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Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1)-mediated Bcl-2 Induction Prolongs Macrophage Survival*

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Background: Mechanisms by which triggering receptor expressed on myeloid cells 1 (TREM-1) amplifies inflammation are not fully defined.
Results: TREM-1 induces anti-apoptotic protein Bcl-2.
Conclusion: Macrophage survival is prolonged by TREM-1.
Significance: TREM-1 activation can propagate inflammation by modulating the survival of inflammatory cells.

Triggering receptor expressed on myeloid cells 1 (TREM-1) is a superimmunoglobulin receptor expressed on myeloid cells that plays an important role in the amplification of inflammation. Recent studies suggest a role for TREM-1 in tumor-associated macrophages with relationship to tumor growth and progression. Whether the effects of TREM-1 on inflammation and tumor growth are mediated by an alteration in cell survival signaling is not known. In these studies, we show that TREM-1 knock-out macrophages exhibit an increase in apoptosis of cells in response to lipopolysaccharide (LPS) suggesting a role for TREM-1 in macrophage survival. Specific ligation of TREM-1 with monoclonal TREM-1 (mTREM-1) or overexpression of TREM-1 with adeno-TREM-1 induced B-cell lymphoma-2 (Bcl-2) with depletion of the key executioner caspase-3 prevents the cleavage of poly(ADP-ribose) polymerase. TREM-1 knock-out cells showed lack of induction of Bcl2 with an increase in caspase-3 activation in response to lipopolysaccharide. In addition overexpression of TREM-1 with adeno-TREM-1 led to an increase in mitofusins (MFN1 and MFN2) and knockdown of TREM-1 decreased the expression of mitofusins suggesting that TREM-1 contributes to the maintenance of mitochondrial integrity favoring cell survival. Investigations into potential mechanisms by which TREM-1 alters cell survival showed that TREM-1-induced Bcl-2 in an Egr2-dependent manner. Furthermore, our data shows that expression of Egr2 in response to specific ligation of TREM-1 is ERK mediated. These data for the first time provide novel mechanistic insights into the role of TREM-1 as an anti-apoptotic protein that prolongs macrophage survival.

Macrophages are the most abundant innate immune cells distributed throughout the body and are implicated in various types of infections/inflammation and cancer (1–5). They also have the plasticity to quickly deal with endangering signals in the organs where they reside. Depending on the signals received, macrophages can become pro- or anti-inflammatory, immunogenic or tolerogenic, and destroying or repairing tissue (6–9). Each phenotype defines macrophage roles with distinct functions. The proinflammatory macrophages like neutrophils are cleared by apoptosis and efferocytosis (10). Clearance of these proinflammatory cells plays a pivotal role in successful resolution of inflammation. When apoptosis of these cells is delayed inflammation persists and perpetuates with continued production of proinflammatory mediators.

Triggering receptor expressed on myeloid cells 1 (TREM-1)[2] is a member of the super immunoglobulin family expressed exclusively on macrophage and neutrophils (11). Blockade of TREM-1 has been shown to improve survival in animal models of infection and sepsis (12–14). We have shown that the functional consequences of TREM-1 silencing in macrophages include an altered availability of key signaling (CD14, IκBα, and MyD88), and effector molecules (MCP-1, IL-1β, and IL-6) downstream of TLR activation. Furthermore, the presence of TLRs is necessary for the expression of TREM-1 in response to specific TLR ligands (15–17). These receptors have thus emerged as potent amplifiers of TLR-initiated inflammatory responses (14, 16, 18). Recent studies have shown that TREM-1 is highly expressed in tumor-associated tissue macrophages in colon, hepatocellular, and lung carcinoma (19–22). Data from these studies indicate that expression of TREM-1 is associated with rapid progression of cancer and worst outcomes. Hence, these studies suggest a role for TREM-1 in tumor growth, however, the mechanisms by which TREM-1 alters cell survival is not defined. We investigated whether TREM-1 alters macrophage cell survival or inhibits apoptosis, which could potentially delay resolution of inflammation.

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[2] The abbreviations used are: TREM-1, triggering receptor expressed on myeloid cells 1; TLR, Toll-like receptor; BMDM, bone marrow-derived macrophages: PARP, poly(ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazole; MFN, mitofusin; Bcl-2, B-cell lymphoma-2.

Apoptosis or programmed cell death is a tightly regulated process, needed to maintain homeostasis by balancing proliferation with cellular demise. The mechanism by which apoptosis is induced and regulated are complex and depend on the specific stimuli and cell type (23, 24). The mitochondrial apoptotic pathway is regulated by the BCL-2 family whose members are classified based on structure and function and comprise of anti-apoptotic proteins such as Bcl-2 and Bcl-xL and pro-apoptotic proteins such as BAX and BAK (24). Loss of balance between pro- and anti-apoptotic Bcl-2 proteins causes calcium influx into mitochondria and decrease in mitochondrial outer membrane permeability, which allows cytochrome *c* to release into the cytoplasm, eventually leading to activation of a cascade of caspases and formation of the apoptosome, causing apoptosis. Caspases are highly conserved proteins that play a central role in the execution of apoptosis and are classified as initiators (caspase-8 or -9) or executioners (caspase-3 and -7) (24–26).

Anti-apoptotic Bcl-2 proteins are commonly up-regulated in human cancers and counteract the activity of their pro-apoptotic relatives. The mechanisms of up-regulation of Bcl-2 depend on the cell type. In T cell and thymocytes Egr2 plays a central role through the up-regulation of Bcl-2 during positive selection of thymocytes and T cells prolonging cell survival (27). The Egr family of zinc finger transcription factors are early response genes that have been shown to regulate cell proliferation, differentiation, and apoptosis by inducing Bcl-2. The expression of Egrs is rapidly induced by stress, injury, mitogens, and differentiation factors (28–30). However, there is no information about the role of Egr2-Bcl-2 signaling in LPS-induced macrophage survival.

In this study we silenced the TREM-1 gene and employed TREM-1 knock-out macrophages to test our hypothesis that TREM-1 may inhibit apoptosis of inflammatory macrophages. Intriguingly we find that TREM-1 activation prolongs macrophage survival by inducing Bcl-2 in an Egr2-dependent manner. TREM-1 overexpression depleted the key executioner caspase-3 thus preventing the cleavage of PARP. Furthermore, overexpression of TREM-1 also led to an increase in mitofusins (MFN1 and MFN2) suggesting that TREM-1 contributes to maintenance of mitochondrial integrity thus favoring cell survival. These data for the first time provide novel mechanistic insight into the role of TREM-1 as an anti-apoptotic protein that prolongs macrophage survival.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6 mice were purchased from Jackson Laboratories. TREM-1/3-deficient mice were kindly provided by Dr. Marco Colonna (31). The studies were approved by the Animal Care Committee and Institutional Biosafety Committee of our institute.

Cell Culture—A murine macrophage cell line RAW264.7 (ATCC, Rockville, MD) and AD293 cell line (Stratagene) were maintained in DMEM supplemented with 10% FBS (HyClone).

Preparation of Bone Marrow-derived Macrophages (BMDM)—BMDM from C57 BL/6 and TREM-1/3-deficient mice were prepared as described previously (32, 33). Briefly mice were euthanized by asphyxiation with CO_2. Cellular material was aspirated from femurs and spun at 400 × *g* at 4 °C for 5 min. Cells were then resuspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 10% L929 cell-conditioned medium containing M-CSF. The cells were allowed to mature into phenotypic macrophages by incubation in the presence of L929 cell-conditioned medium for 5 days before the experiments were done. Purity of the resulting macrophages were confirmed by flow cytometry (>90% $CD11b^+/F4/80^+$).

Preparation of Human Macrophages from Peripheral Blood Monocytes—Human peripheral blood monocytes were isolated from buffy coats (Staedtisches Klinikum Karlsruhe, Germany) by Hypaque-Ficoll density gradient centrifugation. Peripheral blood monocytes were differentiated to macrophages by cultivation in RPMI 1640 with 50 ng/ml of human M-CSF (R&D Systems) for 7 days. Purity of macrophages was controlled by flow cytometry (>90% $CD14^+$). The studies were approved by the Institutional Review Board.

