Am J Physiol Cell Physiol 307: C521–C531, 2014.
First published July 16, 2014; doi:10.1152/ajpcell.00369.2013.

Reduced endoplasmic reticulum stress-induced apoptosis and impaired unfolded protein response in TRPC3-deficient M1 macrophages

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Submitted 3 December 2013; accepted in final form 9 July 2014

Solanki S, Dube PR, Tano JY, Birnbaumer L, Vazquez G. Reduced endoplasmic reticulum stress-induced apoptosis and impaired unfolded protein response in TRPC3-deficient M1 macrophages. *Am J Physiol Cell Physiol* 307: C521–C531, 2014. First published July 16, 2014; doi:10.1152/ajpcell.00369.2013.—Endoplasmic reticulum (ER) stress is a prominent mechanism of macrophage apoptosis in advanced atherosclerotic lesions. Recent studies from our laboratory showed that advanced atherosclerotic plaques in $Apoe^{-/-}$ mice with bone marrow deficiency of the calcium-permeable channel Transient Receptor Potential Canonical 3 (TRPC3) are characterized by reduced areas of necrosis and fewer apoptotic macrophages than animals transplanted with $Trpc3^{+/+}$ bone marrow. In vitro, proinflammatory M1 but not anti-inflammatory M2 macrophages derived from $Trpc3^{-/-}$ $Apoe^{-/-}$ animals exhibited reduced ER stress-induced apoptosis. However, whether this was due to a specific effect of TRPC3 deficiency on macrophage ER stress signaling remained to be determined. In the present work we used polarized macrophages derived from mice with macrophage-specific deficiency of TRPC3 to examine the expression level of ER stress markers and the activation status of some typical mediators of macrophage apoptosis. We found that the reduced susceptibility of TRPC3-deficient M1 macrophages to ER stress-induced apoptosis correlates with an impaired unfolded protein response (UPR), reduced mitochondrion-dependent apoptosis, and reduced activation of the proapoptotic molecules calmodulin-dependent protein kinase II and signal transducer and activator of transcription 1. Notably, none of these pathways was altered in TRPC3-deficient M2 macrophages. These findings show for the first time an obligatory requirement for a member of the TRPC family of cation channels in ER stress-induced apoptosis in macrophages, underscoring a rather selective role of the TRPC3 channel on mechanisms related to the UPR signaling in M1 macrophages.

TRPC channels; macrophage apoptosis; endoplasmic reticulum stress

MACROPHAGE APOPTOSIS is one of the most influential events that shape the characteristics, progression, and fate of atherosclerotic lesions. In early lesion stages macrophage apoptosis, combined with efficient removal of apoptotic cells through efferocytosis, maintains low cellularity and slows lesion growth. In contrast, in advanced plaques where efferocytosis is impaired, accumulation of apoptotic cells contributes to increased necrosis and plaque instability, the preamble of acute coronary syndromes (4, 19, 25). A significant amount of effort in the field is devoted to identifying molecular components associated with those mechanisms, with the hope of pinpointing candidate targets for molecular or pharmacological manipulation of macrophage function that may lead to better therapeutic control of the disease. In vivo, the existence of diverse macrophage populations adds a level of complexity that cannot be ignored. Indeed, a number of studies in human and murine lesions indicate that several macrophage phenotypes exist and that their relative abundance varies with lesion stage (12). Among these, the M1, or inflammatory, and M2, or anti-inflammatory, types, while likely representing extremes of a spectrum of phenotypes, seem to dominate during atherosclerosis. Nevertheless, little is known about specific signaling molecules that might play a role in apoptosis of these macrophage types in the context of atherogenesis.

Transient Receptor Potential (TRP) Canonical (TRPC) channels are among the most important calcium-permeable nonselective cation channels in the cardiovascular system and hematopoietic cells (1). Recent work from our laboratory showed that TRPC3, a member of the TRPC family (1, 26), is an obligatory component of survival mechanisms in murine bone marrow-derived macrophages (BMDMs) (21, 22). In Trpc3-deficient, nonpolarized BMDMs, typical survival signaling was markedly impaired, resulting in increased apoptotic rates, leading to the speculation that macrophage deficiency of TRPC3 would be particularly detrimental in the setting of advanced atherosclerotic lesions (22). Contrarily to this prediction, in recent in vivo studies we found that advanced aortic lesions in $Apoe^{-/-}$ mice with bone marrow deficiency of TRPC3 exhibited a reduction in necrotic core area that correlated with a marked decrease in the number of apoptotic macrophages compared with lesions from control mice (23). This led us to speculate that the features of advanced plaques in these mice might respond to the fact that polarized macrophages dominate in the setting of advanced lesions, and that TRPC3 deficiency may have a different impact in apoptosis of polarized macrophages compared with nonpolarized cells. In line with this interpretation and in contrast with our previous observations in nonpolarized BMDMs (22), M1, but not M2, macrophages derived from $Apoe^{-/-}$ mice with $Trpc3^{-/-}$ bone marrow were less sensitive to endoplasmic reticulum (ER) stress-induced apoptosis than TRPC3-expressing M1 cells (23). Nevertheless, whether this was due to a specific effect of TRPC3 deficiency on macrophage ER stress signaling remained to be determined. To answer this question, in the present work we used BMDMs from mice with macrophage-specific deficiency of TRPC3 to examine potential mechanisms underlying the reduced ER stress-dependent apoptosis of M1 macrophages. Our findings show that the decreased susceptibility of TRPC3-deficient M1 macrophages to ER stress-induced apoptosis is associated with impaired unfolded protein response (UPR), reduced mitochondrion-dependent apoptosis, and reduced ER stress-dependent activation of the proapoptotic molecules calmodulin-dependent

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protein kinase II (CAMKII) and signal transducer and activator of transcription 1 (STAT1). Remarkably, under identical experimental ER stress conditions, these signaling pathways remained unaltered in TRPC3-deficient M2 cells. Our findings are the first to show an obligatory requirement for a member of the TRPC family of cation channels in mechanisms associated with ER stress-induced apoptosis in polarized macrophages. Most notably, the present observations support the notion that the role of TRPC3 in those mechanisms selectively affects M1 macrophages.

MATERIALS AND METHODS

Generation of macrophage-specific TRPC3-deficient mice. Animal handling and use were in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and were approved by the University of Toledo Institutional Animal Care and Use Committee. A Cre/loxP strategy was used to generate myeloid-specific TRPC3-deficient mice. Floxed Trpc3 mice (129SvTrpc3$^{lox/lox}$) were provided by the Comparative Medicine Branch of the National Institute of Environmental Health Sciences (L. Birnbaumer). 129SvTrpc3$^{lox/lox}$ mice carry loxP sites into introns flanking exon 7 of Trpc3, which codes for the pore and the 6th transmembrane regions of the channel (generation of 129SvTrpc3$^{lox/lox}$ mice is described in Ref. 5). LysMCre mice (C57BL/6 background) have macrophage-specific expression of Cre recombinase driven by lysozyme M promoter (Ref. 2; obtained from Jackson Labs). Homozygous Trpc3$^{lox/lox}$ mice were crossed with homozygous LysMCre mice to generate LysMCre$^{+/-}$/Trpc3$^{lox/+}$ mice, which were intercrossed again with Trpc3$^{lox/lox}$ mice to obtain LysMCre$^{+/-}$/Trpc3$^{lox/lox}$ mice. PCR of tail gDNA was used to screen for potential knockouts with the following Trpc3 primers: forward (F): GAT GGC TCA GCA GTT AAA AGC TCT GG, reverse (R): GAA GTC ACT TCA GAC AGT CCA AAT AT. Potential knockouts were identified by the presence of a 461-bp amplicon corresponding to the floxed Trpc3 allele, a 700-bp amplicon corresponding to the mutant LysM Cre recombinase allele, and absence of the wild-type 380-bp Trpc3 amplicon (Fig. 1*A*); the presence of mutant allele LysMCre was tested in separate PCR reactions with primers F: CCCAGAAATGCCAGATTACG and R: TTGGGCTGCCAGAATTTCTC and primers F: CTT GGG CTG CCA GAA TTT CTC and R: TTA CAG TCG GCC AGG CTG AC for the wild-type LysM allele (350 bp), as recommended by Jackson Labs. We selected LysMCre$^{+/-}$/Trpc3$^{lox/lox}$ mice, which had insertion of only one allele of LysMCre construct, to allow functional expression of endogenous lysozyme M from the second allele (as recommended in Ref. 2). Trpc3$^{lox/lox}$ (or Trpc3$^{+/+}$) mice that were negative for mutant LysMCre were used as controls. LysMCre$^{+/-}$/Trpc3$^{lox/lox}$ mice (for simplicity we refer to these mice throughout the text as MacTrpc3KO) showed no gross phenotypes and were good breeders.

