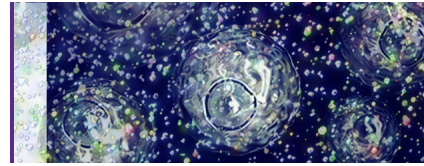


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Cutting Edge: TREM-2 Attenuates Macrophage Activation¹

Isaiah R. Turnbull, Susan Gilfillan, Marina Cella, Taiki Aoshi, Mark Miller, Laura Piccio, Maristela Hernandez, and Marco Colonna²

The triggering receptor expressed on myeloid cells 2 (TREM-2) delivers intracellular signals through the adaptor DAP12 to regulate myeloid cell function both within and outside the immune system. The role of TREM-2 in immunity has been obscured by the failure to detect expression of the TREM-2 protein in vivo. In this study, we show that TREM-2 is expressed on macrophages infiltrating the tissues from the circulation and that alternative activation with IL-4 can induce TREM-2. TREM-2 expression is abrogated by macrophage maturation with LPS of IFN- γ . Using TREM-2^{-/-} mice, we find that TREM-2 functions to inhibit cytokine production by macrophages in response to the TLR ligands LPS, zymosan, and CpG. Furthermore, we find that TREM-2 completely accounts for the increased cytokine production previously reported by DAP12^{-/-} macrophages. Taken together, these data show that TREM-2 is expressed on newly differentiated and alternatively activated macrophages and functions to restrain macrophage activation. The Journal of Immunology, 2006, 177: 3520–3524.

The triggering receptor expressed on myeloid cells-2 (TREM-2)³ is a cell surface receptor and a member of the Ig superfamily with one V-type extracellular domain, a charged transmembrane domain, and short cytoplasmic tail (1, 2). TREM-2 contributes to myeloid cell function within and outside the immune system. The nonimmune function of TREM-2 is demonstrated by the presentation of people deficient for TREM-2. These patients develop Nasu-Hakola disease, which is characterized by bone cysts and demyelinating lesions in the CNS that ultimately result in fatal presenile dementia (3). A role for TREM-2 in the immune system is evidenced by the expression of TREM-2 on in vitro derived macrophages (4) and dendritic cells (1) and by the identification of TREM-2 transcript in macrophage cell lines (2). Additionally, a TREM-2 transcript has been detected in the lungs during experimental mycobacterial infection (5). However, the cell type expressing TREM-2 and its function in the immune system is

unknown. A solution to this problem has been hampered by the failure to detect the TREM-2 protein in the immune system in vivo.

TREM-2 associates with the adaptor DAP12, which is required for surface expression and signaling by TREM-2 (2). DAP12 mediates downstream signaling through a cytoplasmic ITAM domain, which can recruit Syk and activate PI3K, phospholipase C, and Vav signaling cascades (6). In NK cells, DAP12-associated receptors are activating receptors and contribute to target cell lysis (7). In contrast to the activating role of DAP12 in NK cells, Hamerman et al. demonstrated that DAP12^{-/-} macrophages have increased cytokine production as compared with wild type (WT) (8), suggesting an inhibitory role for DAP12. Additional studies have validated this observation in type I IFN-producing cells mediated by NKp44 or Siglec-H (9). Several studies have addressed TREM-2-DAP12 signaling. The cross-linking of TREM-2 transfected into MT2 cells induced NO release (2), and in vitro cross-linking of TREM-2 on dendritic cells caused partial maturation (1). Additionally, blockade of TREM-2 inhibited dendritic cell-mediated activation of NK cells (10). These data suggest that TREM-2 has an activating role. In contrast, in microglial cell culture the knockdown of TREM-2 with short hairpin RNA led to decreased phagocytosis but increased levels of proinflammatory transcripts, suggesting that TREM-2 may inhibit the inflammation (11). Although TREM-2 is reported to be expressed by in vitro cultured macrophages, the role of TREM-2 in macrophage function is unknown.

