

Targeting IRE1 with small molecules counteracts progression of atherosclerosis

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Metaflammation, an atypical, metabolically induced, chronic low-grade inflammation, plays an important role in the development of obesity, diabetes, and atherosclerosis. An important primer for metaflammation is the persistent metabolic overloading of the endoplasmic reticulum (ER), leading to its functional impairment. Activation of the unfolded protein response (UPR), a homeostatic regulatory network that responds to ER stress, is a hallmark of all stages of atherosclerotic plaque formation. The most conserved ER-resident UPR regulator, the kinase/endoribonuclease inositol-requiring enzyme 1 (IRE1), is activated in lipid-laden macrophages that infiltrate the atherosclerotic lesions. Using RNA sequencing in macrophages, we discovered that IRE1 regulates the expression of many proatherogenic genes, including several important cytokines and chemokines. We show that IRE1 inhibitors uncouple lipid-induced ER stress from inflammasome activation in both mouse and human macrophages. In vivo, these IRE1 inhibitors led to a significant decrease in hyperlipidemia-induced IL-1 β and IL-18 production, lowered T-helper type-1 immune responses, and reduced atherosclerotic plaque size without altering the plasma lipid profiles in apolipoprotein E-deficient mice. These results show that pharmacologic modulation of IRE1 counteracts metaflammation and alleviates atherosclerosis.

endoplasmic reticulum stress | unfolded protein response | metaflammation | lipotoxicity | atherosclerosis

Complex molecular interactions between environment, diet, and genetics that take place at the metabolic and immune interface provoke a low-grade, chronic inflammatory response—metaflammation—that engages cells of the immune system (macrophages, neutrophils, and lymphocytes) and metabolic tissues (adipocytes, hepatocytes, and pancreatic cells) (1). An important primer for metaflammation is chronic metabolic overloading of organelles, such as the endoplasmic reticulum (ER) and mitochondria, which results in impairment of their functions (2).

The ER serves essential cellular functions that include the synthesis and folding of secreted and transmembrane proteins, calcium storage, and lipid synthesis for membrane biogenesis or energy storage. Disruption of any of these functions leads to ER stress and the subsequent activation of an elaborate network of adaptive responses, collectively known as the unfolded protein response (UPR) (3). The UPR reestablishes homeostasis through both transcriptional and translational layers of control. The UPR signals through three mechanistically distinct branches that are initiated by the ER-resident protein folding sensors inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (3).

IRE1 controls the phylogenetically most conserved branch of the UPR found from fungi to metazoans. It has an ER-luminal sensor domain that recognizes unfolded peptides and cytosolic kinase and endoribonuclease (RNase) domains that relay the information to downstream effectors (3). On sensing unfolded proteins, IRE1 oligomerizes and *trans*-autophosphorylates, thereby activating its RNase function. Metazoan IRE1 possesses two

functional outputs dependent on its RNase activity. (i) It initiates a nonconventional splicing reaction that processes the mRNA encoding X-box binding protein-1 (*Xbp1*) to allow the translation of its active form, XBP1s, a potent transcription factor that, together with ATF6, drives expression of numerous genes, including those encoding ER-resident chaperones and ER-associated protein degradation machinery (3). (ii) IRE1 selectively degrades ER-bound mRNAs in a process known as regulated inositol-requiring enzyme 1-dependent decay (RIDD) to alleviate ER load (4). By these mechanisms, UPR activation reinstates homeostasis.

Increased ER stress and activation of the UPR are well-documented in atherosclerosis (5). Many metabolic cardiovascular risk factors observed in obesity, including hyperglycemia, hypercholesterolemia, and elevated saturated fats, can induce ER stress in all stages of atherogenesis, the process leading to the development of atherosclerotic plaques. During atherogenesis, a maladaptive inflammatory response is initiated by the deposition of cholesterol-rich lipoproteins in the subendothelial layer of arterial walls (6). Signs of ER stress are most prominent in the atherosclerosis-prone regions of vascular lesions, such as the branching points of arteries, and typically observed in macrophages—among other immune cells—infiltrating these regions (7, 8). Chronic, irremediable ER stress triggers apoptosis in macrophages, contributing to the growth of the necrotic core

Significance

Endoplasmic reticulum (ER) stress is linked to the development of complex metabolic diseases, including diabetes, obesity, and atherosclerosis. Irremediable ER stress can push the unfolded protein response (UPR) toward proinflammatory and proapoptotic signaling. The need to dissociate the adaptive UPR responses from its destructive outputs has become a major challenge for therapeutic strategies aimed at mitigating ER stress that is often observed in chronic diseases. Our findings show that inositol-requiring enzyme 1 (IRE1) plays a critical role in metaflammation and that administering IRE1-specific inhibitors to hyperlipidemic mice counteracts atherosclerosis progression.