In Vitro Experiments—The RAW264.7 cell line, human monocytes matured to macrophages, bone marrow-derived macrophages from wild type, or TREM-1 knock-out mice were used for the *in vitro* experiments. Cells were treated with LPS (100 ng/ml), anti-TREM-1 antibody (mTREM-1) (10 μg/ml), anti-TREM-2 antibody (mTREM-2) (10 μg/ml), IgG (10 μg/ml), and ATP (5 mmol/liter, Sigma) for the specified time points.

Staurosporine-mediated Apoptosis—Staurosporine (2 μM, Sigma) was added to adherent macrophages in media containing serum. 24 h later cells were analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazole (MTT) assay. Cultured cells were pulsed with 25 μl of a 2.5 mg/ml of MTT stock (Sigma) in PBS and incubated for 4 h after which 100 μl of a solution containing 10% SDS, 0.01 mol/liter of HCl was added. Absorbance was measured on a microplate reader (Perkin-Elmer Life Sciences) at a wavelength of 570 nm. Test medium were added to medium alone to provide a blank.

Apoptosis Assessment—Apoptosis was assessed by FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). 10^6 cells per sample were collected, washed twice in PBS, and resuspended with the incubation buffer containing Annexin V antibody at room temperature for 15 min before the addition of propidium iodide. Cells were then analyzed by a fluorescence-activated cell sorter Calibur flow cytometer (BD Biosciences) using CellQuest software.

PCR Array (Apoptosis Pathway)—The PCR microarray for the apoptosis pathway was performed by using RT^2-Profiler PCR Array from SuperArray Bioscience. The PCR array combines the quantitative performance of SYBR Green-based real-time PCR with the multiple gene profiling capabilities of microarray. Ninety-six-well plates containing gene-specific primer sets for apoptosis pathway genes, 5 housekeeping genes, and 2 negative controls were used. After performing thermal cycling (according to the manufacturer's protocol), real-time amplification data were gathered by using ABI Prism 7900HT software. Gene expression was normalized to internal controls (housekeeping genes) to determine the fold-change in gene expression between test and control samples by the $\Delta\Delta C_t$ method (SuperArray Bioscience).

RNA Interference—Cells were transfected with siRNA oligonucleotides targeting TREM-1, Egr2, MEK mRNA, and a non-

related siRNA control (Santa Cruz) through a specific LONZA transfection reagent (LONZA) according to the manufacturer's instructions, respectively. The cells were incubated with siRNA complexes for 48–72 h before analysis.

Recombinant TREM-1 Adenoviral Preparation and Transduction—For TREM-1 overexpression studies TREM-1 adenoviral vectors were generated. Full-length cDNA of mouse TREM-1 was cloned into pAdeno-shuttle plasmid (Stratagene), and then the recombinant adenoviral vector containing TREM-1 was obtained through *in vivo* homologous recombination by using the Adeasy system (Stratagene). The primary stock was propagated four times in AD293 cells to achieve titers of 10^{10} to 10^{12} infectious particles per milliliter. This viral suspension was used to transduce BMDM at an multiplicity of infection of 10.

Quantitative Real-time Polymerase Chain Reaction—Total cellular RNA was extracted using the TRIzol RNA extraction kit (Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized from 1 μg of total RNA using the SuperScript first-strand synthesis system (Fermentas). Real-time PCR was performed with TaqMan® Universal PCR Master Mix (Applied Biosystems) to analyze Bcl-2 expression in macrophages, and primers were also purchased from Applied Biosystems. The expression of actin mRNA was determined for normalization. PCR was conducted using Applied Biosystems (ABI Prism 7900HT) according to the manufacturer's instructions. The cycling conditions were 5 min at 95 °C, 40 cycles of 15 s at 95 °C, 15 s at 60 °C, 15 s at 72 °C, and 5 s at 72 °C. The sequences of the primer pairs will be disclosed upon request. Real-time PCR was performed in triplicate including non-template controls. The data were collected and analyzed using One-Step Software (ABI) according to the $2^{-\Delta\Delta CT}$ method ($\Delta\Delta C_T$).

Flow Cytometric Analysis—An aliquot of 1×10^6 cells was stained with phycoerythrin-conjugated anti-mouse TREM (R&D Systems). Phycoerythrin-conjugated rat IgG_{2A} (R&D systems) was used as isotype antibody control. Stained cells were analyzed on a FACSCalibur machine (BD Biosciences). A minimum of 10,000 cells per sample were analyzed and data stored in list mode. Data analysis was performed with CellQuest software (BD Biosciences).

Western Blot—Protein extraction, electrophoresis, and transfer were performed as previously described (32, 33). The proteins of cell lysates were extracted by RIPA Lysis Buffer (Sigma). Protein content was determined with a Bio-Rad protein assay. Equal amounts of protein (20–25 μg) were analyzed by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore). The membranes were blocked with 5% nonfat dry milk in TBS, 0.1% Tween 20 (TBST) and incubated overnight at 4 °C with antibodies against TREM-1, Bcl-2, Bcl-xL (Santa Cruz Biotechnology), Egr2, caspase-3, cleaved Caspase-3, PARP, cleaved PARP (Cell Signaling Technology), mitofuisn 1 (MFN1), mitofusion 2 (MFN2), caspase-8, cleaved caspase-8, caspase-9, cleaved caspase-9, caspase-1 (p20)(AdipoGen), caspase-11 (Novus Biologicals), or β-actin (Santa Cruz Biotechnology) overnight at 4 °C. Membranes were washed with TBST and incubated with appropriate HRP-conjugated secondary Abs for 1 h at room temperature in TBST. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) detection system (Pierce) on Molecular Imager ChemiDoc XRS system (Bio-Rad). Band densities were quantified by ImageJ and the relative amounts of proteins associated with each specific antibody were normalized to actin bands.

Statistical Analysis—All experiments were repeated at least three times, and data were expressed as mean ± S.D. Statistical analysis was performed using the paired Student's t test and analysis of variance using GraphPad Instat with $p < 0.05$ values considered statistically significant.

RESULTS

TREM-1 Prolongs Macrophage Survival—To address the role of TREM-1 in macrophage survival, we first assessed LPS-induced apoptosis in TREM-1 knockdown cells using siRNA against TREM-1. We reasoned that if TREM-1 is involved in promoting cell survival then elimination of TREM-1 should increase cell death. RAW264.7 cells were transected with control siRNA or TREM-1 siRNA using nucleofaction and the knockdown of TREM-1 protein was confirmed by Western blotting and FACS analysis (Fig. 1, *A* and *B*). After transfecting RAW264.7 cells with control siRNA or TREM-1 siRNA for 48 h cells were treated with *Escherichia coli* LPS (100 ng/ml) for 16 h. The number of apoptotic cells was determined by Annexin V-FITC apoptosis assay. Stained cells were analyzed by FACS. Cells that expressed control siRNA showed apoptosis that was similar to cells treated with LPS alone at 24 h (38%), in contrast, cells that were transfected with TREM-1 siRNA showed an enhanced number of apoptotic cells (80.9%) compared with control cells (Fig. 1*C*). Then BMDM from wild type and TREM-1 knock-out mice were treated with LPS (100 ng/ml) for 16 h. Similar to the TREM-1 knockdown cells, macrophages from TREM-1 knock-out mice showed an enhanced number of apoptotic cells compared with wild type macrophages (Fig. 1*D*). These data suggest that the presence of TREM-1 inhibits apoptosis and prolongs survival of macrophages. To further confirm the role of TREM-1 in cell survival, we examined whether knockdown of TREM-1 altered the sensitivity of cells to staurosporine stress and assessed cell survival by the MTT assay, which measures cell viability by detecting the ability of a mitochondrial enzyme to reduce its substrate. RAW264.7 cells and cells transfected with TREM-1 siRNA were treated with staurosporine. Cell viability was assessed by MTT assay, 24 h after treatment with staurosporine. We found that macrophages expressing endogenous TREM-1 were more resistant to staurosporine-induced apoptosis compared with cells that had endogenous TREM-1 levels knocked down by siRNA (Fig. 1*E*). Collectively, these data suggest that TREM-1 activation inhibits macrophage apoptosis and promotes cell survival signaling.