Herein, we report that TREM-2 is expressed on macrophages that have been recruited to the peripheral tissues but not on myeloid progenitors, circulating cells, or tissue-resident macrophages. We also find that TREM-2 is induced on resident peritoneal cells by IL-4. We have generated TREM-2^{-/-} mice and find that on these cells TREM-2 functions to inhibit cytokine production in response to microbial products. Further, we find no difference between TREM-2^{-/-} and DAP12^{-/-} macrophage cytokine production, demonstrating that TREM-2 is the receptor operative in the increased macrophage cytokine production previously reported for DAP12^{-/-} cells (8).

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110

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² Address correspondence and reprint requests to Dr. Marco Colonna, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110. E-mail address: mcolonna@pathology.wustl.edu

³ Abbreviations used in this paper: TREM-2, triggering receptor expressed on myeloid cells-2; BMDM, bone marrow-derived macrophage; MCSF, macrophage CSF; WT, wild type.

Materials and Methods

Mice

All animal studies were approved by the Washington University Animal Studies Committee (St. Louis, MO). B6.129P2-*TYROBP*^{tm1Tdk} mice (hereafter referred to as DAP12^{-/-}) were generously provided by T. Takai (Tohoku University, Sendai, Japan) (12) and backcrossed to C57BL/6, with background determined by simple sequence length polymorphism typing. STAT6 knockout mice (strain B6.129S2(C)-*Stat6*^{tm1GrwJ}) were from The Jackson Laboratory.

Generation of *TREM-2*^{-/-} mice

The *TREM-2* targeting construct was designed to delete a portion of the transmembrane and cytoplasmic domains encoded by exons 3 and 4. Two genomic fragments (4.6 and 5.4 kb) flanking this region were amplified by PCR and cloned into the targeting vector pMC1neo. The targeting construct was transfected into E14.1 embryonic stem cells. Correctly targeted clones were injected into C57BL/6 blastocysts, and chimeras were bred to transgenic mice expressing Cre under the CMV promoter to delete the neomycin resistance gene, resulting in the strain of mice designated as B6.129P2-*TREM2*^{tm1cln} (hereafter referred to as *TREM-2*^{-/-}). These mice were backcrossed to C57BL/6 until >99% of the loci were derived from the C57BL/6 strain by simple sequence length polymorphism typing. We find that the *TREM-2* mutation is transmitted with the expected Mendelian ratios and that the mice have no gross abnormalities.

Ab generation

A Wistar rat was immunized with recombinant protein consisting of the extracellular domain of *TREM-2* fused to the human Ig constant domain. The spleen was harvested and fused with Sp2/0 myeloma cells. Ab-producing clones were selected by ELISA and screened against Jurkat cells that had been transiently transfected with *TREM-2*.

Macrophage characterization

To elicit primary macrophages, mice were treated with 1.5 ml of 2% thioglycollate medium i.p. injected; cells were isolated by peritoneal lavage. To generate bone marrow-derived macrophages (BMDM), total bone marrow was cultured in DMEM supplemented with 10% bovine calf serum, 5% horse serum, and 6 ng/ml recombinant human CSF-1 (R&D Systems). Cells were cultured for 5–6 days, and adherent cells were detached with 1 mM EDTA in PBS. Cells were stained with commercially available Abs: anti-CD11b, anti-CD40, anti-GR1 (BD Pharmingen), and F4/80 (Caltag Laboratories).

Allergen-induced pulmonary inflammation

BALB/c mice were sensitized to OVA by immunization on days 1 and 14 with 20 μ g of OVA mixed with 2.25 mg of aluminum hydroxide (alum). On day 17, mice were challenged intranasally with 20 μ l of 1% OVA, and 48 h later the recruited cells were isolated by bronchioalveolar lavage.

TLR stimulations and cytokine determination

BMDM were replated and allowed to adhere for 4 h at 37°C, and then TLR agonists were added. LPS (*Salmonella abortus-equi*), zymosan (*Saccharomyces cerevisiae*), and CpG 1826 DNA were from Sigma-Aldrich. Cell culture supernatant were recovered 24 h after stimulation (or as indicated). TNF- α and IL-6 were assayed by cytometric bead array (BD Biosciences mouse inflammation kit).