Preparation of bone marrow-derived macrophages. Preparation of BMDMs and in vitro differentiation to the M1 or M2 phenotype was performed essentially as described by us in References 22 and 23. Polarization to the M1 or M2 phenotype was confirmed by semiquantitative real-time PCR (qRT-PCR) with primers for markers of M1 (iNOS, TNFα) and M2 (ArgI, mannose receptor) phenotypes, as we described in Reference 23.

In vitro TUNEL assay. Apoptosis was assayed by using the In Situ Cell Death Detection Kit, TMR red (Roche) as we described in Reference 23.

Cell lysis and immunoblotting. Cell lysis and immunoblotting were performed essentially as we described in References 22 and 24. Briefly, after cell lysis, solubilized proteins were separated in 10% acrylamide gels, electrotransferred to polyvinylidene difluoride (PVDF) membranes, and immunoblotted with the indicated primary antibody. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham). Primary antibodies used were inositol-requiring enzyme 1α (IRE1), C/EBP-homologous protein (CHOP), cleaved poly(ADP-ribose) polymerase (PARP), phospho-CAMKII (Thr286), phospho-STAT1 (Ser727), and total STAT1, all from Cell Signaling; glyceraldehyde-3-phosphate dehy-

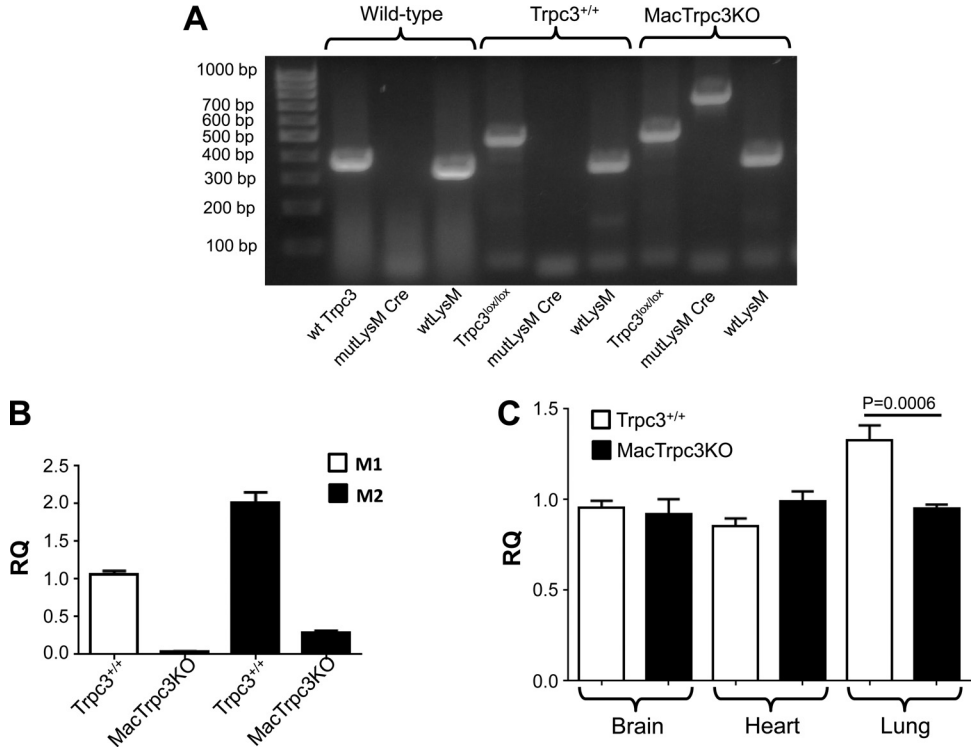


Fig. 1. *A*: genotyping of macrophage-specific knockout (MacTrpc3KO) mice using gDNA from tail clips. Genotyping of wild-type (wt) (C57BL/6) and Trpc3$^{+/+}$ mice is shown for comparison. MacTrpc3KO animals are characterized by the presence of a 461-bp amplicon corresponding to the floxed Trpc3 gene (Trpc3$^{lox/lox}$), a 700-bp amplicon corresponding to the mutant LysM Cre recombinase allele (mutLysMCre), a 350-bp amplicon corresponding to the wild-type LysM allele (wtLysM), and absence of the 380-bp amplicon that corresponds to wild-type Trpc3. *B*: semiquantitative real-time PCR (qRT-PCR) on cDNA from M1 and M2 macrophages prepared from bone marrow of Trpc3$^{+/+}$ and MacTrpc3KO mice. *C*: qRT-PCR on cDNA prepared from brain, heart, and lungs of Trpc3$^{+/+}$ and MacTrpc3KO mice. RQ, relative quantitation.

drogenase (GAPDH) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Real-time PCR. Real-time PCR (RT-PCR) was performed essentially as we described in Reference 23. cDNA was evaluated with qRT-PCR using TrueAmp SYBR Green qPCR supermix (Applied Biosystems). The relative amount of mRNA was calculated by comparison to the corresponding standards and normalized relative to Gapdh. Results are expressed as means ± SE relative to $Trpc3^{+/+}$-M1 controls [relative quantitation (RQ)]. Sequences of primers used are as follows: arginase I (F: CAGAAGAATGGAAGAGTCAG; R: CAGATATGCAGGGAGTCACC), iNOS (F: TGCATGGACCAGTATAAGGCAAGC; R: TTCTGGTCGATGTCATGAGCAA), TNFα (F: CAGGCGGTGCCTATGTCTC; R: CGATCACCCCGAAGTTCAGTAG), mannose receptor (F: CTCTGTTCAGCTATTGGACGC; R: CGGAATTTCTGGGATTCAGCTTC), TRPC3 (F: TTCAAGACTTTGTTTTGGTCCAT; R: AGCAATTAGCATGTTGAGTAAAACG, sequences designed and kindly provided by Dr. Nancy Rusch, Univ. of Arkansas for Medical Sciences), PKR-like eukaryotic initiation factor 2α kinase (PERK) (F: TGGAGTCCCTGCTCGAATCTT; R: ACTGTAAGCACTGAGTCCGTA), IRE1 (F: ACACCGACCACCGTATCTCA; R: CTCAGGATAATGGCCATGTC), CHOP (F: AAGCCTGGTATGAGGATCTGC; R: TTCCTGGGGATGAGATATAGGTG), ER oxidoreductase-1α (ERO1α) (F: AAGCCAACCGCATTGAAGAAT; R: CTGTCTGACGAATCATCATGCT), Bcl-2 homologous antagonist killer (Bak) (F: GCCCTGTACGTCTACCAGC; R: TGGCGATGTAATGATGCAGTATG), Bcl-2 interacting mediator of death (Bim) (F: CCCGGAGATACGGATTGCAC; R: GCCTCGCGGTAATCATTTGC), spliced X-box binding protein 1 (s-XBP1) (F: CTGAGTCCGAATCAGGTGCAG; R: GTCCTAGGGAAGATGTTCTGG), TRPC1 (F: GTCGCACCTGTTATTTTAGCTGC; R: TGGGCAAAGACACATCCTGCTGGGCAAAGACACATCCTGC), TRPC2 (F: GTCATCATGTGGCACTAACACC; R: TGTTCACAATCTCAGTCCAGTTG), TRPC4 (F: TGACTTCGTTGAGTGCCAAAG; R: TCAGTTACAAACGCCATAGCC), TRPC5 (F: GTGTATCCAGTTCGGAGGTAGA; R: CCTCGCTTGATAAGGCAATGA), TRPC6 (F: GCTTCCGGGGTAATGAAAACA; R: GTATGCTGGTCCTCGATTAGC), TRPC7 (F: TCCTGGACTCGGCTGAGTATG; R: GCAGTTGAAATTGAGGGTCTTGG), TRP Vanilloid (TRPV)2 (F: CGGCACTTCCTCTCT; R: GTCGGTCACGGTCAA), Melastatin-Related TRP (TRPM)2 (F: CAGATCCCAACCTACATTGACG; R: GAAGGTGTAGTTGAACATGGCGA), TRPM4 (F: CCCTGAGGATGGTGTTGAGT; R: AGGAGCACTGGGATGTCAAT), and Gapdh (F: AGGTCGGTGTGAACGGATTTG; R: TGTAGACCATGTAGTTGAGGTCA). PrimerBank (18, 27) identification numbers are TNFα: 133892368c1; mannose receptor: 224967061c1; PERK: 124001563c3; CHOP: 160707928c1; IRE1: 13249351a1; ERO1α: 322506110c2; TRPC1: 2052504a1; TRPC2: 6755887a1; TRPC4: 254540209c2; TRPC5: 118130397c2; TRPC6: 160333369c2; TRPC7: 118129964c1; Gapdh: 6679937a1; Bak: 111955301c3; Bim: 6753192a1. Primers for arginase I and iNOS were as in Reference 7, for s-XBP1 as in Reference 28, and for TRPV2, TRPM2, and TRPM4 as in Reference 9.