Anti-apoptotic Proteins Are Induced by TREM-1—Because TREM-1 knockdown increased apoptosis we reasoned that TREM-1 ligation might alter the balance of anti- and pro-apoptotic proteins. A comprehensive microarray of apoptosis-related proteins validated that TREM-1 knockdown decreased expression of Bcl-2, Bcl-xL, Egr2, Mcl, and ATF-5 genes (Tables 1 and 2) (34). Bcl-2 and related proteins are currently perceived to be the most important anti-apoptotic proteins (35). Because there was a robust decrease in gene expression of Bcl-2 and

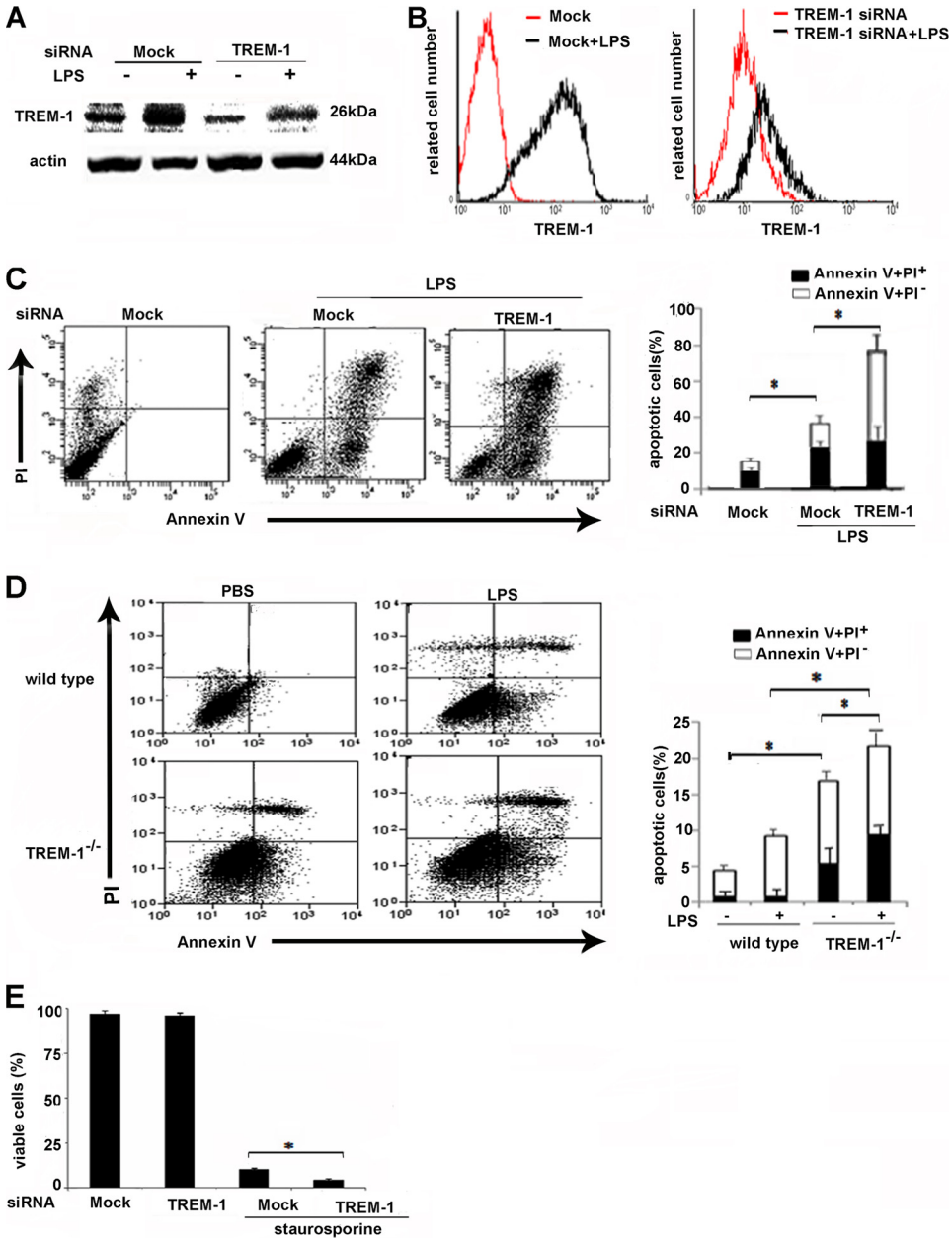

FIGURE 1. **TREM-1 prolongs macrophage survival.** RAW264.7 cells were transected with control siRNA or TREM-1 siRNA for 48 h using nucleofection and knockdown of TREM-1 protein was confirmed by Western blotting (*A*) and FACS analysis (*B*). *C*, apoptosis of cells after transfection of siTREM-1 or control siRNA assessed by FITC-Annexin V shows that cells transfected with TREM-1 knockdown have an enhanced number of apoptotic cells after treatment with LPS (100 ng/ml) for 16 h as compared with cells transfected with control siRNA. *D*, bone marrow-derived macrophages from TREM-1 knockout mice also show an enhanced number of apoptotic cells compared with the wild type macrophages following LPS (100 ng/ml) treatment. *E*, cell survival by the MTT assay shows that macrophages expressing endogenous TREM-1 are more resistant to staurosporine-induced apoptosis compared with cells that have endogenous TREM-1 levels knocked down by siRNA ($n = 3–4$; *, $p < 0.01$).

Bcl-xL in TREM-1 knockdown cells we performed additional experiments to define the relationship of protein induction of Bcl-2 and Bcl-xL in TREM-1-silenced cells. Cells transfected with siTREM-1 or control siRNA were treated with LPS (100 ng/ml). Western blotting for Bcl-2 and Bcl-xL confirmed a significant reduction in Bcl-2 (Fig. 2*A*) and Bcl-xL (Fig. 2*B*) protein levels in the TREM-1 knockdown cells. We then performed experiments to define the protein expression of Bcl-2 in relationship to specific ligation of TREM-1 using monoclonal antibodies that only activate TREM-1 (mTREM-1). Expression of Bcl-2 was significantly increased in response to specific ligation of TREM-1 compared with control antibody (Fig. 2*C*). To further confirm the relationship of the Bcl-2 induction in response to TREM-1 activation we overexpressed TREM-1 using adenoviral vectors and transected macrophages from wild type mice and compared the expression of Bcl-2 with TREM-1 knock-out macrophages. Macrophages were transected with adenoviral vectors expressing TREM-1 or lacZ for 48 h following which expression of Bcl-2 was determined by Western blotting in response to treatment with LPS (100 ng/ml) at 16 h. Macrophages transfected with adeno-TREM-1 vector showed an enhanced induction of Bcl-2 protein compared with cells that

TABLE 1

Genes up-regulated

Microarray data for apoptotic proteins from RAW264.7 cells transfected with TREM-1 siRNA or control siRNA. RNA was extracted after treatment with LPS and microarray analysis was performed.

	Gene symbol	Description	Average fold-change
1	Akt1	Thymoma viral proto-oncogene 1	12.27
2	Bag3	BCL-2-associated athanogene 3	4.73
3	Bcl2l1	BCL-2-like 1	7.29
4	Nod1	Nucleotide-binding oligomerization domain containing 1	11.33
5	Casp14	Caspase-14	28.71
6	Casp3	Caspase-3	21.59
7	Nme5	NME/NM23 family member 5	6.22
8	Prdx2	Peroxiredoxin 2	3.08
9	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	5.31
10	Cd40	CD40 antigen	8.16
11	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	17.28

TABLE 2

Genes down-regulated

Microarray data for apoptotic proteins from RAW264.7 cells transfected with TREM-1 siRNA or control siRNA. RNA was extracted after treatment with LPS and microarray analysis was performed.