Results and Discussion

TREM-2 is expressed on infiltrating (but not resident) macrophages

To establish the distribution of *TREM-2*, we generated a mAb specific for murine *TREM-2*. mAb 178 is specific for *TREM-2*, because it bound to cells cotransfected with *TREM-2* but not to control cells transfected with the related receptor *TREM-1* (data not shown). We were unable to find surface expression of *TREM-2* on peripheral blood cells or in the bone marrow. We also measured *TREM-2* in peripheral organs including liver, spleen, and lung by flow cytometry; we found no expression of *TREM-2* in any tissue studied.

We then measured *TREM-2* expression on peritoneal macrophages. Mice were injected with thioglycollate, and peritoneal exudate cells were harvested either before or 1, 2, or 3 days after injection. We found that *TREM-2* is not expressed on res-

ident peritoneal macrophages. However, macrophages recruited to the peritoneum by thioglycollate expressed *TREM-2*, and the fraction of *TREM-2*⁺ cells increased over time after the injection of thioglycollate (Fig. 1A). These cells were characterized for other markers expressed on infiltrating macrophages. The *TREM-2*⁺ cells uniformly expressed the macrophage markers CD11b, F4/80, and CD115.

Based on the fraction of cells expressing *TREM-2* (19% by 24 h), the effect we observe cannot be due simply to the induction of *TREM-2* on the resident cells, because even if *TREM-2* was induced on all resident peritoneal cells, that could not account for the fraction of *TREM-2*⁺ cells, as thioglycollate induces a >5-fold increase in the number of macrophages recovered from the peritoneum. Although we cannot exclude the possibility that the injection of thioglycollate induces expression of *TREM-2* on resident cells, *TREM-2* must also be expressed on recently infiltrated cells.

TREM-2 is expressed during allergen-induced pulmonary inflammation

Considering that *TREM-2* is expressed on macrophages recruited to the peritoneum by thioglycollate, we hypothesized that *TREM-2* may be generally expressed on macrophages that are infiltrating tissue. We tested this hypothesis in a second model involving macrophage recruitment during allergen-induced pulmonary inflammation. WT mice were sensitized to OVA by immunization in the presence of alum. We then measured the expression of *TREM-2* on macrophages recruited to the air space after intranasal challenge. Consistent with prior studies (13), challenge with OVA caused significant increase in macrophages in the airspaces of sensitized mice. We found that the cells that were recruited to the alveolar spaces of the lung after challenge in the sensitized mice expressed *TREM-2* (Fig. 1B). Importantly, we noted that the *TREM-2*⁺ cells were also

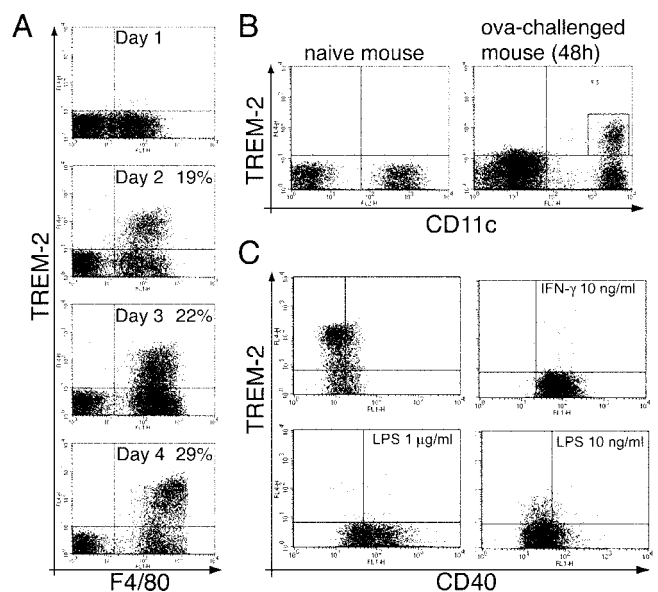


FIGURE 1. *TREM-2* is expressed on macrophages. *A*, Peritoneal macrophages were isolated before (day 1) or after (days 2–4) injection of thioglycollate. Thioglycollate-recruited macrophages express *TREM-2*. *B*, Mice were sensitized to OVA by immunization and then challenged intranasally with OVA. Macrophages recruited to the alveolar space in the sensitized mouse express *TREM-2*. *C*, The murine macrophage cell line RAW264 express *TREM-2*, which is down-regulated after overnight activation by IFN- γ or LPS at indicated doses. *D*, *TREM-2* is highly expressed on BMDM from WT mice.