Statistical analysis. Values are shown as means ± SE for the corresponding *n* values indicated. Comparison of mean values between groups was performed with a two-tailed Student's *t*-test using Prism GraphPad version 6 for Windows 2007 (GraphPad Software, San Diego, CA). *P* values of <0.05 were considered significant.

RESULTS

We generated mice with macrophage-specific deletion of Trpc3 by crossing $Trpc3^{lox/lox}$ (or $Trpc3^{+/+}$) mice with mice having myeloid-specific expression of Cre recombinase (LysMCre) to generate $LysMCre^{+/-}/Trpc3^{lox/lox}$ mice (for simplicity, MacTrpc3KO; see MATERIALS AND METHODS for details; Fig. 1*A*). Trpc3 mRNA levels were drastically decreased (>90%) in both M1- and M2-type BMDMs from MacTrpc3KO mice compared with control macrophages (Fig. 1*B*). TRPC3 deficiency was specific for macrophages and not observed in brain, heart, or lung (Fig. 1*C*). The ~25% decrease in Trpc3 mRNA observed in lungs from MacTrpc3KO mice likely reflects the high macrophage content of this tissue. Importantly, deletion of TRPC3 did not affect the expression level of the other members of the TRPC family (Fig. 2*A*) or that of other Ca^{2+}-permeable TRP superfamily members, such as TRPV2, TRPM2, and TRPM4 (Fig. 2*B*), known to be expressed in mouse primary macrophages (25). In both M1 and M2 macrophages TRPC3 deficiency correlated with a >90% reduction in constitutive barium influx, a hallmark of TRPC3 channels operating in constitutive mode (not shown).

In previous work using macrophages from mice with bone marrow-specific deficiency of TRPC3 (23) we found a marked reduction of ER stress-induced apoptosis in M1 but not M2 macrophages deficient in TRPC3 compared with control, TRPC3-expressing cells. To determine whether this was also the case in BMDMs from $Trpc3^{lox/lox}$ ($Trpc3^{+/+}$) and MacTrpc3KO mice, and thus to assess their utility as a model to study the impact of TRPC3 deficiency on ER stress signaling, we first examined whether their response to ER stress-induced apoptosis was similar to that observed in our previous work. To that end, BMDMs from $Trpc3^{+/+}$ and MacTrpc3KO mice were induced to differentiate in vitro to the M1 or M2 phenotype. As in our previous observations in macrophages from mice with $Trpc3^{-/-}$ bone marrow (23), TRPC3 deficiency did not affect expression of specific M1 or M2 phenotypic markers (Fig. 3). Macrophages were serum starved or treated with thapsigargin (1 μM) for 24 h to induce ER stress, and apoptosis was then evaluated by an in vitro terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Under these conditions and similarly to our previous observations in polarized macrophages from mice with $Trpc3^{-/-}$ bone marrow (23), apoptosis was markedly reduced in $Trpc3^{-/-}$-M1 macrophages compared with controls (Fig. 4*A*); under identical conditions, the susceptibility of $Trpc3^{-/-}$-M2 macrophages to these proapoptotic stimuli was not different from that of $Trpc3^{+/+}$-M2 cells (Fig. 4*B*). In addition, when macrophage ER stress was induced by inhibiting protein glycosylation with tunicamycin (5 μg/ml, 24 h) or by promoting accumulation of free cholesterol by treatment with acetylated LDL (40 μg/ml) in the presence of the acyl coenzyme A:cholesterol acyl transferase (ACAT) inhibitor SA58035 (10 μg/ml), apoptosis was evident in M1 and M2 macrophages derived from control and MacTrpc3KO mice (Fig. 4, *A* and *B*); however, this effect was markedly reduced in M1 but not M2 macrophages deficient in TRPC3. The reduction in apoptosis exhibited by $Trpc3^{-/-}$-M1 macrophages was directly correlated with a decrease in the levels of cleaved PARP compared with $Trpc3^{+/+}$ cells (not shown). In addition, compared with $Trpc3^{+/+}$ macrophages, M1 but not M2 cells lacking TRPC3 also showed reduced susceptibility to the proapoptotic actions of staurosporine (Fig. 5, *A* and *B*), which induces apoptosis mostly through the mitochondrial pathway and independently from the UPR. Indeed, even at the highest concentrations used staurosporine did not induce expression of the ER stress markers IRE1 or CHOP in M1 or M2 macrophages (Fig. 5*C*). The mRNA levels of Bim and of Bak, two proapoptotic members of the Bcl-2 family that are involved in apoptosis induced by ER stress and the mitochondrial pathway,