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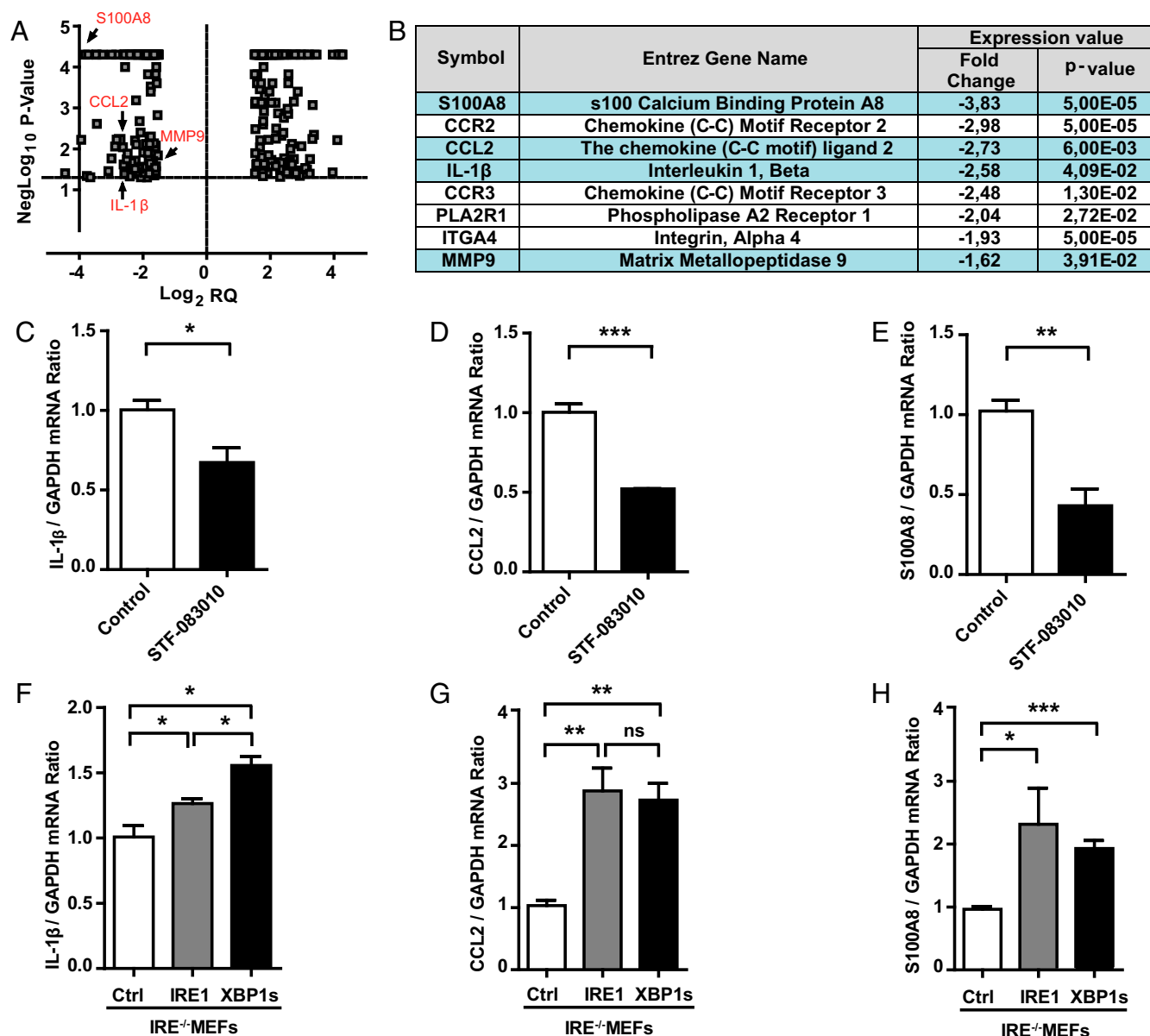


Fig. 1. IRE1 regulates the expression of proatherogenic genes. (A) RNA-seq analysis in BMDMs treated with 60 μ M STF-083010 or DMSO (control) for 6 hours. Volcano plot of differentially expressed mRNAs. (B) Analysis of atherosclerosis-related mRNAs using the IPA tool (details are in the text). (C–E) Confirmation of IRE1-dependent atherogenic gene regulation in mouse BMDMs treated with STF-083010 or DMSO (control) by qRT-PCR. (F–H) qRT-PCR analysis of atherogenic gene expression in IRE1^{−/−} MEFs on forced expression of XBP1s or restoring IRE1's function. Data: mean values \pm SEM; $n = 3$. Student's t test. Ctrl, control; ns, not significant. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

production of proatherogenic cytokines in both mouse and human macrophages.

Inflammasome Activation Depends on IRE1 During Lipotoxicity. Because IRE1 inhibition leads to a strong suppression of IL-1 β secretion, we reasoned that IRE1 may contribute to the lipid-induced activation of the Nod-like receptor family, pyrin domain-containing protein-3 (NLRP3) inflammasome, a multi-component platform that contains caspase-1 and induces the caspase-1-dependent secretion of the proinflammatory cytokines IL-1 β and IL-18 (42, 43). Previous studies showed that ER stress induces inflammasome activation through several mechanisms, including calcium mobilization and the release of reactive oxygen species from damaged mitochondria (mtROS) (44). Because earlier studies from our laboratory and the laboratories of others showed that treatment of macrophages with saturated

fatty acids activates IRE1 and because these lipids specifically activated the NLRP3 inflammasome through inducing mtROS production, we sought to investigate this connection further (42, 43, 45). To this end, we first measured mtROS production in cells exposed to lipotoxic stress in the presence of IRE1 inhibitors. We observed that lipid-induced ER stress in BMDMs resulted in a dramatic elevation of mtROS, which was completely blocked by 4 μ 8c treatment as well as XBP1 knockdown (Fig. 3 A and B and Fig. S4A).