CD11c⁺CD11b^{low} (data not shown). These markers are specific for alveolar macrophages (13), suggesting that TREM-2 is induced on resident macrophages.

In pursuit of these studies we have also assayed TREM-2 expression during several other models of immune function. We have assayed the lymph node cells after viral infection with HSV or EBV and splenocytes after infection with murine CMV; we have also assayed the draining lymph nodes after peripheral injection of mycobacteria or CFA plus OVA. In none of these conditions was TREM-2 observed. Consistent with prior studies, we found that TREM-2 is expressed on the murine macrophage cells line RAW264 (4). Additionally, expression was rapidly down-regulated in response to activation with LPS or IFN- γ (Fig. 1C). This activation-induced down-regulation may explain our failure to detect TREM-2 in the other models. In all of these experimental systems microbial products are present to activate macrophages, which would rapidly down-regulate TREM-2.

These data show that TREM-2 is expressed on macrophages recruited to the peritoneum (by thioglycollate) or into the air spaces (by OVA in sensitized mice). Importantly, we did not find TREM-2 expression in the blood or bone marrow after administration of thioglycollate, suggesting that the expression of TREM-2 is not induced centrally (e.g., in the bone-marrow) but instead is induced locally during the transition from the circulation to the extravascular space or by cytokine-mediated activation.

Based on the expression of TREM-2 during allergen induced pulmonary inflammation, we hypothesized that alternative macrophage activation by IL-4 or IL-13 may up-regulate TREM-2. To test this hypothesis, we isolated resident peritoneal cells, which do not express TREM-2 (see Fig. 1A) from WT mice. These cells were cultured with or without IL-4 for 48 h. We found that alternative activation of resident peritoneal macrophages with IL-4 induced TREM-2 (Fig. 2A).

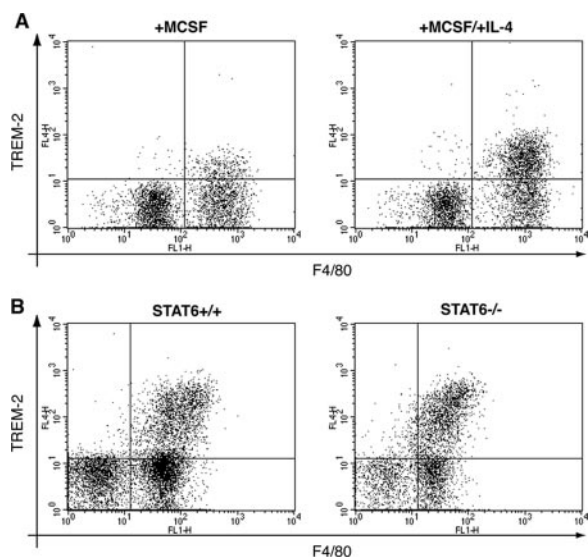


FIGURE 2. Alternative activation is sufficient but not necessary for TREM-2 expression. *A*, Resident peritoneal cells were isolated from WT mice and cultured for 48 h in the presence of MCSF with or without IL-4. Culture with IL-4 induced TREM-2 expression of resident peritoneal macrophages. *B*, Thioglycollate-recruited peritoneal cells were isolated from WT and STAT6^{-/-} mice, and TREM-2 expression was assayed by FACS. TREM-2 is expressed independently of STAT6 expression.

To determine whether the expression of TREM-2 that we had previously observed on thioglycollate recruited macrophages was the result of endogenous type-II cytokines, we measured TREM-2 expression on peritoneal exudate cells recruited to thioglycollate in WT vs STAT6^{-/-} mice. STAT6 is required for the signaling of both IL-4 and IL-13 (14), the two cytokines that have been reported to induce alternative activation of macrophages (15). We find that TREM-2 was well expressed on thioglycollate-elicited cells independently of STAT6, demonstrating that type II activation is sufficient but not necessary for TREM-2 expression by macrophages (Fig. 2B).

