced TNF. Wild-type macrophages were infected with a lentivirus encoding a TREM-2 shRNA (5) or a nonspecific shRNA (15). *A*, TREM-2 knockdown in GFP-expressing transduced cells. The mean fluorescence intensity (MFI) of the anti-TREM-2 mAb staining on the GFP-expressing cells is shown. Lentivirus-infected cells were treated with CpG DNA (*B*) or zymosan (*C*) and analyzed (see Fig. 1). Data are representative of five independent experiments.

Thus, macrophages express a TREM-2 ligand. These data explain the ability of TREM-2 to cause inhibition in macrophage cultures, as well as the apparent ligand-induced, ITAM-dependent internalization or intracellular retention of TREM-2 seen upon expression of the TREM-2+DAP12 chimera in macrophages.

TREM-2 is expressed in osteoclasts, macrophages, myeloid dendritic cells and microglia (7). It has sequence homology to TREM-1 and NKp44 (8). As association with DAP12 would predict, ligation of TREM-2 using mAbs induced NO production by a macrophage cell line (8) and promoted partial maturation of human immature monocyte-derived dendritic cells (9). TREM-2 signaling through DAP12 is required for efficient osteoclast development and function (10–14), and TREM-2 mAb-mediated cross-linking enhances the formation of osteoclasts in vitro (5).

In mouse microglia, mAb-induced TREM-2 cross-linking resulted in cytoskeletal rearrangements (15). This same study correlated TREM-2 expression with the ability of microglia to phagocytose apoptotic neurons-TREM-2 overexpression increased phagocytosis, whereas TREM-2 knockdown decreased phagocytosis. Of note, TREM-2 overexpression in microglia led to reduced amounts of TNF and iNOS mRNA after coculture with apoptotic neurons, whereas TREM-2 knockdown resulted in an almost two-fold increase in TNF and iNOS mRNA (15). These data suggest that TREM-2 signaling positively regulates phagocytosis, but negatively regulates inflammatory responses in microglia, as we have shown in macrophages. Apoptotic neurons may express TREM-2 ligands, which can give either activating or inhibitory signals. Alternatively, the microglia may themselves express TREM-2 ligands, similar to macrophages. Other DAP12-associated receptors have been implicated in inhibition of TLR responses in IFN-producing cells (IPC). Signaling through Siglec-H in mouse IPC and NKp44 in human IPC inhibits CpG DNA-induced type I IFN production, and DAP12-deficient IPC secrete increased type I IFNs in response to CpG DNA (16, 17).

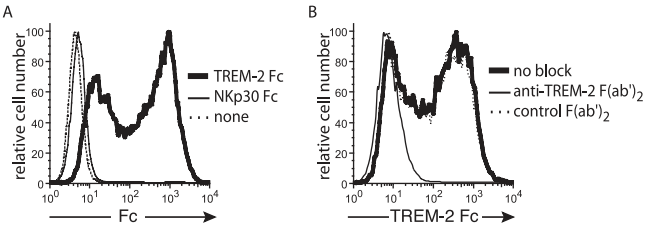

FIGURE 5. Macrophages express a TREM-2 ligand. *A*, Macrophages were stained with a TREM-2-Fc fusion protein (thick line), an irrelevant fusion protein (NKp30 Fc) (thin line), or with secondary Ab alone (dashed line). Data are representative of six independent experiments. *B*, Macrophages were stained with TREM-2 Fc as in *A* after pretreatment of the TREM-2 Fc with no blocking Ab (thick line), a $F(ab')_2$ of a control mAb (dashed line), or $F(ab')_2$ of anti-TREM-2 (thin line). Data are representative of three independent experiments.

In humans, mutation of either DAP12 or TREM-2 results in Nasu-Hakola syndrome, a rare disease characterized by bone cysts and presenile dementia (10, 18). This phenotype implies a role for the TREM-2-DAP12 receptor complex in osteoclast and microglia differentiation and function. Because TREM-2 and DAP12 have been implicated in the activation of myeloid cells (8, 9), one might predict immunodeficiencies in humans lacking these molecules. In contrast, although these patients live 40–50 years, they do not have increased susceptibility to infections. Thus, the predominant role of DAP12 and its associated receptors, such as TREM-2, might be in the inhibition, rather the activation, of the innate immune system.

Acknowledgments

We thank Michael McManus, Amy Heidersbach, and the Diabetes/Sandler Center Lentiviral Core for lentivirus stocks and discussions, and Shigenari Hayashi and Steven Spusta for valuable reagents.

Disclosures

The authors have no financial conflict of interest.

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