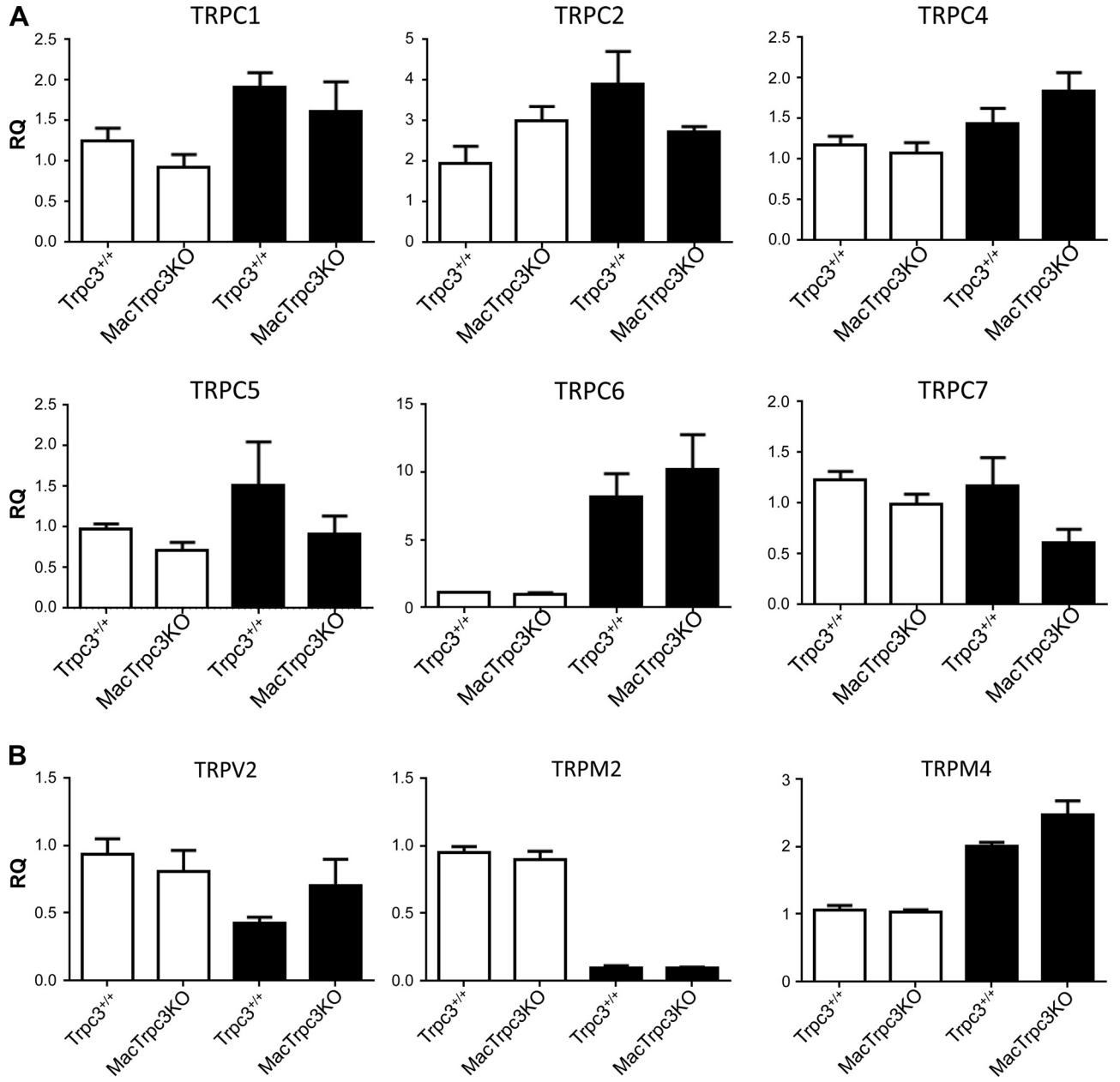


Fig. 2. Expression level of mRNA of members of the Transient Receptor Potential (TRP)C (*A*) and TRP Vanilloid (TRPV) and Melastatin-Related TRP (TRPM) (*B*) families was examined by qRT-PCR on cDNA from M1 and M2 macrophages prepared from bone marrow of $Trpc3^{+/+}$ and MacTrpc3KO mice. Sequences of the primers used are provided in MATERIALS AND METHODS. Graphs represent data (means ± SE) of 3 independent experiments performed in triplicate.

respectively (8, 14), were also markedly decreased in $Trpc3^{-/-}$-M1 macrophages subjected to tunicamycin-induced ER stress compared with $Trpc3^{+/+}$ cells [Bim: 0.49 ± 0.040 vs. 0.21 ± 0.03 ($P = 0.0002$), Bak: 0.61 ± 0.03 vs. 0.41 ± 0.02 ($P < 0.0001$) ($n = 3$–5), for normalized mRNA levels in $Trpc3^{+/+}$ vs. MacTrpc3KO macrophages, respectively].

We next evaluated the expression level of markers of UPR signaling in M1 and M2 macrophages derived from both groups of animals. As in other eukaryotic cells, three major sensor proteins drive UPR signaling in macrophages: PERK, IRE1, and activating transcription factor-6 (ATF6) (6). M1 and M2 macrophages were serum starved or exposed to thapsigargin or tunicamycin for 24 h, and the expression level of IRE1 was examined by Western blot. As shown in Fig. 6, total protein levels of IRE1 were markedly reduced in $Trpc3^{-/-}$-M1 macrophages compared with control cells, whereas TRPC3 deficiency did not affect total IRE1 levels in M2 macrophages. IRE1 activation can be assessed indirectly by the extent of autophosphorylation on Ser724 or by its endoribonuclease activity, which derives in selective splicing of the mRNA encoding for the transcription factor X-box binding protein 1 (XBP1) into an active spliced form (s-XBP1) (17). Notably, in $Trpc3^{-/-}$-M1 macrophages mRNA levels for s-XBP1 were reduced both under basal and ER stress conditions, supporting the notion of a correlation between reduced total IRE1 expression and activity [basal: 0.87 ± 0.05 vs. 0.27 ± 0.06 ($P = 0.0005$), thapsigargin treatment: 2.34 ± 0.04 vs. 1.60 ± 0.20 ($P = 0.03$) ($n = 3$), for normalized mRNA levels in $Trpc3^{+/+}$

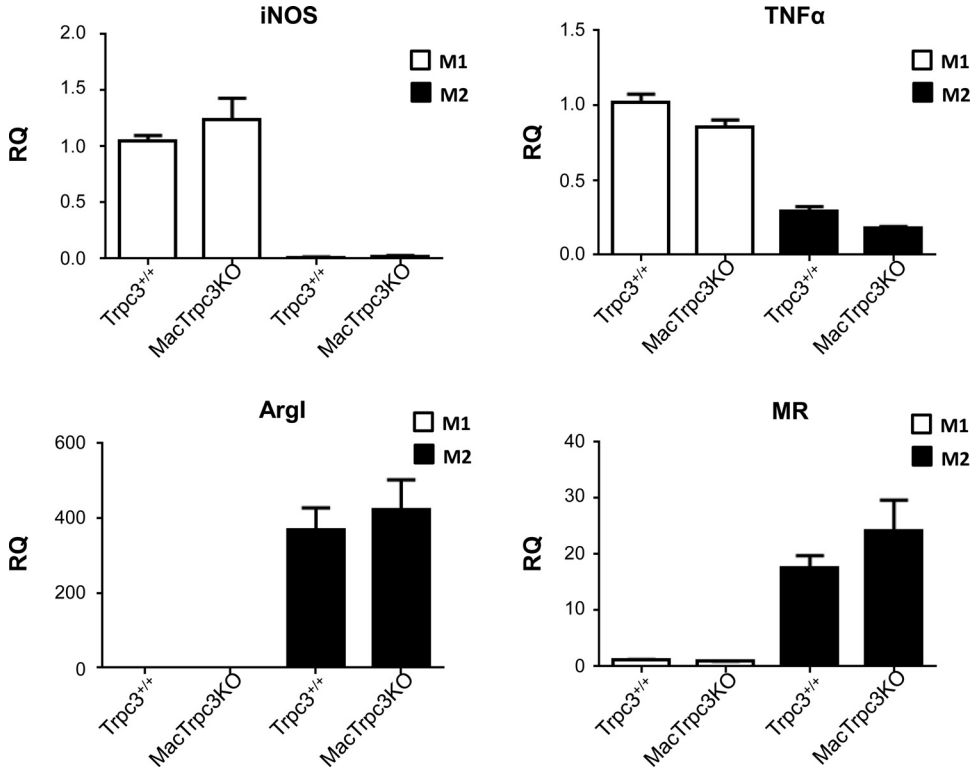


Fig. 3. Bone marrow-derived macrophages from Trpc3$^{+/+}$ or MacTrpc3KO mice were polarized to M1 or M2 phenotype, and RNA and cDNA were prepared as described in MATERIALS AND METHODS. Expression levels of specific markers for M1 or M2 macrophage phenotypes were examined by qRT-PCR as described in MATERIALS AND METHODS. iNOS, inducible nitric oxide synthase; TNFα, tumor necrosis factor α; ArgI, arginase I; MR, mannose receptor. Graphs represent data (means ± SE) of at least 3 independent experiments performed in triplicate.

vs. MacTrpc3KO macrophages, respectively]. After tunicamycin treatment, PERK mRNA levels were also decreased in M1 but not M2 macrophages (Fig. 6*B*). In addition, M1, but not M2, cells lacking TRPC3 also exhibited reduced expression levels of CHOP (Fig. 7*A*) and of ERO1α, a transcriptional target of CHOP (Fig. 7*B*).