The impact of IRE1 signaling on inflammasome activation has been postulated to be mediated by the IRE1-dependent accumulation of the thioredoxin-interacting protein (TXNIP), a thioredoxin inhibitor with increased levels that promote activation of the NLRP3 inflammasome (35). In stark contrast to these earlier findings, which used cells treated with canonical ER poisons, lipid-induced ER stress led to a profound suppression of

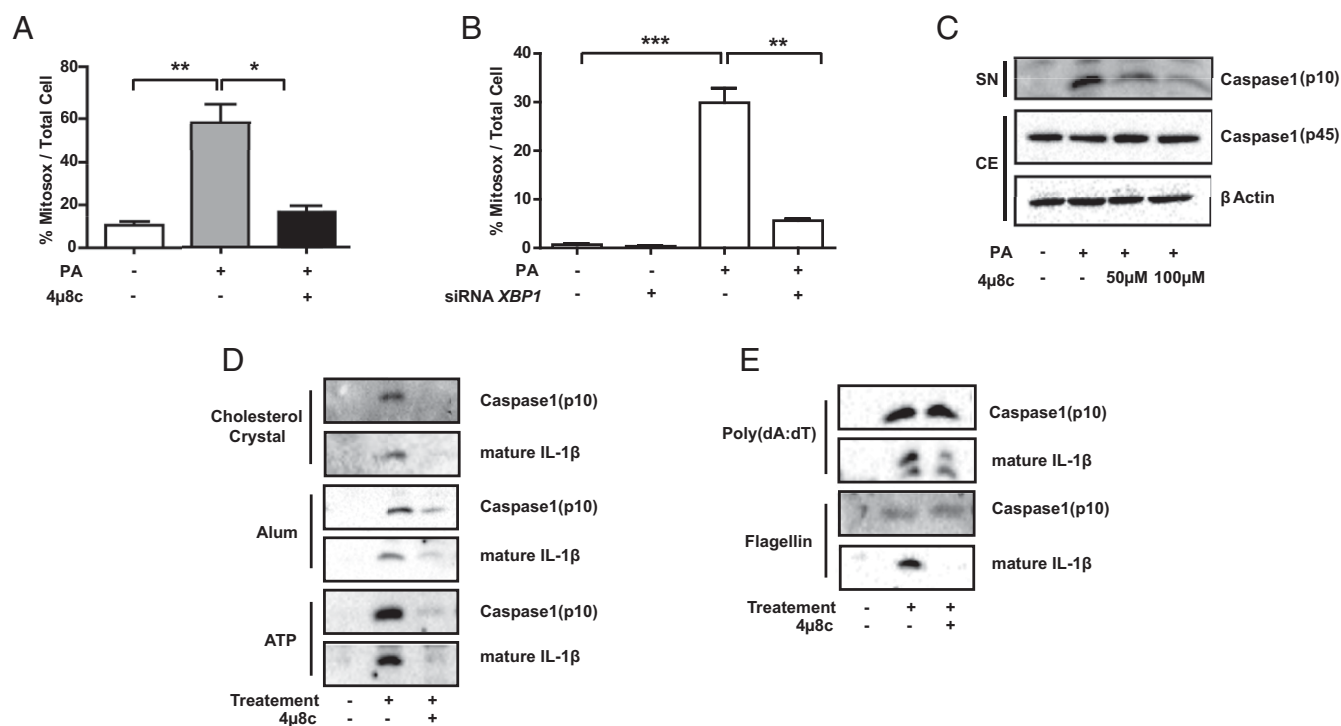


Fig. 3. IRE1 inhibitors block lipid-induced mtROS release and inflammasome activation. (A and B) mtROS production was measured in LPS-primed, PA-stimulated mouse BMDMs after (A) 100 μM 4μ8c or DMSO (control) treatment or (B) transfection with scrambled or *Xbp1*-specific siRNAs. (C–E) Immunoblots of the levels of the zymogen (p45) and mature (p10) forms of caspase-1 in LPS-primed mouse BMDMs pretreated with 4μ8c (at the indicated doses) or DMSO (control) and stimulated with (C) PA, (D) other NLRP3 agonists (5 nM ATP, 200 μg/mL alum, or 400 μg/mL cholesterol crystals), or (E) specific activators of other inflammasome complexes [5 μg/mL poly(dA:dT) and 1 μg/mL flagellin] according to previously published protocols (described in detail in *Experimental Procedures*). Blots shown are representative of three independent experiments. Statistics are the same as in Fig. 1. CE, cell extract; SN, supernatant. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

group (Fig. 4B). Furthermore, when we evaluated the impact of STF-083010 on plaque development in the aortic root, we observed a significant reduction (21.4%; $P < 0.001$) in the foam cell area (visualized by Oil Red O staining) in the inhibitor-treated group compared with control mice (Fig. 4C).

Analogous experiments using 4μ8c in the same animal model [using previously published doses that showed no toxicity (33, 37, 40, 48)] produced similar results (Fig. 4D–F): 4μ8c treatment led to a significant reduction (45.2%; $P < 0.001$) in atherosclerotic lesion area in en face aorta preparations (Fig. 4E) and a significant reduction in the spliced *Xbp1* mRNA (Fig. S7A) ($P < 0.05$) but no change in IRE1 phosphorylation in the spleens (Fig. S7B). Furthermore, 4μ8c treatment led to a reduced foam cell area (Fig. 4F) without overt differences in body weight, blood glucose levels (Table S4), liver morphology, and plasma ALT activity between the inhibitor-treated and control mice (Fig. S7C and D). These in vivo findings show that pharmacological inhibition of IRE1 can effectively mitigate plaque development in mice.