Generation of TREM-2^{-/-} mice and expression of TREM-2 on bone marrow-derived macrophages (BMDM)

To address the biological function of TREM-2, we established TREM-2 knockout mice (Fig. 3A). We generated BMDM from WT and TREM-2^{-/-} mice and measured the expression of TREM-2 and other markers of macrophage differentiation. We found that TREM-2 is expressed on WT BMDM but not on TREM-2-knockout BMDM (Fig. 3B). We found no difference in CD11b, F4/80, CD40, or GR-1 expression levels when comparing WT and TREM-2^{-/-} cells (data not shown). Whereas Humphery et al. recently reported that TREM-2 expression is induced during *in vitro* osteoclastogenesis induced by culture BMDM with macrophage CSF (MCSF) and the receptor activator of NF- κ B ligand (4), we find that differentiation of macrophages in MCSF alone leads to expression of TREM-2.

TREM-2 skews cytokine production in response to TLR agonists

To determine the function of TREM-2 in macrophages, WT and TREM-2^{-/-} BMDM were treated with the TLR agonists zymosan (TLR2/6), LPS (TLR4), CpG DNA (TLR9), poly(I:C) (TLR3), and imiquimod (TLR7/8) (16). After 24 h, cytokines levels were measured. We found that TREM-2^{-/-} macrophages produce increased TNF- α in response to LPS, zymosan, and CpG (Fig. 4A). However, there was no difference when the cells were stimulated with imiquimod or poly(I:C)

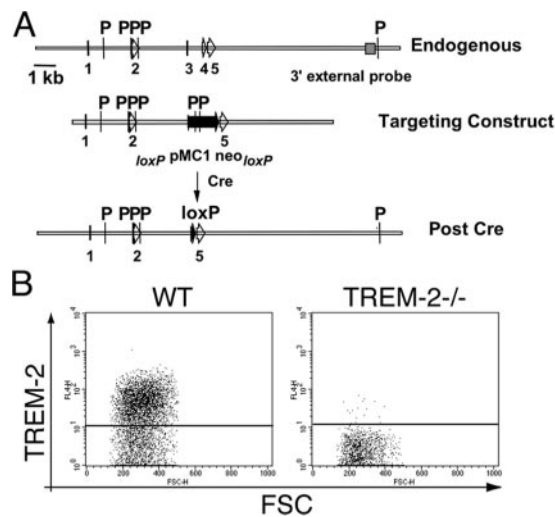


FIGURE 3. Generation of TREM-2^{-/-} mice. *A*, The TREM-2 genomic locus was deleted by homologous recombination with a construct eliminating portions of exons 3 and 4 (P represents a PST1 restriction site). *B*, Macrophages were derived from WT or TREM2^{-/-} and stained with mAb 178. TREM-2 was well expressed on WT cells but absent on the TREM2^{-/-} cells.