CAMKII and STAT1 are important components of the mechanisms mediating ER stress-induced apoptosis in macrophages (10). In previous work we found that TRPC3-mediated constitutive Ca^{2+} influx modulates CAMKII function in macrophages (21, 24). Thus we next examined the activation status of CAMKII and also that of STAT1 in M1 and M2 macrophages derived from Trpc3$^{+/+}$ and MacTrpc3KO mice when exposed to conditions that promote ER stress. Similar to our previous findings in polarized macrophages from mice with bone marrow deficiency of TRPC3 (23), basal and ER stress (serum starvation, thapsigargin or tunicamycin treatments)-induced activation of CAMKII and STAT1, as indicated, respectively, by the extent of phosphorylation of Thr286 (CAMKII) and of Ser727 (STAT1), were severely impaired in TRPC3-deficient M1 macrophages compared with control cells (Fig. 8). Basal and ER stress-induced phosphorylation status of CAMKII and STAT1 were comparable between Trpc3$^{+/+}$ and Trpc3$^{-/-}$ M2 macrophages (Fig. 8). In elicited peritoneal macrophages CAMKII is a major contributor to the activation of STAT1 through phosphorylation of Ser727 on STAT1 (10). In M1 macrophages pretreated with the CAMKII inhibitor KN-93 (10 μM) STAT1 phosphorylation on Ser727 was not significantly affected, suggesting that in these cells STAT1 phosphorylation on Ser727 does not require CAMKII (not shown). Interestingly, in M1 cells the phosphorylation status of CAMKII on Thr286 under basal conditions was completely abrogated by TRPC3 deficiency, suggesting that TRPC3, presumably through its constitutive function, supports the mechanisms underlying tonic activity of this kinase. In agreement with this notion, treatment of Trpc3$^{+/+}$-M1 macrophages with the selective TRPC3 blocker pyrazole-10 (Pyr10; Ref. 16) resulted in almost complete abrogation of CAMKII Thr286 phosphorylation (Fig. 9*A*). Inducing ER stress under conditions of free cholesterol loading also resulted in activation of CAMKII and STAT1 in TRPC3-expressing M1 and M2 macrophages (Fig. 9, *A* and *B*, respectively). Remarkably, whereas in Trpc3$^{+/+}$-M1 macrophages the effect of free cholesterol loading on CAMKII and STAT1 phosphorylation was drastically reduced by Pyr10, the selective TRPC3 inhibitor had no effect on M2 cells (Fig. 9*B*).

DISCUSSION

In recent in vivo studies we found that advanced lesions in Apoe$^{-/-}$ mice with bone marrow deficiency of TRPC3 had, compared with control animals, reduced areas of necrosis and fewer apoptotic macrophages (23). This correlated with reduced ER stress-induced apoptosis, in vitro, of M1 but not M2 macrophages derived from Trpc3$^{-/-}$Apoe$^{-/-}$ animals (23). The question then arose of whether this was due to a specific effect of TRPC3 deficiency on macrophage UPR. To answer this question, in the present work we used polarized macrophages derived from mice with macrophage-specific deficiency of TRPC3 (MacTrpc3KO) and examined the expression level of signaling components of the UPR pathway and the activation status of typical mediators of macrophage apoptosis.

Our findings show that, similarly to our previous observations in polarized macrophages from mice with Trpc3$^{-/-}$ bone marrow (23), M1 macrophages derived from MacTrpc3KO mice exhibit a marked reduction in ER stress-induced apopto-

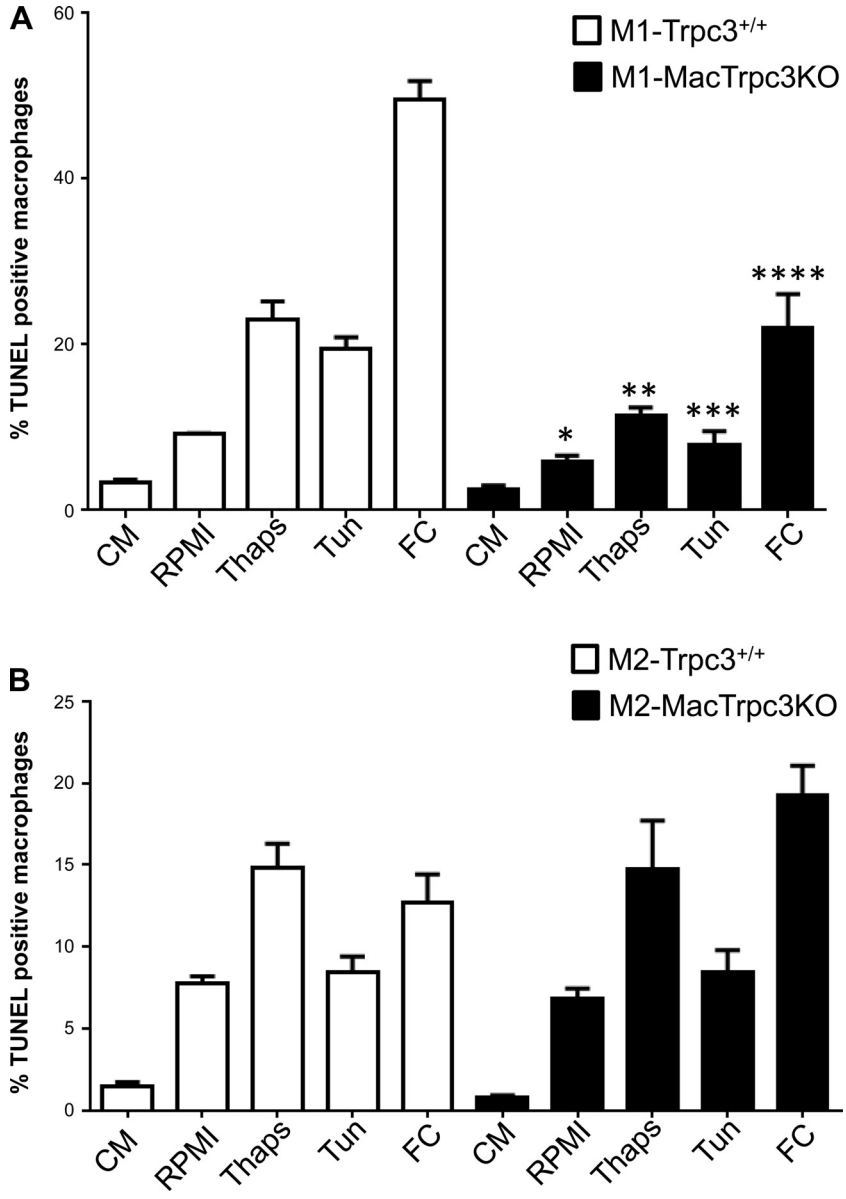
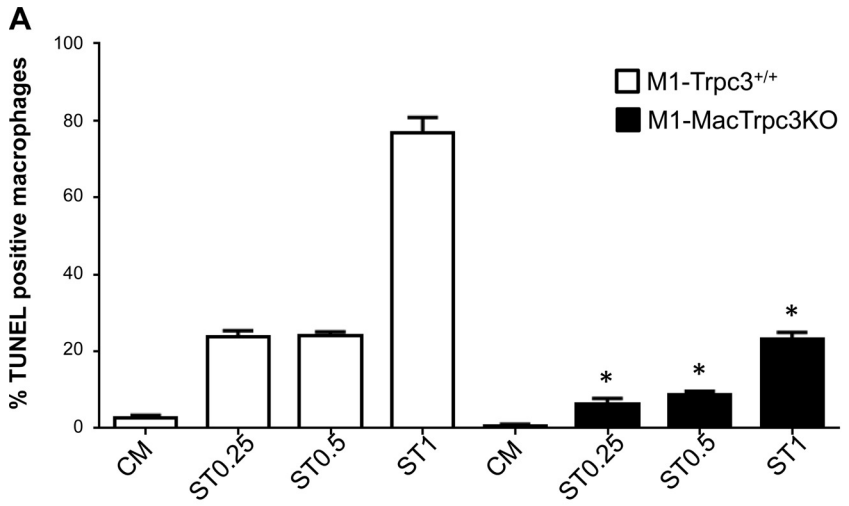