Pharmacological Inhibition of IRE1 Alters Plaque Composition. Endothelial cells, vascular smooth muscle cells (VSMCs), and immune cells, such as lymphocytes, dendritic cells, neutrophils, and macrophages, play important roles in the development of atherosclerotic plaques in the arterial wall. The UPR is activated in many of these cell types and at all stages of atherosclerotic plaque development. This increase in ER stress is also associated with plaque progression, vulnerability to rupture, and acute coronary syndrome in humans (7). Given that IRE1 inhibitors alleviated atherosclerosis in *ApoE*^{−/−} mice, we next analyzed the impact of these inhibitors on the cellular composition of the lesions. STF-083010 treatment led to a significant reduction (35%; $P < 0.01$) in macrophages [as visualized by monocyte/macrophage marker-2 (MOMA-2) staining] infiltrating the aortic root plaques (Fig. 5A).

This reduction in macrophage numbers was not the product of increased apoptosis as determined by TUNEL (in situ cell death detection) assays in macrophage-enriched areas of the plaques (Fig. 5B). Furthermore, there were no differences in the necrotic core area between the treatment and control groups (Fig. S8A). During plaque formation, VSMCs migrate from adventitia to intima, secreting collagen and sealing the fibrous cap of the plaque. The analysis of the lesions in STF-083010-treated mice (with Masson's Trichrome staining) showed that there is a significant increase in collagen content that is responsible for tensile strength and elasticity of the plaques (22%; $P < 0.05$) without changes in the numbers of the VSMCs infiltrating the lesions (Fig. S8B and D). However, we did not observe significant differences in the fibrous cap thickness between the treatment and control groups (Fig. S8C). Finally, STF-083010 treatment did not alter CD3⁺ T-cell numbers in the adventitia/lesions (Fig. S8E). Taken together, these results indicate that the major consequences of IRE1 inhibition include a reduction in macrophages and an increase in collagen deposition in atherosclerotic plaques.

Last, we sought in vivo evidence for the observed inhibition of IL-1β by IRE1 inhibition in macrophages (Figs. 1 and 2). We observed that STF-083010 treatment reduced the expression of IL-1β in the aortic root lesions stained with a specific antibody against IL-1β (Fig. 5C). Together, these results validate our earlier in vitro findings and show that the antiatherogenic effect of IRE1 inhibitors involves a blockage of inflammation in the lesions.

IRE1 Inhibitors Suppress Hyperlipidemia-Induced Th-1 Immune Responses. Atherosclerosis initiation and progression depend on both innate and adaptive immunity pathways. T cells orchestrate adaptive immunity, whereas macrophages bridge innate and adaptive immune processes that contribute to lesion development. Th cells form the majority of lymphocytes in the atherosclerotic