	Gene symbol	Description	Average fold-change
1	Apaf1	Apoptotic peptidase activating factor 1	−9.91
2	Atf5	Activating transcription factor 5	−11.60
3	Bad	BCL-2-associated agonist of cell death	−2.27
4	Bak1	BCL-2-antagonist/killer 1	−4.30
5	Bax	BCL-2-associated X protein	−1.10
6	Bcl-10	B cell leukemia/lymphoma 10	−4.36
7	Bcl-2	B cell leukemia/lymphoma 2	−45.76
8	Bcl-2l10	Bcl2-like 10	−5.88
9	Bcl-2l2	BCL2-like 2	−10.01
10	Naip1	NLR family, apoptosis inhibitory protein 1	−11.68
11	Naip2	NLR family, apoptosis inhibitory protein 2	−3.63
12	Birc5	Baculoviral IAP repeat-containing 5	−5.79
13	Bnip3	BCL-2/adenovirus E1B interacting protein 3	−18.22
14	Bok	BCL-2-related ovarian killer protein	−25.30
15	Card10	Caspase recruitment domain family, member 10	−2.70
16	Casp2	Caspase 2	−23.05
17	Dad1	Defender against cell death 1	−2.63
18	Dapk1	Death associated protein kinase 1	−10.34
19	Ltbr	Lymphotoxin B receptor	−2.18
20	Mcl1	Myeloid cell leukemia sequence 1	−23.13
21	Pim2	Proviral integration site 2	−3.23
22	Pycard	PYD and CARD domain containing	−7.17
23	Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	−5.00
24	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	−2.11
25	Cd70	CD70 antigen	−3.35

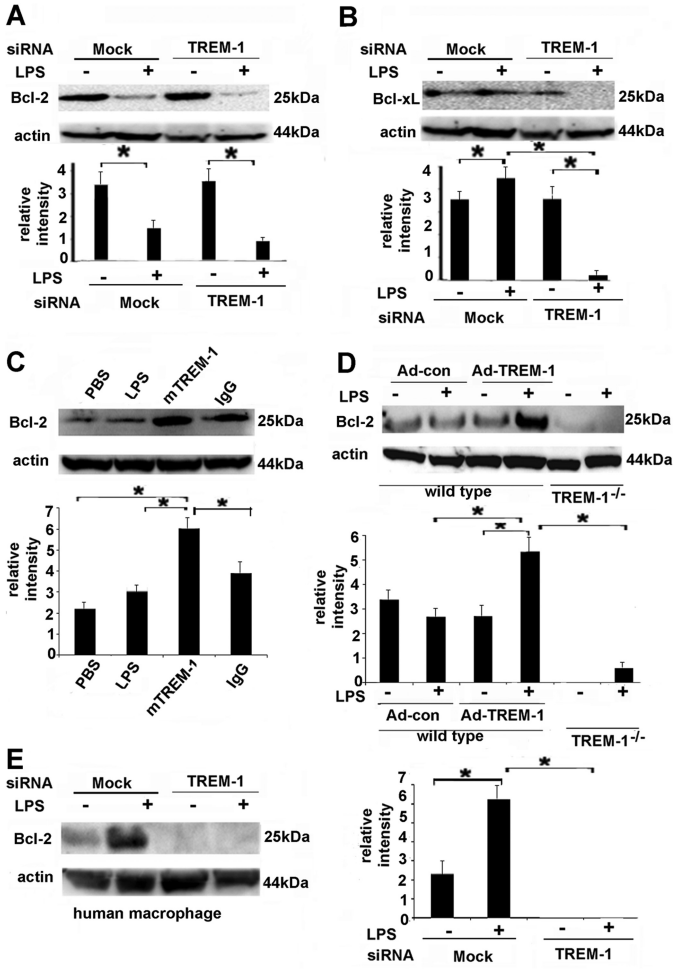

FIGURE 2. **Anti-apoptotic proteins are induced by TREM-1 in macrophages.** RAW264.7 cells were transfected with TREM-1 or control siRNA for 48 h and then treated with LPS (100 ng/ml) for 16 h. *A,* Western blotting for Bcl-2, and *B,* Bcl-xL confirmed a significant reduction in Bcl-2 and Bcl-xL proteins in tTREM-1 knockdown cells transfected with siTREM-1. *C,* Bcl-2 protein expression by Western blot analysis in response to specific ligation of TREM-1 using monoclonal TREM-1 (mTREM-1) confirmed that expression of Bcl-2 was significantly increased in response to mTREM-1 compared with control antibody. *D,* Western blotting of Bcl-2 protein in cells that overexpress TREM-1 by transfection using adenoTREM-1 or adeno-lacZ in wild type bone marrow-derived macrophages. Macrophages transfected with adenoviral vectors expressing TREM-1 or control adenovirus (lacZ) for 48 h were treated with LPS (100 ng/ml). Bcl-2 expression was determined by Western blotting. Macrophages transfected with adeno-TREM-1 showed an enhanced induction of Bcl-2 protein compared with cells treated with control vectors. Bcl-2 was significantly attenuated in bone marrow-derived macrophages from TREM-1 knockout mice. *E,* Bcl-2 expression by Western blot analysis in macrophages from normal human volunteers. Human macrophages were transfected with TREM-1 siRNA or control siRNA prior to treatment with LPS (100 ng/ml). Induction of Bcl-2 was attenuated in cells that were transfected with TREM-1 siRNA ($n = 3–4$; *, $p<0.05$).

were treated with control vectors (Fig. 2*D*). On the other hand induction of Bcl-2 was significantly attenuated in the TREM-1 knock-out macrophages (Fig. 2*D*). Similar experiments were performed using monocyte/macrophages extracted from normal human volunteers. Human macrophages were transfected with TREM-1 siRNA or control siRNA prior to treatment with LPS (100 ng/ml). Bcl-2 induction was determined by Western blot analysis 16 h after treatment with LPS. Induction of Bcl-2 was attenuated in cells that were transfected with TREM-1 siRNA (Fig. 2*E*). Together, these data show that the anti-apoptotic attributes of TREM-1 activation are directly related to induction of Bcl-2.

Bcl-2 Induction by TREM-1 in Macrophages Is Egr2 Dependent—Next we investigated the mechanism by which TREM-1 induces Bcl-2. Induction of Bcl-2 has been shown to be regulated by Egr2 during positive selection and survival of thymocytes (27, 29). Egr proteins comprise a family of transcriptional regulators (Egr1–4) that modulate gene expression involved in the growth, differentiation, and survival of a variety

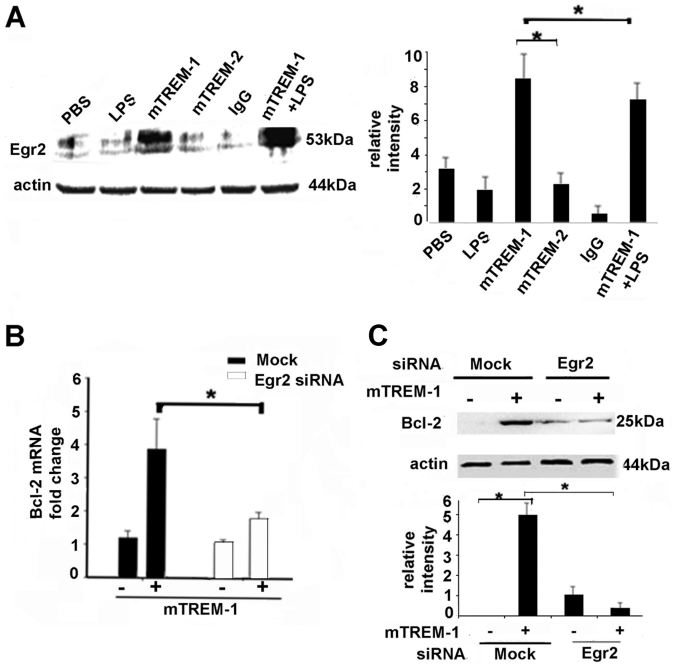

FIGURE 3. **Bcl-2 induction by TREM-1 in macrophages is Egr2 dependent.** BMDM from wild type mice were treated with LPS, mTREM-1, mTREM-2, or isotype antibodies for 16 h. *A*, Western blot analysis for Egr2 protein demonstrating increased expression of Egr2 in response to mTREM-1 ligation, which is accentuated in the presence of LPS. RAW264.7 cells were transfected with siEgr2 or control RNA for 48 h and then treated with mTREM-1 for 16 h. *B*, Bcl-2 mRNA (determined by quantitative PCR), and *C*, Western blot analysis demonstrating attenuated Bcl-2 induction in Egr2-silenced cells ($n = 4–5$; *, $p < 0.01$).