Fig. 4. Bone marrow-derived macrophages from Trpc3$^{+/+}$ or MacTrpc3KO mice were polarized to the M1 (*A*) or M2 (*B*) phenotype and incubated (24 h) in complete growth medium (CM), serum-free RPMI (RPMI), or RPMI containing thapsigargin (Thaps, 1 μM) or tunicamycin (Tun, 5 μg/ml) or under conditions that result in cellular free cholesterol loading [FC: 40 μg/ml acetylated LDL + 10 μg/ml acyl coenzyme A:cholesterol acyl transferase (ACAT) inhibitor SA58035] and then processed for in vitro terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. In *A*, $*P = 0.014$, $**P = 0.003$, $***P = 0.002$, $****P = 0.001$ for the difference between equivalent treatments in MacTrpc3KO vs. Trpc3$^{+/+}$ macrophages. In both Trpc3$^{+/+}$ or MacTrpc3KO macrophages differences between treatment conditions and corresponding controls were statistically significant ($P < 0.01$). In *B*, there were no significant differences between MacTrpc3KO and Trpc3$^{+/+}$ macrophages. All differences between treatment conditions and corresponding controls had $P < 0.001$. In both *A* and *B* all values are means ± SE ($n = 3$–5).

sis subsequent to serum starvation or thapsigargin treatment. In addition, inducing ER stress with the *N*-glycosylation inhibitor tunicamycin or by subjecting macrophages to conditions of free cholesterol loading also resulted in less apoptosis in TRPC3-deficient M1 macrophages compared with control cells. In all instances, TRPC3 deficiency selectively affected M1 macrophages, as apoptosis of M2 cells lacking TRPC3 was comparable to that in Trpc3$^{+/+}$-M2 macrophages. In Trpc3$^{-/-}$-M1 macrophages there was a correlation between reduced apoptosis and reduced levels of cleaved PARP, a direct substrate of caspase-3, indicating that lack of TRPC3 seriously compromises the execution stages of macrophage apoptosis.

Whereas the stimuli that promote macrophage apoptosis in vivo in the setting of inflammation are quite diverse, persistent and unresolved ER stress and the subsequent UPR signaling is a common underlying mechanism. The present findings show that compared with Trpc3$^{+/+}$ cells the expression levels of the UPR components IRE1 and PERK are markedly reduced in TRPC3-deficient M1 macrophages. Activation of IRE1 is in part a consequence of its dissociation from chaperone proteins, e.g., BiP, and binding to unfolded proteins in the ER (15). Thus our immunoblot experiments, although not reflecting the activation status of IRE1, show that TRPC3 deficiency severely impairs expression of this ER stress sensor molecule. Selective splicing of the mRNA encoding for the transcription factor XBP1 into the active s-XBP1 form is a specific indicator of IRE1 endoribonuclease activity (17). Our findings showing a reduction of s-XBP1 mRNA in Trpc3$^{-/-}$-M1 macrophages under both basal and ER stress conditions are in line with the decreased IRE1 expression in these cells. Interestingly, reduced expression of IRE1 has been associated with improved survival rates of mammalian cells under conditions of prolonged ER stress (Ref. 20 and references therein).

The finding that levels of the proapoptotic transcription factor CHOP were reduced in M1 but not M2 macrophages lacking TRPC3 is in concordance with the overall impaired



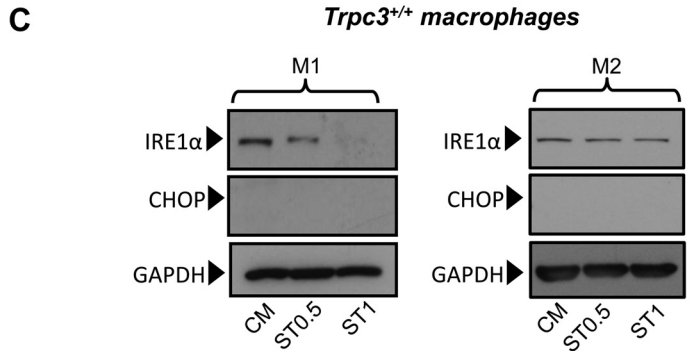


Fig. 5. Bone marrow-derived macrophages from Trpc3$^{+/+}$ or MacTrpc3KO mice were polarized to the M1 (*A*) or M2 (*B*) phenotype and incubated for 24 h in complete growth medium or RPMI containing staurosporine (ST; 0.25, 0.5, or 1 μM) and then processed for in vitro TUNEL assay. In *A*, $*P < 0.0001$ for the differences between equivalent treatments in MacTrpc3KO vs. Trpc3$^{+/+}$ macrophages. In *B*, $*P = 0.003$, $**P = 0.03$, $***P < 0.0001$ for the differences in respect to the equivalent treatments in Trpc3$^{+/+}$ macrophages. In all instances, differences between staurosporine treatments and their corresponding controls had $P < 0.001$. All values are means ± SE (n = 3–5). In *C*, bone marrow-derived macrophages from Trpc3$^{+/+}$ mice were polarized to the M1 or M2 phenotypes and incubated for 24 h in complete growth medium or RPMI containing staurosporine (0.5 or 1 μM) and then processed for immunodetection of inositol-requiring enzyme 1α (IRE1α; ~130 kDa) or C/EBP-homologous protein (CHOP; ~27 kDa) in whole cell lysates. Membranes were reprobed for GAPDH (~37 kDa) to control for protein loading. Blots are representative of 3 independent experiments.

UPR response of the M1 cells, as CHOP can be induced by all branches of the UPR. One important mechanism of CHOP-induced apoptosis is augmented ER oxidative stress by the CHOP transcriptional target ERO1α (13). In agreement with the reduction in CHOP expression, ERO1α levels were also decreased in TRPC3-deficient M1, but not M2, macrophages. ERO1α is also thought to promote calcium release from ER through luminal activation of inositol trisphosphate receptor type-1 followed by calcium-dependent activation of the pro-apoptotic CAMKII (20). It remains to be determined whether the impaired activation of CAMKII observed in TRPC3-deficient M1 macrophages is in part related to the reduced expression of the CHOP/ERO1α axis in these cells. In previous work we found that TRPC3-mediated constitutive calcium influx can modulate CAMKII function in macrophages (21, 24). Thus it is also possible that if a pool of proapoptotic CAMKII exists in