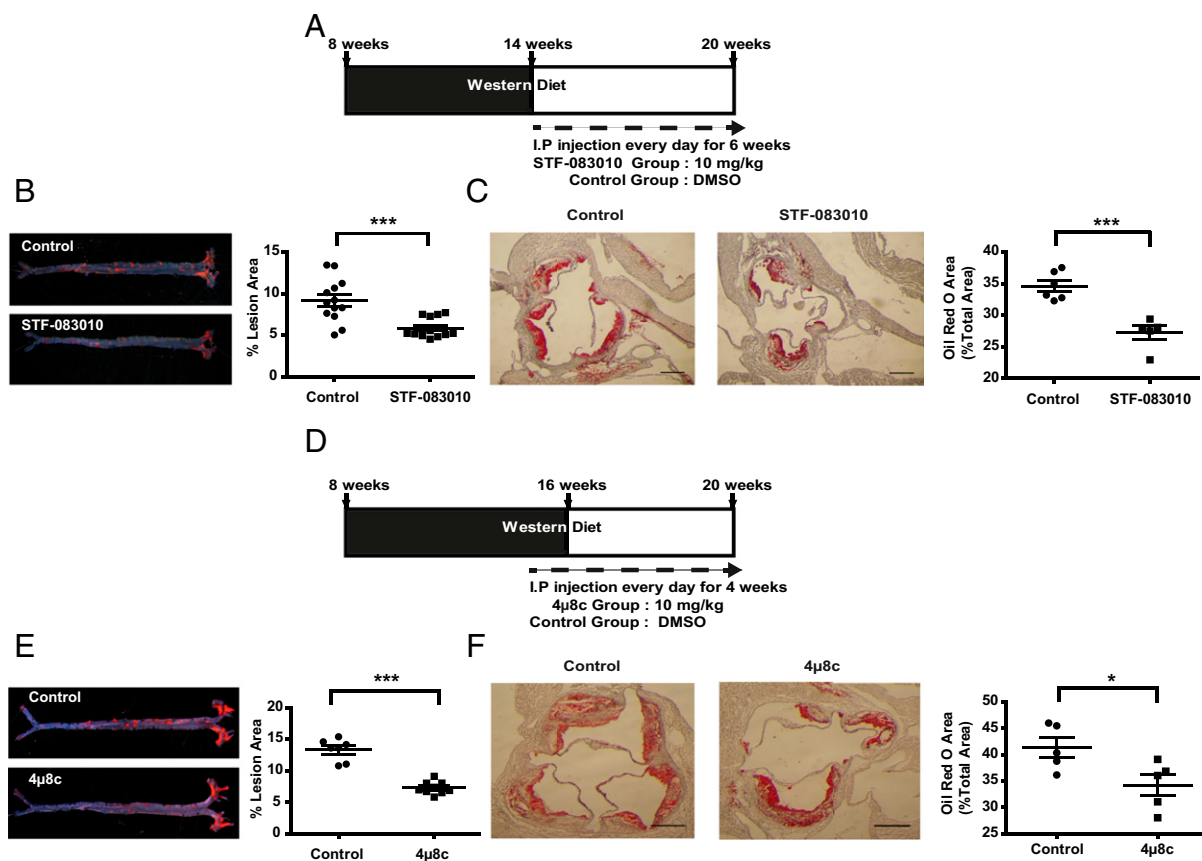


Fig. 4. IRE1 inhibitors reduce plaque area in a mouse model of atherosclerosis. (A) Experimental design in ApoE^{-/-} mice using STF-083010 (10 mg/kg per day). (B) En face aorta analysis of atherosclerotic plaques in ApoE^{-/-} mice. (Left) Sudan IV staining of atherosclerotic plaques. (Right) Quantification of plaque area ($n = 13$ –14). (C) Analysis of aortic sinus plaque area in the animals in A and B. (Left and Center) Oil Red O staining of aorta cross-sections. (Right) Quantification of plaque area ($n = 5$ –6). (D–F) Experimental design and data for analogous experiments in ApoE^{-/-} mice using 4μ8c (10 mg/kg per day; E, $n = 7$ –9; F, $n = 5$). Statistics are the same as in Fig. 1. (Scale bar: 350 μm.) * $P \leq 0.05$; *** $P \leq 0.001$.

plaques. Th-1 cells are proinflammatory, produce high amounts of IFN- γ , and contribute to the progression of atherosclerosis. Two other types of lymphocytes implicated in atherosclerosis progression include Th-2 cells, which produce IL-4, and Th-17 cells, which produce IL-17 (49–51). The inflammasome-induced cytokines IL-18 and IL-1 β play an important role in the polarization of Th-1 and Th-17 responses (52). Because inhibition of IRE1 suppressed inflammasome activation (Fig. 3C and Fig. S4D and E) and IL-1 β production in lipid-challenged macrophages (Fig. 2) as well as lesions and tissues (Fig. 5C and Fig. S9A and B), we next assessed the impact of IRE1 inhibition on systemic IL-18 levels and Th cell differentiation in hyperlipidemic mice. ApoE^{-/-} mice (on Western diet) that were treated with STF-083010 displayed a significant decrease in plasma IL-18 levels (Fig. 6A) ($P < 0.05$) and a marked reduction in the secretion of IFN- γ —but not IL-4 or IL-17—from splenocytes ($P < 0.001$) (Fig. 6B–D and Fig. S9C–E). We did not observe changes in the overall T-cell counts in atherosclerotic lesions after STF-083010 treatment (Fig. S8E), indicating that decreased lymphokine production is intrinsic to intracellular signaling and does not result from a decline in the infiltrating immune cells that produce them. In conclusion, the reduced inflammasome activity in these mice (as measured by IL-1 β and IL-18 levels in Figs. 5C and 6A) after STF-083010 treatment correlates with the suppression of the Th-1 inflammatory response that is known to promote atherosclerosis development.

Discussion

Studies in mice and humans suggest that chronic ER stress plays an important role in atherosclerosis progression. Therefore, pharma-

cological manipulation of the UPR—the network of signaling pathways that respond to ER stress—represents a promising therapeutic approach to manage atherosclerosis (7, 14, 53). The recent discovery of highly selective UPR modulators provides unique opportunities to investigate the contribution of individual UPR branches to the pathogenesis of this disease. Using small molecules that target IRE1, we showed that modulating IRE1 signaling counteracts atherosclerotic plaque formation in mouse models.

First, IRE1 inhibition altered plaque cellular composition mainly by reducing the numbers of macrophages in the atherosclerotic lesions without altering apoptosis. We infer that this effect is likely to stem from reduction in CCL2, a strong macrophage chemoattractant, consistent with our observations in macrophages treated with IRE1 inhibitors. Alternatively, IRE1 modulators could impact macrophage clearance from lesions by phagocytosis of dying cells. We observed no change in the apoptotic cell counts in lesions, arguing against this possibility. Nevertheless, more detailed future studies are required to discriminate between these two possibilities.

Second, IRE1 inhibitor-treated mice displayed an increased collagen content in atherosclerotic lesions, which imparts tensile strength and elasticity to the plaques (54). However, we did not observe an increase in fibrous cap thickness on IRE1 inhibitor treatment. Because we observed no differences in the number of VSMCs in the lesions, the increased collagen deposition may be related to increased collagen folding and secretion, which is consequential to enhanced ER function coupled to reduced cleavage by MMPs. In fact, early in our study, we observed that MMP9 is regulated by IRE1, whereas another study reported RIDD-dependent