of cell types. Whether Egr2 can regulate the expression of Bcl-2 in macrophages is not known. Because microarray data showed a significant increase in the expression of Egr2 in response to TREM-1 ligation (34) we questioned if Egr2 contributed to the expression of Bcl-2. We first confirmed that Egr2 is induced by specific ligation of TREM-1. Bone marrow-derived macrophages from wild type mice were treated with LPS, or monoclonal antibodies that specifically ligate TREM-1. Control cells were treated with mTREM-2 or isotype antibody. We found a robust induction of Egr2 in response to TREM-1 ligation, which was accentuated in the presence of LPS (Fig. 3*A*). To define if Bcl-2 induction is regulated by Egr2, we silenced the Egr2 gene in macrophages using a siRNA approach. Egr2 knockdown cells were treated with monoclonal TREM-1 and expression of the Bcl-2 gene was determined in response to mTREM-1. We found that expression of the Bcl-2 protein and message was significantly attenuated in Egr2-silenced cells (Fig. 3, *B* and *C*). These data suggest that induction of Bcl-2 in response to TREM-1 ligation is regulated by Egr2 in macrophages.

We next wanted to determine the mechanisms of induction of Egr2 by TREM-1. In T cells the expression of Egr2 is dependent on ERK and calcineurin signaling (36). Because TREM-1 ligation activates MAP kinase signaling (37), we examined if MAP kinases are involved in Egr2 expression. BMDM from wild type mice were treated with monoclonal mouse TREM-1 (10 μg/ml). Within 10 min of treatment we were able to detect the expression of phosphorylated p38 and ERK1/2 (data not shown). We then treated cells with SB203560 (p38i), UO126, and PD325901 (10 μmol/liter) MEK inhibitors prior to treatment with monoclonal TREM-1 antibody (10 μg/ml). Treatment with MEK inhibitors particularly U0126 resulted in significant inhibition of Egr2 expression with ligation of TREM-1 (Fig. 4, *A* and *B*). These experiments were also performed in human monocyte/macrophages and similar to the results from BMDM we found that the expression of TREM-1 induced by mTREM-1 was attenuated by the MEK inhibitor (U0126) (Fig. 4*C*). To conclusively define the role of MEK in the induction of Egr2 we performed experiments in macrophages with MEK siRNA. Macrophages were transfected with MEK or control siRNA prior to treatment with mTREM-1 (10 μmol) or IgG. The expression of Egr2 was significantly lower in MEK-silenced cells in response to mTREM-1. Collectively, these data suggest that expression of Egr2 in macrophages in response to specific ligation of TREM-1 is dependent on the MEK/MAP kinase signaling pathway.

TREM-1 Depletes Executionary Caspase-3 with Decreased Proteolytic Cleavage of PARP—A central step in the execution of apoptosis is the activation of caspases, a family of cysteine proteases that are ubiquitously expressed as inactive precursors (zymogens) with little or no protease activity. We therefore determined the effects of TREM-1 signaling on caspases. The caspase family is subdivided into initiator and effector caspases. Effector caspases execute apoptosis after they are proteolytically processed by initiator caspases. To determine the effects of TREM-1 on executionary caspases, TREM-1 knockdown cells and control cells were treated with *lps* (100 ng/ml). Caspase-3, PARP, and cleaved PARP (c-PARP) expression were determined by Western blotting. We found that TREM-1 knockdown cells showed an increased expression of caspase-3 and cleavage of PARP suggesting that the presence of TREM-1 is necessary for the functional activation of caspase-3 (Fig. 5, *A* and *B*). To further confirm the role of TREM-1 in the expression of caspases and PARP, bone marrow-derived macrophages were treated with LPS and monoclonal TREM-1 antibody to specifically activate TREM-1. Cells that were treated with mTREM-1 showed depletion of caspase-3, whereas control cells treated with LPS, anti-TREM-2, or IgG showed intact caspase-3 (Fig. 5*C*). We then performed experiments where we overexpressed TREM-1 in wild type BMDM by using adeno-TREM-1. BMDM from wild type mice were transfected with adeno-TREM-1 or control adenovirus for 48 h prior to treatment with LPS (100 ng/ml). Cells treated with adeno-TREM-1 showed depletion of caspase-3, whereas cells treated with control adenoviruses showed intact caspase-3 (Fig. 5*D*). BMDM from TREM-1 knock-out mice showed an increased expression of caspase-3 (Fig. 5*D*) with lack of PARP cleavage (data not shown). These data are in agreement with studies from TREM-1-silenced cells. Because caspase-3 is initiated by caspase-9, we determined the expression of caspase-9 in response to mTREM-1 and LPS. BMDM from wild type mice were treated with LPS (100 ng/ml) and mTREM-1. Levels of pro-caspase-9 were decreased in the presence of LPS and mTREM-1 (Fig. 5*E*). There were no significant differences in the levels of caspase-8 expression. In addition we were also unable to detect differences in the expression of Bax and Bad (Fig. 6*A*) from cells treated with mTREM-1. Together, these data suggest that TREM-1-induced anti-apoptotic changes are exhibited by inhi-