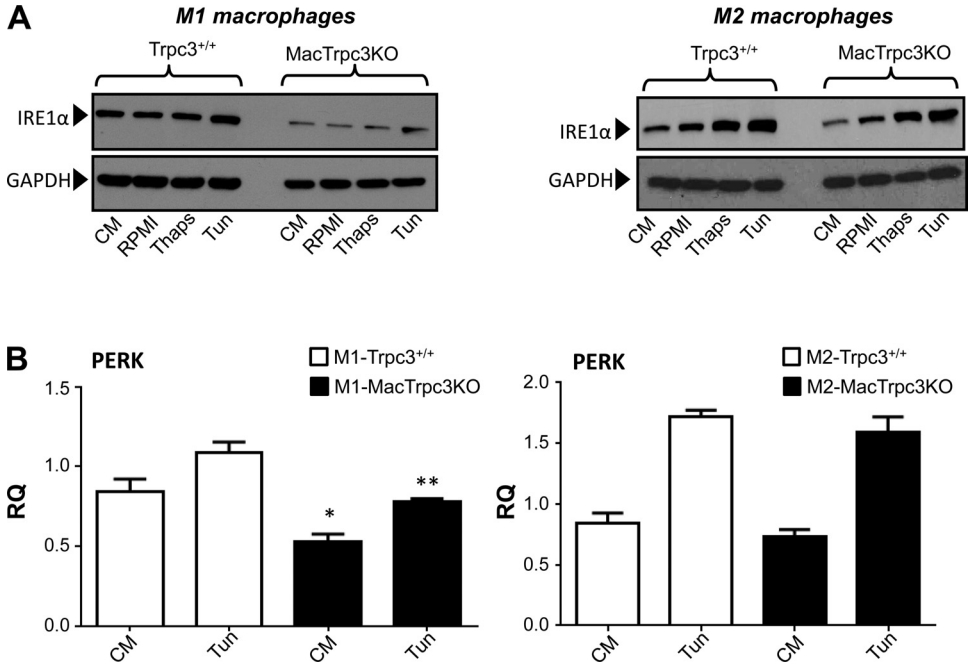


Fig. 6. *A*: bone marrow-derived macrophages from Trpc3$^{+/+}$ or MacTrpc3KO mice were polarized to the M1 or M2 phenotype and incubated (24 h) in complete growth medium, serum-free RPMI, or RPMI containing thapsigargin (1 μM) or tunicamycin (5 μg/ml) and then processed for immunodetection of IRE1α (~130 kDa) in whole cell lysates. Membranes were reprobed for GAPDH (~37 kDa) to control for protein loading. Blots are representative of 3 independent experiments. In *B*, M1 or M2 macrophages derived from bone marrow of Trpc3$^{+/+}$ or MacTrpc3KO mice were incubated (24 h) in complete growth medium or in RPMI containing tunicamycin (5 μg/ml). RNA and cDNA were prepared as described in MATERIALS AND METHODS, and mRNA levels of PKR-like eukaryotic initiation factor 2α kinase (PERK) were measured by qRT-PCR. Graphs represent data (means ± SE) of 3 independent experiments performed in triplicates. *P = 0.017, **P = 0.003 for the difference between equivalent treatments in MacTrpc3KO vs. Trpc3$^{+/+}$ cells. There were no significant differences in PERK mRNA levels between M2-MacTrpc3KO and M2-Trpc3$^{+/+}$ macrophages.

close proximity to the channel, calcium entering through TRPC3 can contribute to activation of this kinase. This notion is strongly supported by our findings showing that in M1 macrophages the phosphorylation status of CAMKII on Thr286 under basal conditions is abolished by TRPC3 deficiency or by treating TRPC3-expressing M1 cells with the TRPC3-selective blocker Pyr10. In the context of macrophage apoptosis STAT1 is another key component of proapoptotic mechanisms downstream of ER stress (10). In peritoneal macrophages CAMKII has been shown to contribute to activation

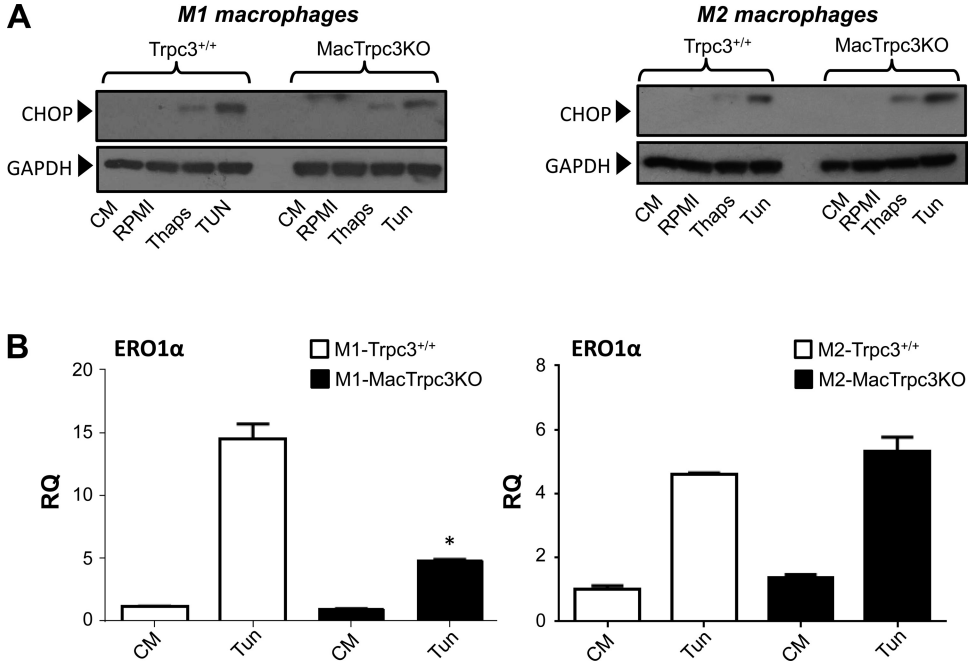


Fig. 7. *A*: bone marrow-derived macrophages from Trpc3$^{+/+}$ or MacTrpc3KO mice were polarized to the M1 or M2 phenotype and incubated (24 h) in complete growth medium, serum-free RPMI, or RPMI containing thapsigargin (1 μM) or tunicamycin (5 μg/ml) and then processed for immunodetection of CHOP (~27 kDa) in whole cell lysates. Membranes were reprobed for GAPDH (~37 kDa) to control for protein loading. Blots are representative of 3 independent experiments. In *B*, M1 or M2 macrophages derived from bone marrow of Trpc3$^{+/+}$ or MacTrpc3KO mice were incubated (24 h) in complete growth medium or in RPMI containing tunicamycin (5 μg/ml). RNA and cDNA were prepared as described in MATERIALS AND METHODS, and mRNA levels of ER oxidoreductase-1α (ERO1α) were measured by qRT-PCR. Graphs represent data (means ± SE) of 3 independent experiments performed in triplicate. *P = 0.0009 for the difference between equivalent treatment in MacTrpc3KO vs. Trpc3$^{+/+}$ cells. All differences between tunicamycin treatments and their corresponding controls have $P < 0.0001$. There were no significant differences in ERO1α mRNA levels between M2-MacTrpc3KO and M2-Trpc3$^{+/+}$ macrophages.

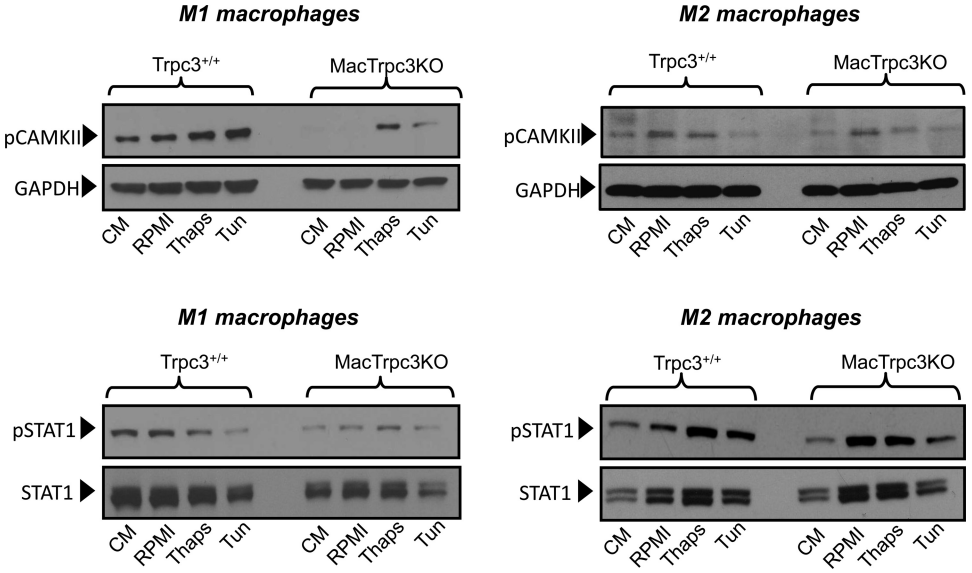


Fig. 8. Bone marrow-derived macrophages from Trpc3$^{+/+}$ or MacTrpc3KO mice were polarized to the M1 or M2 phenotype and incubated (24 h) in complete growth medium, serum-free RPMI, or RPMI containing thapsigargin (1 μM) or tunicamycin (5 μg/ml) and then processed for immunodetection of phospho-calmodulin-dependent protein kinase II (CAMKII) (Thr286) or phospho-signal transducer and activator of transcription 1 (STAT1) (Ser727) in whole cell lysates, as indicated. Membranes were reprobed for GAPDH or total STAT1 to control for protein loading. Blots are representative of 3 independent experiments.