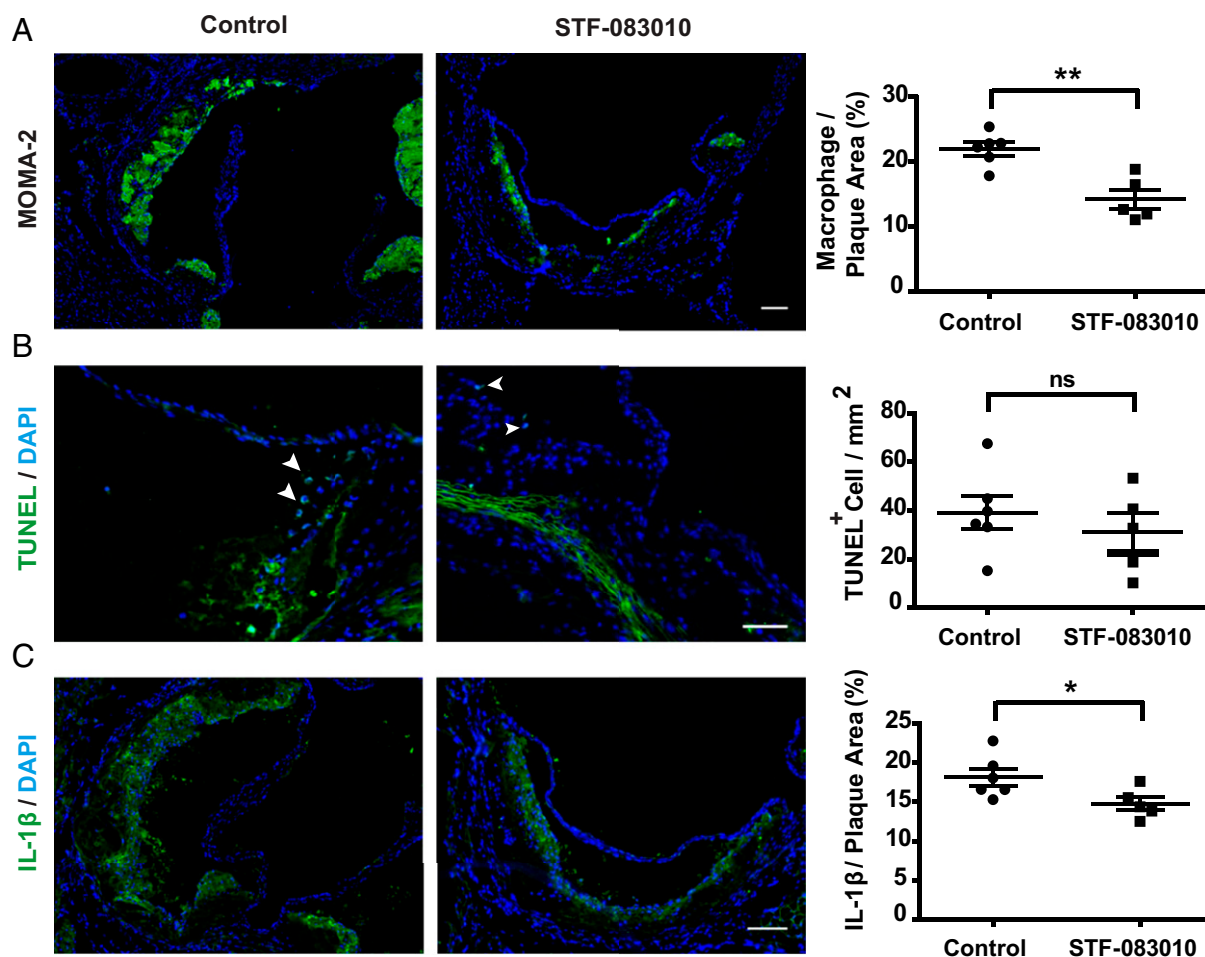


Fig. 5. IRE1 inhibitors alter plaque composition and inflammation. Immunohistochemical and TUNEL assay analyses of proximal aorta cryosections from ApoE^{-/-} mice (Fig. 4) treated with an IRE1 inhibitor. In each case, a representative image is shown in *Left* and *Center*, and the quantification of the data appears on in *Right*. (A) MOMA-2. (Scale bar: 100 μ m.) (B) TUNEL assay (apoptotic cells are shown with arrowheads). (Scale bar: 50 μ m.) (C) IL-1 β . (Scale bar: 100 μ m.) Statistics are the same as in Fig. 1. ns, not significant. * $P \leq 0.05$; ** $P \leq 0.01$.

collagen degradation during ER stress (55). Both observations lend support to our findings and substantiate our hypotheses.

Third, the results from our RNA-seq analyses in macrophages that were treated with IRE1 inhibitors strongly hinted at IRE1's involvement in the production of several proatherogenic cytokines, chemokines, and their receptors, including IL-1 β , CCL2, and chemokine receptor 2. Indeed, IRE1, through XBP1s, regulates *Il-1 β* and *Ccl2* mRNA induction in lipid-stressed macrophages. Moreover, oxidative stress can activate NF- κ B and production of CCL2, resulting in the recruitment of monocytes to a growing plaque (7).

Fourth, cholesterol crystals, saturated fatty acids, and ROS accumulate in plaque areas and provide activation signals for activation of the NLRP3 inflammasome and subsequent secretion of IL-1 β and IL-18 (44). These cytokines generate Th-1-type immune responses that promote plaque progression and unstable lesions (52). Treatment of macrophages with the IRE1 inhibitors suppressed lipid-induced mtROS production, activation of the NLRP3 inflammasome, and subsequent IL-1 β secretion. Our results, therefore, implicate IRE1 activation in the perpetuation of lipid-induced mitochondrial oxidative stress upstream of NLRP3 inflammasome activation, but our data show that this effect is independent of TXNIP induction or calcium mobilization (35, 44). Macrophage mitochondrial oxidative stress plays an important role in atherogenesis by amplifying inflammation (56). Our results reveal that IRE1 is crucial for the regulation of mtROS generation during lipid-induced ER stress; however, future detailed studies

will be needed to uncover how this is achieved by IRE1. In addition to our findings in cells, we confirmed the inhibitory effect of IRE1 inhibitors on hypercholesterolemia-induced IL-1 β and IL-18 production in vivo in plaques and plasma, respectively. Consistent with these observations, treatment with IRE1 inhibitors led to a marked suppression of hyperlipidemia-induced Th-1 immune responses in these mice. We observed no differences in T-cell numbers in adventitia/lesions between the IRE1 inhibitor treatment and control groups, and previous studies have shown the IRE1 inhibitor used in this study does not affect Treg cells (33, 37). Collectively, these findings show that prevention of inflammasome-associated cytokine production by IRE1 inhibitors in vivo has dramatic effects on counteracting atherosclerotic disease progression. Our findings correlating suppression of IL-1 β with atheroprotection are timely in view of a major ongoing clinical trial on the effect of an anti-IL-1 β trial in coronary artery disease (by Cantos). Although our data show a clear impact of IRE1 inhibition on macrophage inflammatory functions, activation of the UPR also occurs in many other lesion-resident cell types. Thus, our results do not exclude the possibility that the antiatherogenic effects of IRE1 inhibition could also involve other lesion-resident cell types that contribute to atherogenesis.