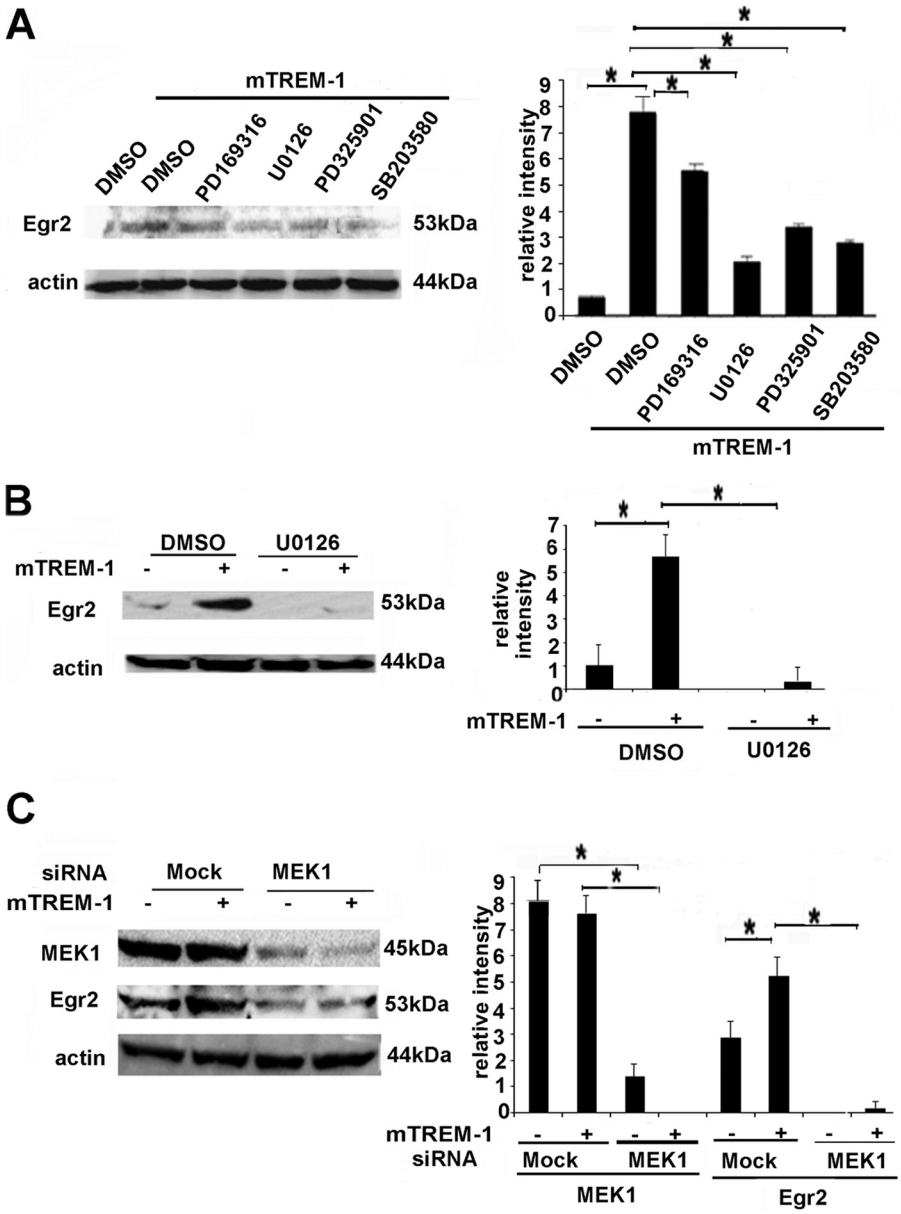

FIGURE 4. **Induction of Egr2 in macrophages in response to ligation of TREM-1 is dependent on MAP kinase.** RAW264.7 cells were treated with MAP kinase inhibitors PD169316, SB203560, U0126, and PD325901 (10 μmol/liter) for 1 h prior to treatment with mTREM-1 (10 μg/ml). *A,* Western blot analysis of Egr2 showed an attenuation of expression of Egr2 protein with MAPK inhibitors. *B,* human monocytes matured to macrophages treated with MEK1/2 inhibitor (U0126) (10 μmol) 1 h prior to treatment with mTREM-1 at 16 h shows lack of Egr2 activation. *C,* RAW264.7 cells transfected with MEK siRNA showed an attenuated expression of Egr2 protein determined by Western blotting compared with cells transfected with control siRNA ($n = 3–5$; *, $p < 0.05$).

bition of executioner caspase-3. Features of apoptotic cell death are also shared by pyroptosis, which is initiated by activation of caspase-1 and caspase-11 (38, 39). We therefore investigated if TREM-1 silencing alters the expression of caspase-1 and caspase-11. We were unable to detect differences in the activation of caspse-1 and caspase-11 in response to LPS and ATP in wild type and TREM-1 knock-out macrophages (Fig. 6*B*) suggesting that TREM-1-mediated anti-apoptotic effects are not related to NLRP3-associated pyroptosis.

TREM-1 Enhances Mitochondrial Integrity by Inducing Mitofusins—Interactions between anti-apoptotic and pro-apoptotic proteins exist in a delicate balance at the mitochondrial membrane that determines cell fate. Recent reports suggest that mitochondrial fragmentation is a central mechanism of apoptosis. Mitochondrial outer membrane permeabilization causes mitochondrial fragmentation and leads to the release of apoptotic factors (37). The pivotal role of mitochondria in control of apoptosis has been extensively investigated. Mitochondria are highly mobile and dynamic organelles that continually fuse and divide and play an important role in executing apoptosis (40, 41). Multiple proteins have been identified that mediate mitochondrial fission and fusion processes. Dynamin-related GTPases, MFN are associated with the mitochondrial outer membranes and maintain the integrity of the mitochondrial membrane. Mammalian cells have two mitofusin isoforms, MFN1 and MFN2 (42). Mitochondrial homeostasis is vital to

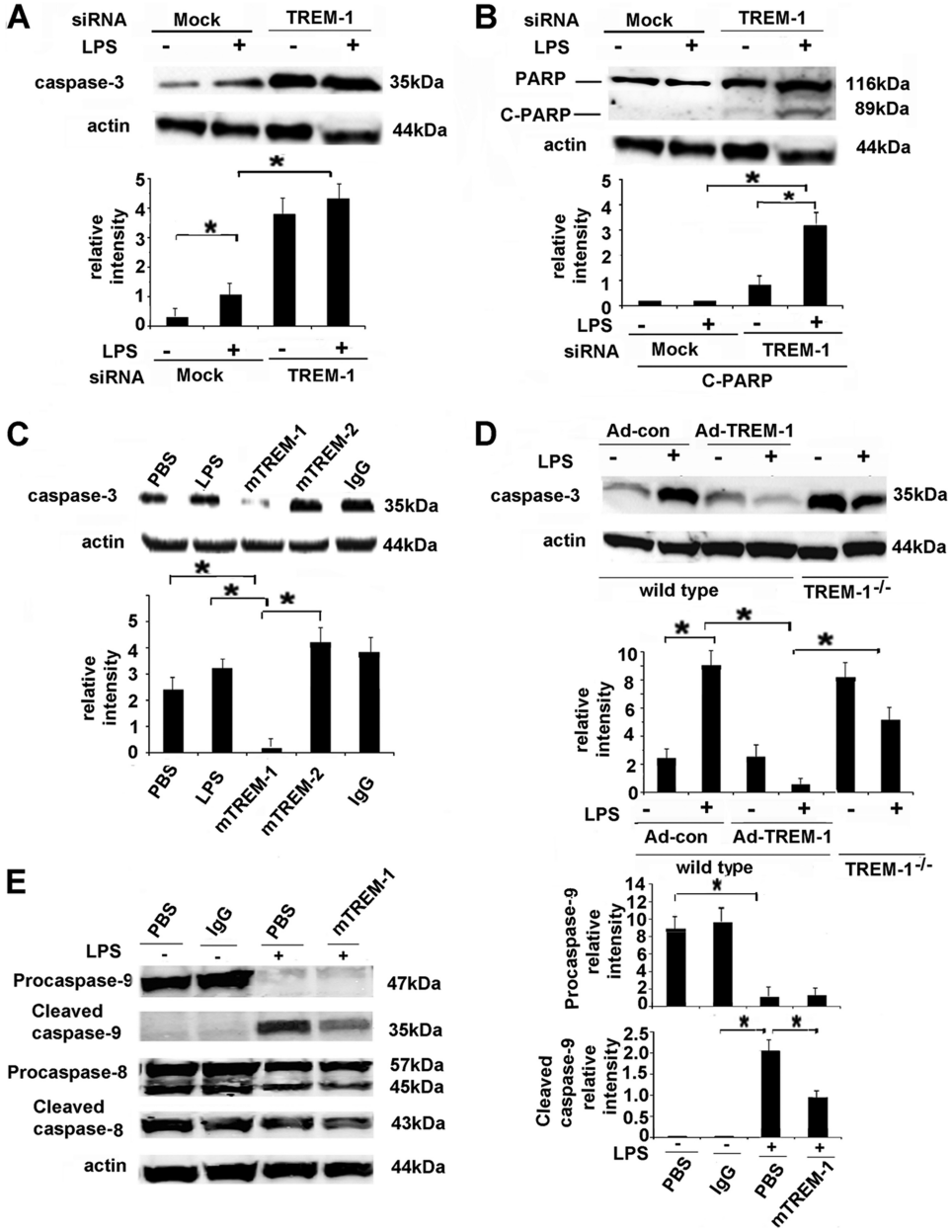

FIGURE 5. **TREM-1 depletes executioner caspase-3 with decreased proteolytic cleavage of PARP**. RAW264.7 cells transfected with siTREM-1 or control siRNA for 48 h were treated with LPS (100 ng/ml). *A*, caspase-3. *B*, PARP and cleaved PARP (*c-PARP*) expression was determined by Western blotting. *C*, wild type BMDM treated with mTREM-1 showed depletion of caspase-3, whereas control cells treated with anti-TREM-2 or IgG showed intact caspase-3. *D*, Western blotting for caspase-3 from BMDM. BMDM from wild type mice were transfected with adeno-TREM-1 or control adenovirus (10^7 pfu) followed by treatment with LPS. BMDM from TREM-1 knockout mice were treated with LPS. *E*, Western blotting for procaspase-8, -9, and cleaved caspase-8 and -9 from BMDM treated with LPS (100 ng/ml) and mTREM-1 (10 μg/ml) ($n = 3$; *, $p < 0.05$).

the survival of cells. In most experimental paradigms a fused mitochondrial phenotype seems to protect cells from programmed cell death, forced activation of organelle fission favors apoptosis by facilitating the release of cytochrome *c* from the mitochondria with the subsequent activation of effector caspases. Recent studies suggest that, during apoptosis, BCL proteins regulate mitochondrial morphology by interacting with the mitofusion proteins (43, 44). We therefore investigated the effects of TREM-1 on mitochondrial integrity by determining the effects of TREM-1 activation on mitofusins. TREM-1 knockdown cells and wild type cells were treated with LPS (100 ng/ml). Expression of mitofusins was determined by Western blot analysis. As shown in Fig. 7*A*, TREM-1 knockdown cells showed a loss in MFN1 and MFN2 suggesting that the presence of TREM-1 promotes mitochondrial fusion and integrity thus making cells resistant to apoptosis. To further confirm these findings we determined the expression of MFN1 and MFN2 in cells that overexpress TREM-1 by employing adenoTREM-1. Overexpression of TREM-1 increased the expression of MFN1 and MFN2 (Fig. 7*B*). Together these data suggest that anti-apoptotic attributes of TREM-1 may be conferred by enhancing mitochondrial integrity, thus promoting mitochondrial fusion in macrophages.