of STAT1 by directly phosphorylating Ser727 on STAT1 (10). Our findings show that whereas both CAMKII and STAT1 activation in M1 macrophages are markedly dependent on TRPC3 expression, TRPC3 deficiency seems to have a more drastic effect on the activation of CAMKII than that of STAT1. In addition, we did not observe changes in STAT1 Ser727 phosphorylation under conditions of pharmacological inhibition of CAMKII. Altogether, these findings suggest that mechanisms other than CAMKII should account for Ser727 phosphorylation of STAT1 in the polarized macrophage.

In vitro, M1 macrophages are generated by inducing differentiation of cultured nonpolarized macrophages established from a heterogeneous population of bone marrow cells. In our previous studies M1 macrophages were generated from bone marrow obtained either from mice with global deficiency of TRPC3 or from transplanted mice that received bone marrow from Trpc3$^{-/-}$ donors (23). In that scenario, it can be argued that the impact of TRPC3 deficiency on M1 macrophages may also be influenced by any effect that TRPC3 deficiency might have on nonmyeloid cells present in the culture, which contribute to medium conditioning and to induction of macrophage-specific gene programs. The present results showing that the response to ER stress-induced apoptosis of M1 macrophages derived from MacTrpc3KO mice is similar to that of macrophages from mice with either global or bone marrow deficiency of TRPC3 (23) thus validate the utility of this new mouse model to expand not only into mechanistic studies on the role(s) of TRPC3 in ER stress signaling and apoptosis but most importantly as a new model to examine the in vivo relevance of our in vitro findings.

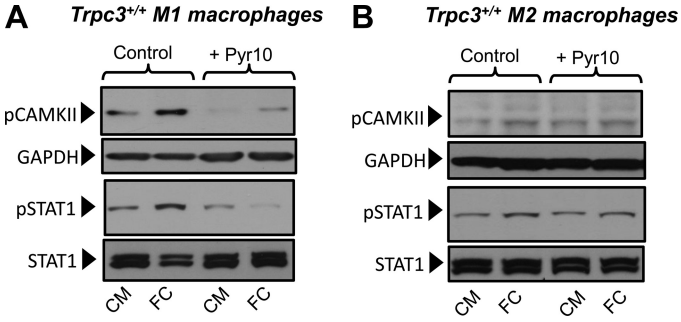


Fig. 9. Bone marrow-derived macrophages from Trpc3$^{+/+}$ mice were polarized to the M1 (*A*) or M2 (*B*) phenotype and incubated (24 h) in complete growth medium or subjected to free cholesterol loading (40 μg/ml acetylated-LDL + 10 μg/ml ACAT inhibitor SA58035), in the absence or presence of the selective TRPC3 blocker pyrazole-10 (Pyr10; 2 μM) as indicated and then processed for immunodetection of phospho-CAMKII (Thr286) or phospho-STAT1 (Ser727) in whole cell lysates. Membranes were reprobed for GAPDH or total STAT1 to control for protein loading. Blots are representative of 3 independent experiments.

Our findings also indicate that TRPC3-deficient M1 macrophages are more resistant than TRPC3-expressing cells to apoptosis induced by the alkaloid staurosporine. Whereas the mechanism by which staurosporine induces apoptosis is not fully understood, it is well established that it takes place independently of the UPR and is mediated, for the most part, by the mitochondrial apoptotic pathway (11). Recent studies by Feng et al. (3) showed the existence of functional TRPC3 channels in the inner mitochondrial membrane and showed that calcium entry through these channels contributes to the overall mitochondrial calcium uptake. Mechanisms that lead to exacerbated mitochondrial calcium uptake can reduce the mitochondrial transmembrane potential and increase mitochondrial membrane permeability, two critical early events in the mitochondrial apoptotic pathway. Although further studies are required to determine whether this is also the case in macrophages, it is tempting to speculate that the reduced proapoptotic effect of staurosporine in TRPC3-deficient M1 cells could be in part due to reduced—or delayed—calcium uptake into the mitochondria.

Altogether, the findings presented in this work show that genetic or pharmacological inhibition of TRPC3 results in a rather selective protection of M1 macrophages from ER stress-induced apoptosis with no effect on macrophages of the M2 type. At this time, the nature of such selectivity is unclear and warrants further studies. Functional TRPC channels are tetrameric, and TRPC3 can form homo (all 4 subunits are TRPC3)- and hetero [at least 1 subunit is TRPC3 + other TRPC member(s)]-tetramers (26). The final channel arrangement can

significantly affect not only channel regulatory and pharmacological properties but also its interactions with signaling partners. It is possible that differences exist between M1 and M2 macrophages in terms of TRPC3 channel makeup (i.e., homo- vs. heterotetrameric) or in the repertoire of potential signaling or adaptor proteins coupling TRPC3 to intracellular pathways that are expressed by each macrophage type. This may include coupling of TRPC3 function to mechanisms related to regulated expression of UPR components and/or to calcium-dependent signaling mediating ER stress-induced apoptosis. In this context, the finding that basal and ER stress-induced activation of CAMKII and STAT1 are severely impaired in TRPC3-deficient M1 but not M2 cells, and almost completely abolished by the selective TRPC3 blocker Pyr10 in $Trpc3^{+/+}$-M1 but not in $Trpc3^{+/+}$-M2 cells, suggests that coupling of TRPC3 function to these proapoptotic signaling molecules may indeed be phenotype specific.

In both human and murine atherosclerotic lesions the presence of both M1 and M2 macrophage populations is well documented. Although significant progress has been made in our understanding of mechanisms controlling macrophage apoptosis, little is known about specific signaling molecules that might play a role in apoptosis of specific macrophage types. This is important considering that the impact of apoptosis of M1 vs. M2 macrophages on lesion progression and plaque stability can be quite different depending upon their relative abundance at each lesion stage. Therefore, identification of targets that might selectively affect apoptosis of one particular macrophage phenotype may represent a unique interventional opportunity to manipulate lesional macrophages in a phenotype-selective manner. In this context, our findings indicating a selective role of TRPC3 channel on mechanisms related to ER stress-induced apoptosis of M1 macrophages represent a unique first step toward reaching that goal.

ACKNOWLEDGMENTS

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GRANTS

This work was supported by National Institutes of Health (NIH) Grant R01 HL-111877-01 (to G. Vazquez), the Intramural Research Program of the NIH Project Z01-ES-101864 (to L. Birnbaumer), and the University of Toledo College of Medicine.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.S., P.R.D., and G.V. conception and design of research; S.S., P.R.D., and J.-Y.T. performed experiments; S.S. and G.V. analyzed data; S.S. and G.V. interpreted results of experiments; S.S., P.R.D., J.-Y.T., L.B., and G.V. approved final version of manuscript; G.V. prepared figures; G.V. drafted manuscript; G.V. edited and revised manuscript.

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