Fifth, the reduction in plaque inflammation and size occurred independent of a correction of elevated plasma lipid levels in IRE1 inhibitor-treated ApoE^{-/-} mice. This notion contrasts with results of a previous study, in which mice bearing a liver-specific deletion

Western Blot Analysis. Cells were lysed in lysis buffer [50 mM Hepes, pH 7.9, 100 mM sodium chloride, 10 mM EDTA, 10 mM sodium fluoride, 4 mM tetrasodium diphosphate, 1% Triton X-100, 2 mM sodium orthovanadate, 1 mM PMSF (phenylmethylsulfonyl), 1× phosphatase inhibitor mixture (Sigma-Aldrich), 1× protease inhibitor mixture (Sigma-Aldrich)]. Lysates were cleared by brief centrifugation followed by the addition of 5× SDS loading dye. For the detection of cleaved caspase-1 (active p10 form) in the cell medium, cell culture supernatants were collected, mixed with 5× SDS loading dye, and heated at 95 °C for 5 min before loading on SDS/PAGE gels. Proteins were subjected to SDS/PAGE separation and transferred onto PVDF membranes. Blocking and antibody incubation were carried out in TBS (Tris-buffered saline) with 0.1% (vol/vol) Tween-20 and 5% (wt/vol) dry milk or BSA and visualized by ECL in a BioRad Imager.

Transfection. IRE1^{−/−} MEFs that reached 60–80% confluency were transfected with the indicated plasmids (2 µg DNA for every 4.5 × 10⁵ cells) and polyethylenimine (Sigma-Aldrich), and BMDMs were electroporated using a Neon Electroporator (Invitrogen) according to the protocols provided by the manufacturer.

RNAi. BMDMs were transfected with 50 nM siRNA against *ire1* (SI0099588; Qiagen), 70 nM siRNA against *Xbp1* (SI01473227; Qiagen), or scrambled siRNAs (1027281; Qiagen); 24 hours after transfection, the cells were treated with indicated reagents.

RNA Isolation and qRT-PCR. Trisure (Bioline) was used to isolate total RNA from cells and reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (K1691; Thermo Scientific) according to the manufacturer's protocols. cDNAs were amplified using specific primers on a Rotor Gene (Qiagen) real-time PCR instrument. Roche SYBR Green was used for qRT-PCR. Quantifications were performed using the $\Delta\Delta C_t$ (threshold cycle) method, and gene expression levels were normalized to *Gapdh* transcript levels using the following expression: (primer efficiency)^{− $\Delta\Delta C_t$} , where $\Delta\Delta C_t$ means ΔC_t (target gene) − ΔC_t (reference gene). We analyzed the results from three or more independent experiments using the Student's *t* test.

RNA-Seq Library Preparation and Sequencing. Total RNA was isolated from control and IRE1-inhibited (with STF-083010) BMDM samples using Trisure (Bioline). To remove genomic DNA contamination, the RNA samples were treated with 20 U DNase I (New England Biolabs). rRNA was depleted from 5 µg total RNA using Ribo-Zero Gold rRNA Removal Kit (Epicentre Biotechnologies) following the manufacturer's recommendations. Sequencing libraries for whole-transcriptome analysis were prepared using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre Biotechnologies) following the manufacturer's recommendations. After 3'-terminal tagging, the di-tagged cDNA was purified using column concentrators (DNA clean-up and concentrators; Zymo Research). The cDNA libraries were bar-coded to allow sample multiplexing using ScriptSeqTM Index PCR Primers (Epicentre Biotechnologies). The libraries were amplified by 12 cycles of PCR, and the amplified libraries were size-selected and purified using 8% TBE (Tris/borate/EDTA) acrylamide gels. The libraries were quantified using Agilent Technologies 2100 Bioanalyzer. Up to four RNA-seq libraries were then multiplexed in a single lane of an Illumina HiSeq2500 Deep-Sequencer Flow Cell (University of California, San Francisco Center for Advanced Technologies) and sequenced using 50-bp single-end sequencing chemistry.

RNA Sequencing Data Processing. The 3' adapter sequences (AGATCGGAA-GAGCACAGTCTGAAC) were removed from the sequenced libraries using the FastQ/A clipper found in the FastX Toolkit (hannonlab.cshl.edu/fastx_toolkit/) after d-multiplexing, and only reads longer than 20 nt were kept for alignment. The adapter-stripped reads were then aligned with the Bowtie indices for the mouse genome reference version 10 of the University of California, Santa Cruz Genome Browser (mm10) using the splice junction mapper Tophat2 v2.0.9 and the sequence aligner Bowtie 2 V2.2.3.0 with default parameters. The transcript assembler Cufflinks V2.1.1 was then used on the list of mapped reads to assemble and quantify transcripts using an mm10 reference annotation and masking mitochondrial, rRNA, and tRNA sequences. To estimate the changes in gene expression levels, the number of sequenced reads that align to a gene of interest was then compared among biological samples using Cuffdiff (default parameters). Changes in the levels of expression of normalized Cufflinks-quantified transcripts are expressed as fragments per kilobase of transcript per million mapped reads (FPKM).