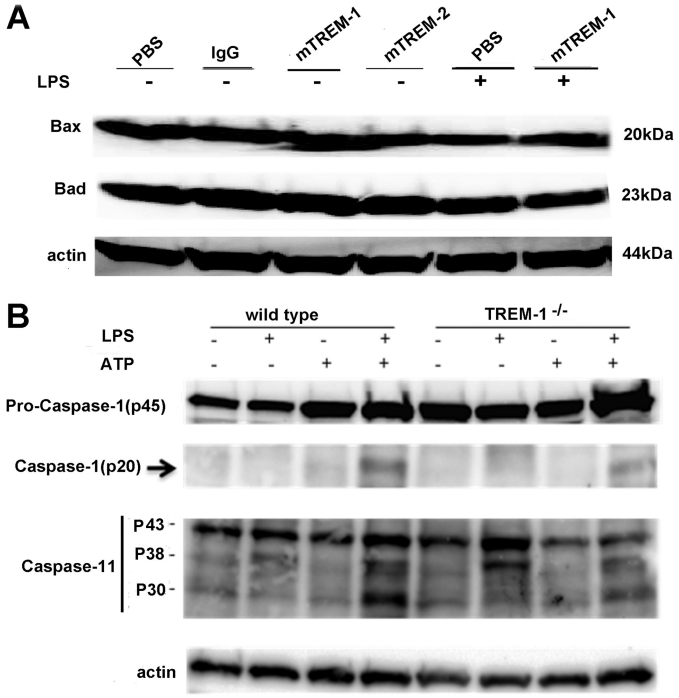

FIGURE 6. **TREM-1 ligation does not alter Bax and Bad expression.** *A,* Western blot for Bax and Bad from BMDM treated with mTREM-1, with and without LPS (100 ng/ml). No significant differences were detected in the expression of Bax and Bad protein in cells treated with and without mTREM-1. *B,* Western blot analysis for caspase-1 and caspase-11. BMDM from wild type and TREM-1 knockout mice were treated with LPS (100 ng/ml) for 4 h and pulsed with ATP (5 mM) during the last 30 min of incubation. There were no significant differences detected in caspase-1 and caspase-11 between the wild type and knockout macrophages.

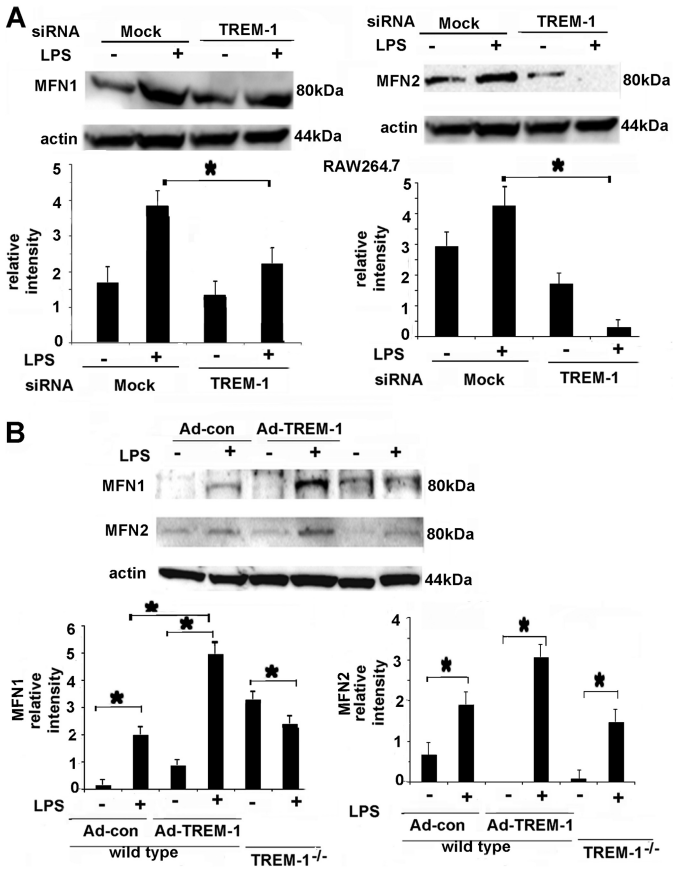

FIGURE 7. **TREM-1 enhances mitochondrial integrity by inducing mitofusins.** RAW264.7 cells transfected with TREM-1 siRNA or control siRNA for 48 h were treated with LPS (100 ng/ml). *A,* Western blot for mitofusin 1 and 2 (*MFN1* and *MFN2*) was performed at 16 h after treatment with *lps.* TREM-1 knockdown cells showed a decrease in MFN1 and MFN2. *B,* BMDM from wild type mice were transfected with adeno-TREM-1 or control adenovirus (10^7 pfu) followed by treatment with LPS (100 ng/ml). Western blot analysis for MFN1 and MFN2 showed an increased expression of mitofusins in cells overexpressing TREM-1 ($n = 3–5$; *, $p < 0.05$).

DISCUSSION

In this study we have shown a novel role for TREM-1 in inhibition of macrophage apoptosis. We show that specific ligation of TREM-1 induces key anti-apoptotic proteins Bcl-2 and Bcl-xL, whereas TREM-1 knock-out macrophages exhibit an increase in apoptosis of cells suggesting a role for TREM-1 in macrophage survival. Mechanistically we show that induction of Bcl-2 in response to ligation of TREM-1 is Egr2 mediated and that expression of Egr2 is ERK dependent. Additionally, specific ligation of TREM-1 or overexpression of TREM-1 depletes key executioner caspase-3, preventing the cleavage of PARP, providing further evidence of anti-apoptotic attributes of TREM-1. Furthermore, overexpression of TREM-1 led to an increase in mitofusins (MFN1 and MFN2) suggesting that TREM-1 contributes to the maintenance of mitochondrial integrity thus favoring cell survival. These data for the first time provide novel mechanistic insights into the role of TREM-1 as an anti-apoptotic protein that prolongs macrophage survival.

Macrophages are the most abundant immune cells in most tissue parenchyma. Because of their crucial role in immunity, regulation of the monocyte/macrophage life span is important in both physiological and pathological processes. The accumulation of macrophages in inflammatory processes reflects both an enhanced recruitment of macrophages and their resistance to apoptosis (3, 6, 45). Proinflammatory macrophages play a pivotal role in perpetuation of inflammation by synthesizing and secreting a wide array of cytokines such as IL-1β, IL-6, and TNF-α, chemokines, and arachidonic metabolites to initiate and amplify inflammatory responses, whereas they also recruit other immune cells and phagocytes including neutrophils. Although understanding the macrophage phenotype as pro- and anti-inflammatory is being defined the mechanisms of resistance to apoptosis are not well understood. Apoptosis plays fundamental roles in embryogenesis, immune system homeostasis (8, 45), and in diverse pathological conditions. Evading apoptosis by up-regulation of antiapoptotic or down-regulation of proapoptotic proteins is an important step in altering cell survival (23). This study highlights an important role for TREM-1 in prolonging survival of inflammatory macrophages.