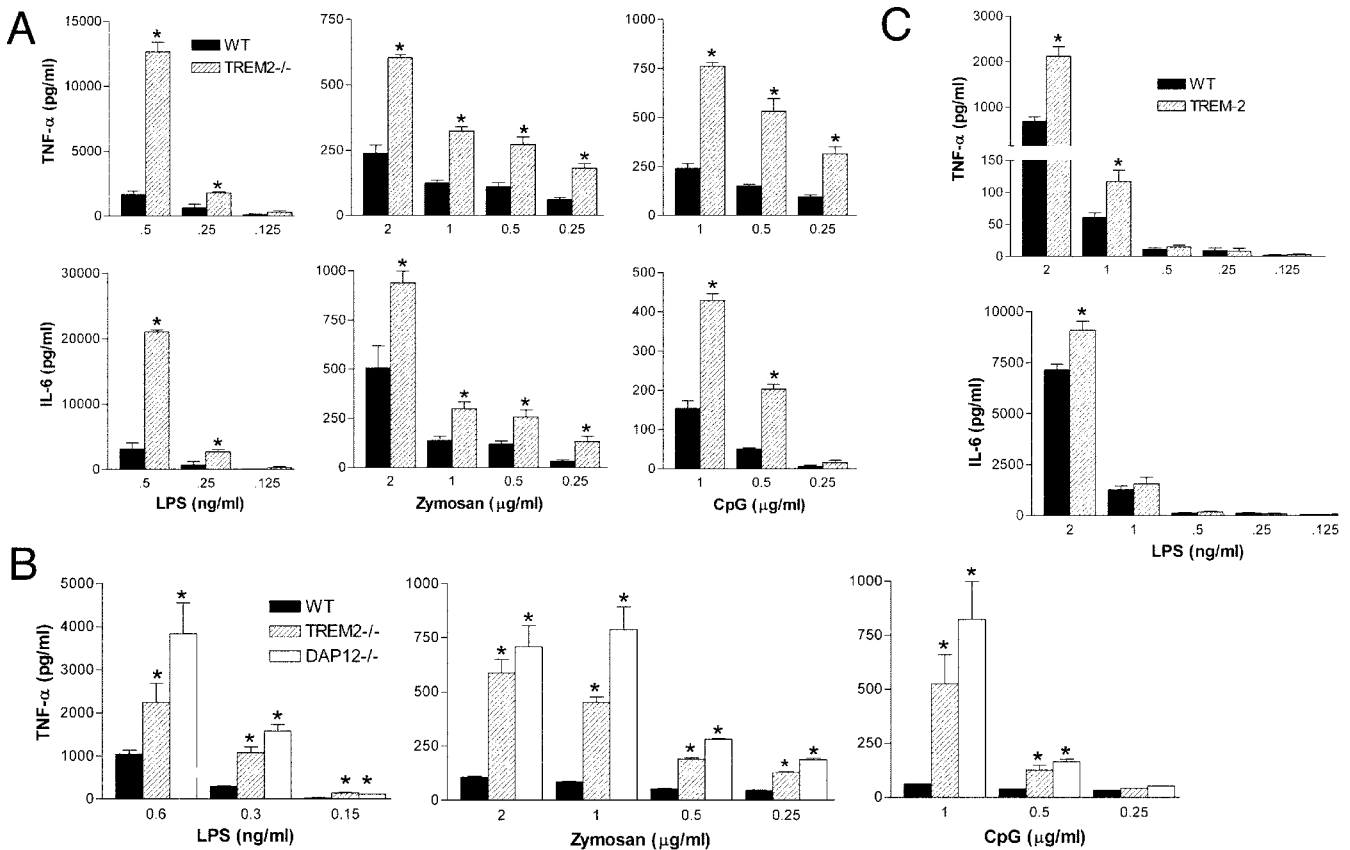


FIGURE 4. TREM-2 attenuates macrophage cytokine production. Macrophages were stimulated with the indicated concentration of LPS, zymosan, or CpG, and the levels of TNF- α and IL-6 in the supernatant were measured 24 h later. *A*, WT vs TREM-2^{-/-} BMDM. TREM-2^{-/-} cells produce increased cytokine as compared with WT cells. *, $p < 0.05$; data are representative of at least three independent experiments. *B*, TREM-2 accounts for the decreased cytokine response by DAP12^{-/-} cells. WT, TREM-2, and DAP12^{-/-} BMDM were stimulated as indicated. Both WT and DAP12^{-/-} cells produced increased cytokine as compared with WT. There was no difference between TREM-2 and DAP12^{-/-} cells. *, $p < 0.05$; data are representative of at least two independent experiments. *C*, TREM-2 inhibits the primary macrophage response to LPS. Peritoneal macrophages were isolated from WT or TREM-2^{-/-} mice and stimulated with LPS at the indicated doses for 24 h. TREM-2^{-/-} cells produced increased TNF- α and IL-6 in response to LPS. *, $p < 0.05$.

(data not shown). We also find a significant but more modest increase in IL-6 production in response to LPS, zymosan, and CpG in the absence of TREM-2 (Fig. 4*A*). We found no reproducible difference between WT and TREM-2^{-/-} cells with regard to IL-10 production, although there was a trend toward increased IL-10 in the TREM-2^{-/-} cells (data not shown); we found no IL-12p70 or IFN- γ produced by the cells in our study.