Flow Cytometric Analysis of Intracellular Cytokine Staining. Fresh splenocytes were prepared from mice spleen, and erythrocytes were removed using RBC (red blood cell) lysis buffer as described earlier (43). Cells were stimulated for 4 hours with phorbol-myristate-acetate (PMA; 50 ng/mL; Abcam) and ionomycin (1 µg/mL; Abcam) in the presence of Golgi stop (BD Biosciences). Live cells were discriminated from dead ones by using Zombie Green (Bio Legend). Cell surfaces were stained with PerCP-Cy5.5-conjugated anti-CD4 antibody (BD Biosciences) followed by incubation in Cytofix/Cytoperm Solution (BD Biosciences) at room temperature for 15 min. Then, intracellular cytokines were stained with allophycocyanin (APC)-conjugated IFN- γ , phycoerythrin (PE)-conjugated IL-17A, and PE-conjugated IL-4 antibodies (all from BD Biosciences). Data were analyzed on BD Accuri C6 software.

Measurement of Secreted IL-1 β and IL-18 and CCL2 Cytokines. An IL-1 β Elisa Kit (Abcam) was used for detecting IL-1 β ; a Mouse IL-18 ELISA Kit (Medical & Biological Laboratories) and a Mouse CCL2 Elisa Kit (Abcam) were used for detecting the cytokines in mouse plasma or conditioned medium, respectively, as indicated according to the manufacturer's instructions.

Plasma Measurements. FPLC was used for analyzing the size distribution of lipoproteins. All measurements were carried out at the Mouse Metabolic Phenotyping Center at the University of Cincinnati. For the resolution of major lipoprotein classes from plasma; the columns were equilibrated in 50 mM PBS. Using a microtiter plate enzyme-based assay, the major lipoprotein classes were measured in cholesterol or triglyceride assays from collected fractions.

Staining of Cryosections. Cryosections (7-µm thick) were cut from the aortic root of the frozen heart tissue with a cryostat (Leica CM1850) and stained with Oil Red O, anti-MOMA-2 (monocyte/macrophage marker) (ab33451; Abcam), anti-CD3-Alexa488 (1:400; Biolegend), TUNEL Kit (11684795910; Roche), anti- α -SMA (ab5694; Abcam), and IL-1 β antibody (ab9722; Abcam). Immunofluorescent staining was mounted with an antifade reagent including DAPI (GR211467-2; Abcam). Representative images were taken with a Zeiss Fluorescent Microscope. Collagen content of the lesions and fibrous cap thickness were determined in Masson's Trichrome-stained lesions (as per the manufacturer's protocol; Bio-Optica). Fibrous cap thickness was calculated according to the published protocol (59). Heart tissue sections were stained with Oil Red O stain for plaque area quantifications and H&E for necrotic core quantification in accord with previously published protocols (43). All quantifications were performed using ImageJ (NIH). Percentage of average cross-sectional stained area per leaflet was calculated from all three valves.

Atherosclerotic Lesion Analysis. Aortas were pinned on a black wax surface, and atherosclerotic lesions were analyzed in the aortic arch and descending aorta by Sudan IV staining as described earlier (43). Areas were quantified using ImageJ and expressed as the percentages of the total aorta area.

Quantification of mtROS. mtROS production was measured with MitoSOX Red Mitochondrial Superoxide Indicator (M36008; Life Technologies) according to the protocol provided by the company. Representative images were acquired with a confocal microscope (LSM 510; Zeiss) and analyzed with ImageJ.

Mitochondrial Calcium Measurement. RHOD-2 AM (cell-permeable fluorescent calcium indicator) (Thermo Scientific) was used for mitochondria-specific calcium measurement according to previously published protocols (60).

Mice and Treatments. ApoE^{−/−} mice in a C57BL/6 background (Charles River WIGA GmbH) were used in atherosclerosis experiments. Starting from 8 weeks of age, male mice were fed a Western diet (TD88137 mod. containing 21% fat and 0.2% cholesterol; Ssniff) for 6 weeks. Then, the mice were injected with STF-083010 (10 mg/kg) or DMSO, both given in 16% (vol/vol) Cremophor EL (Sigma-Aldrich) saline solution via i.p. injections as described previously, for 6 more weeks while mice were continued on the Western diet (21). The other ApoE^{−/−} mice that were used in atherosclerosis experiments were fed a Western diet for 8 weeks. Then, they were injected with 4 μ 8c (10 mg/kg) or DMSO, both given in 16% (vol/vol) Cremophor EL saline solution via i.p. injections as described previously (33), for 4 more weeks while mice were continued on Western diet. Weights were measured every other day, whereas blood glucose concentrations were measured before and after treatments. At the end of the experiment, mice were anesthetized, and blood was collected by cardiac puncture. Bone marrow, spleen, and liver tissues were collected, frozen immediately into liquid nitrogen, and stored at −80 °C. Perfusion was performed with ice-cold PBS and heparin (1,000 U/mL) followed by 10% formalin solution (Sigma). After fixation, the aorta was

dissected intact, immersed immediately in 10% formalin, and stored at 4 °C until analysis. The heart was removed at the proximal aorta, placed into a tissue mold, covered with OCT (optimal cutting temperature compound) (Tissue-Tek), frozen in cold isobutene solution, and stored at –80 °C. All animal experiments were performed according to protocols approved by the Experimental Animal Care Committee at Bilkent University.

Statistical Analysis. Values are expressed as mean ± SEM. No samples were treated as outliers and left out of analysis. Statistical significance was evaluated using the Student's *t* test or the Mann-Whitney test (for *in vivo* analysis as indicated in the figures). *P* < 0.05 was considered as significant.

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