TREM-1 is a member of the superimmunoglobulin receptor that is expressed on monocytes and neutrophils (11, 12, 31, 37, 46, 47). TREM-1 is a glycoprotein with a single extracellular immunoglobulin-like domain, a transmembrane region with a charged lysine residue, and a short intracellular region (48, 49), which binds to its as yet unidentified ligand. Following ligation it associates with a signal transduction molecule, DAP12, triggering that initiates the secretion of inflammatory cytokines. TREM-1 has emerged as an important amplifier of TLR-in-

duced inflammatory responses activating transcription factors that regulate the expression of proinflammatory cytokines and chemokines (14, 16). This study provides yet another mechanism by which TREM-1 can perpetuate inflammation by inhibiting apoptosis of macrophage by activating the key anti-apoptotic proteins of the Bcl-2 family.

Apoptosis is a self-destructive cellular process that has pivotal roles in tissue development and immune regulation that generally culminates with the sequential activation of caspases, the cysteine proteases responsible for cleavage of specific proteins that ultimately results in cellular demise (50, 51). B-cell lymphoma-2 (Bcl-2) and related proteins are currently perceived to be the most important proteins that control apoptosis. Their function is related to their ability to interfere with mitochondrial apoptosis pathways (24, 52, 53). Family members include anti-apoptotic Bcl-2 and Bcl-xl and pro-apoptotic Bax, Bad, Bid, and Bim. Interactions between these anti-apoptotic and pro-apoptotic Bcl-2 proteins exist in a delicate balance at the mitochondrial membrane that ultimately determines cell fate. Because the Bcl-2 proteins play a pivotal role in apoptosis and cell death their role in cancer pathogenesis and progression has been extensively defined. However, their contribution to inflammatory cell survival is less well studied (54). Our data for the first time shows that specific ligation of TREM-1 activates Bcl-2 thus prolonging macrophage survival. On the other hand TREM-1 knockdown cells and macrophages from TREM-1 knock-out mice show an enhanced apoptosis in response to LPS. These studies implicate an important role for TREM-1-induced Bcl2 induction in prolonging life of inflammatory cells.

We also show that Bcl-2 induction in response to TREM-1 ligation is mediated by activation of Egr2. Egr proteins comprise a family of transcriptional regulators (Egr1–4) that modulate gene expression involved in the growth, differentiation, and survival of a variety of cell types (55–58). Egr1 and Egr2 have been implicated in regulating differentiation of inflammatory cells (57, 59). The expression of Egr2 is dependent on ERK and calcineurin signaling; the endogenous repressor Nab2 inhibits Egr2 in neurons and T cells (30, 36, 60–62). Induction of anti-apoptotic protein Bcl-2 is regulated by Egr2 during positive selection and survival of thymocytes (27, 29). Egr2 polymorphisms have been associated with altered immune response in SLE and neuropathies related to survival of inflammatory cells (63–65). In this study we demonstrate that activation of TREM-1 induces Bcl-2 and Egr2. Our data shows that Bcl-2 induction is Egr2 dependent as silencing of Egr2 led to abrogation of Bcl-2 in response to activation of TREM-1. Furthermore, we show that Egr2 expression is dependent on MEK, which was confirmed by using MEK inhibitors and silencing of MEK led to attenuated induction of Egr2.

Caspases are known to act as important mediators of apoptosis and to contribute to the overall apoptotic morphology through the cleavage of various cellular substrates. We therefore investigated the anti-apoptotic attributes of TREM-1 activation and our data demonstrates that ligation of TREM-1 with mTREM-1 led to depletion of caspase-9 and executioner caspase-3 with prevention of PARP cleavage. We did not detect differences in activation of caspase-1 and caspase-11 in TREM-1 knock-out macrophages suggesting that TREM-1 may not contribute to pyroptosis (38). Interestingly, recent studies have shown that TREM-1 is expressed in tumor-associated macrophages in colon, lung, and liver cancer and that the levels of TREM-1 expression correlate with the aggressiveness of tumor (19, 21, 22). The mechanisms by which TREM-1 contributes to tumor progression are not defined. This study for the first time provides important clues into the role of TREM-1 signaling in cell survival. Further studies will define the mechanisms by which TREM-1 expression in tumor tissue leads to tumor growth and progression.

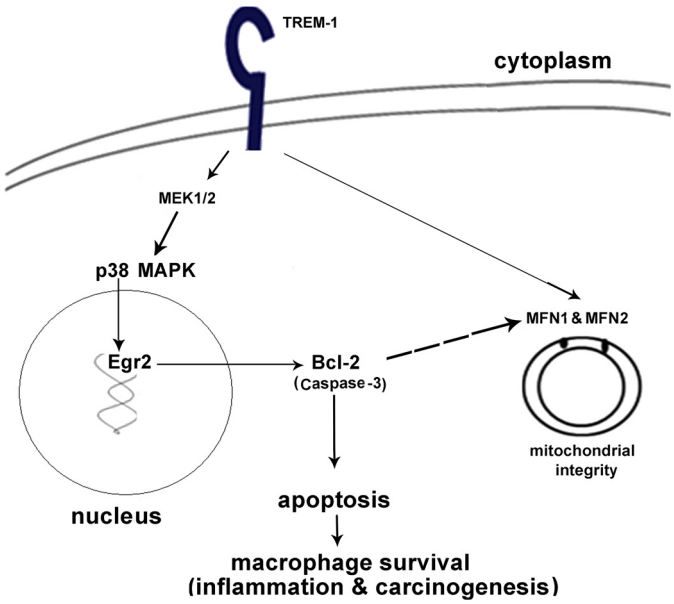

FIGURE 8. **Proposed schematic by which TREM-1 induces anti-apoptotic proteins to prolong macrophage survival.** TREM-1 ligation induces MAP kinases MEK1/2 and p38 leading to induction of Egr2 and Bcl-2. Induction of anti-apoptotic Bcl-2 prevents apoptosis by inhibiting the executionary caspase-3 and maintaining the mitochondrial integrity through expression of mitofusins.

The mechanisms by which Bcl-2 proteins mediate anti-apoptotic effects are not fully defined but recently the involvement of Bcl-2 family proteins in mitochondrial shape changes and organelle localization has been described (66, 67). This has been related to the fundamental underlying function of Bcl-2 proteins, possibly in mitochondrial energetics (68), which influences mitochondrial morphology and function. Although the mechanism through which Bcl-2 family proteins regulate mitochondrial morphogenesis is not well understood, studies suggest close interactions between Bcl-2 family proteins and the dynamin-like GTPases Drp1 and MFN1/2 that physically mediate mitochondrial outer membrane fission and fusion, respectively (67, 69, 70). In this study we show that TREM-1 induces Bcl-2 and increases the expression of MFN1 and MFN2, which increases mitochondrial integrity and thus inhibits apoptosis. Although we have not directly investigated the relationship between Bcl-2 and mitofusin expression in response to TREM-1, our data suggests that anti-apoptotic attributes of TREM-1 are reflected in the ability of Bcl-2 anti-apoptotic proteins to interfere with mitochondrial apoptosis pathways.

In summary, data from our study defines a novel role for TREM-1 as an anti-apoptotic molecule prolonging the survival of inflammatory macrophages. TREM-1 knock-out macro-

phages exhibited an increase in apoptosis of cells with lack of induction of Bcl-2 and an increase in caspase-3 activation in response to LPS. Specific ligation of TREM-1 with monoclonal TREM-1 or overexpression of TREM-1 induced Bcl-2 with depletion of the key executioner caspase-3 preventing the cleavage of PARP. In addition, ligation of TREM-1 and overexpression of TREM-1 also led to an increase in mitofusins (MFN1 and MFN2) suggesting that TREM-1 contributes to the maintenance of mitochondrial integrity favoring cell survival (Fig. 8). Lack of clearance of pro-inflammatory macrophages can potentially delay the resolution of inflammation. Our studies provide a novel mechanism by which TREM-1 activation can propagate inflammation by modulating the survival of inflammatory cells and highlight a therapeutic role for blockade of TREM-1 in inflammatory diseases and perhaps in carcinogenesis.

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