Cutting Edge: TREM-2 Attenuates Macrophage Activation

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Cutting Edge: TREM-2 Attenuates Macrophage Activation

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

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.
Materials and Methods

Mice

All animal studies were approved by the Washington University Animal Studies Committee (St. Louis, MO). B6.129P2-TyroBPm1Gru/J mice (hereafter referred to as DAP12−/−) were generously provided by T. Takai (Tohoku University, Sendai, Japan) (12) and backcrossed to C57BL6, with background determined by simple sequence length polymorphism typing. STAT6 knockout mice (strain B6.129S2(C)-Stat6tm1Gru/J) 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 C57BL6 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-TREM2tm1Gru (hereafter referred to as TREM-2−/−). These mice were backcrossed to C57BL6 until greater than 99% of the loci were derived from the C57BL6 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. Abs-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 where sensitized to OVA by immunization on days 1 and 14 with 20 μg of OVA mixed with 2.25 mg of aluminum hydroxide (alum). On day 17, mice were sensitized intranasally with 20 μl of 1% OVA, and 48 h later the recruited cells were isolated by bronchoalveolar 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 resident 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...
CD11c<sup>+</sup>CD11b<sup>low</sup> (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 cell 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 readily 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).

**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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> cells (data not shown). Whereas Humphrey 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-κB ligand (4), we find that differentiation of macrophages in MCSF alone leads to expression of TREM-2.

**TREM-2 skew cytokine production in response to TLR agonists**

To determine the function of TREM-2 in macrophages, WT and TREM-2<sup>−/−</sup> 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<sup>−/−</sup> 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)

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**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<sup>−/−</sup> mice, and TREM-2 expression was assayed by FACS. TREM-2 is expressed independently of STAT6 expression.

**FIGURE 3.** Generation of TREM-2<sup>−/−</sup> 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<sup>−/−</sup> and stained with mAb 178. TREM-2 was well expressed on WT cells but absent on the TREM2<sup>−/−</sup> cells.
We also find a significant but more modest increased in IL-6 production in response to LPS, zymosan, and CpG in the absence of TREM-2 (Fig. 4A). 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 and TREM-2/−/−, and DAP12/−/− macrophages. We found no difference between TREM2/−/− and DAP12/−/− macrophages in response to zymosan, CpG, and LPS (Fig. 4B), 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 response to LPS, zymosan, and CpG in the absence of TREM-2 (Fig. 4C). 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. 4C), 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 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 BioXell, which retains the rights to TREM-2, but did not support this work.

References