TREM-2 completely accounts for the skewed cytokine production seen in DAP12^{-/-} cells

TREM-2 signals through the adaptor DAP12 (1), and previous studies have shown that DAP12^{-/-} cells produce increased cytokines in response to TLR agonists. To determine whether TREM-2 was the receptor accounting for these effects, we compared TNF- α production by WT, TREM2^{-/-}, and DAP12^{-/-} macrophages. We found no difference between TREM2^{-/-} and DAP12^{-/-} macrophages in response to zymosan, CpG, and LPS (Fig. 4*B*), demonstrating that TREM-2 alone accounts for the inhibition of TLR activation previously reported.

TREM-2 inhibits the response to LPS by primary macrophages

To establish the role of TREM-2 on primary macrophages, we measured cytokines produced by peritoneal macrophages in re-

sponse to TLR agonists. Cells were recruited by the injection of thioglycollate and assayed for cytokine production in response to LPS and zymosan. We found that, consistent with results from BMDM, peritoneal macrophages from TREM-2^{-/-} mice produced increased TNF- α and IL-6 in response to LPS (Fig. 4*C*), but there was no difference in response to zymosan (data not shown). The phenotype observed in primary cells is thus much less dramatic than that of BMDM derived in vitro. In contrast to the virtually uniform expression of TREM-2 observed on BMDM, only one of three peritoneal macrophages express TREM-2 (Fig. 1, *A* vs *D*). This likely accounts for the increased parity in the responses of WT and TREM-2^{-/-} peritoneal cells.

Concluding remarks

In this study, we found that TREM-2 is expressed by recently differentiated macrophages and that it functions to attenuate the macrophage response to microbial products. We also found that type II inflammation induces TREM-2, both in vivo during allergen-induced pulmonary inflammation and ex vivo by the treatment of resident peritoneal cells with IL-4. We do not find any expression of TREM-2 on other cell populations, including progenitors in the bone marrow, circulating monocytes, or tissue-resident macrophages. TREM-2 is rapidly down-regulated in response to either IFN- γ or LPS. We found that similar to DAP12^{-/-} cells, TREM-2^{-/-} macrophages

produce increased levels of inflammatory cytokines when exposed to TLR agonists. Importantly, we see no difference between DAP12^{-/-} and TREM-2^{-/-}, demonstrating that TREM-2 completely accounts for the increased cytokine response previously reported (8).

The mechanisms by which TREM-2 attenuates macrophage cytokine production (or, generally, by which the ITAM motif of DAP12 inhibits cells) remains unclear. Hamerman et al. reported increased phosphorylation of ERK by DAP12^{-/-} cells in response to LPS as compared with WT cells (8). In contrast to these results, we observe no increase of ERK activation by LPS in the TREM2^{-/-} cells (data not shown), suggesting that there may other receptors (in addition to TREM-2) contributing to the difference in MAPK activation observed when WT and DAP12^{-/-} macrophages were previously compared. Previous studies have demonstrated that PI3K can inhibit TLR activation through activation of the inhibitory apoptosis signal-regulating kinase ASK (17), and DAP12 is known to activate PI3K (6). It is possible that DAP12-mediated activation of PI3K may account for the inhibitory effects of TREM-2. In our studies we were unable to detect any defects in PI3K activity; however, this pathway merits further study.

What is the origin of the TREM-2⁺ cells? In the context of type II inflammatory responses, alternative activation by IL-4 or IL-13 likely up-regulates TREM-2 on resident cells such as alveolar macrophages. We also found that TREM-2 is expressed on infiltrating macrophages independent of alternative activation. Transmigration has been shown to induce differentiation of monocytes, with circulating cells becoming macrophages as they transmigrate across an endothelial layer (18), which could provide a stimulus for induction of TREM-2. Overall, we conclude that TREM-2 is expressed in two contexts: 1) in the presence of type-II inflammation TREM-2 is induced on tissue resident macrophages; and 2) as monocytes leave the circulation and differentiate into macrophages, TREM-2 is induced.

Disclosures

M. Colonna and Washington University have a financial interest in BioXcell, which retains the rights to TREM-2, but did not support this work.

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