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 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-1β 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 demonstrate that pharmacologic modulation of IRE1 counteracts metaflammation and alleviates atherosclerosis.

Endoplasmic reticulum stress | unfolded protein response | metabolic stress | inflammation | lipotoxicity

INTRODUCTION

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 re-establishes 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 IRE1 (inositol-requiring enzyme 1), PERK (protein kinase RNA-like ER kinase), and ATF6 (activating transcription factor 6) (3).

IRE1 controls the phylogenetically most conserved branch of the UPR, found from fungi to metazoans. It has an ER-lumenal sensor domain that recognizes unfolded pep-
Fig. 1. IRE1 regulates the expression of pro-atherogenic genes. (A) RNA-seq analysis in BMDMs treated with 60 μM STF-083010 or DMSO (control) for 6 hours. Volcano plot of differentially expressed mRNAs. (B) Analysis of atherosclerosis-related mRNAs using the IPA tool (see text for details). (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 upon forced expression of XBP1s or upon restoring IRE1’s function. Data: mean values ± SEM; n=3; ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, n.s. = not significant; Student’s t test.

Recent drug discovery efforts have opened the door to approaches that entail selective modulation of UPR signaling. This quest has led to the identification of several new small molecules that target the enzymatic activities of specific UPR regulators (19-23). Specifically, blocking IRE1 or XBP1s function has been shown to be beneficial for restraining tumor progression in mouse models (21, 24), highlighting that the specific targeting of the UPR can have beneficial impact in disease.

Several lines of evidence support the notion that selective pharmacological targeting of IRE1 is a desirable therapeutic approach for treatment of atherosclerosis. First, a profound increase in IRE1 phosphorylation and XBP1s expression is observed in atherosclerotic plaques of mice and humans (8, 10). Second, mechanical sheer stresses activate IRE1, while cardiovascular disease risk factors, such as oxidized phospholipids and homocysteine, induce both IRE1 and PERK (5, 25-29). Third, experimentally sustained XBP1 mRNA splicing in the vessel wall promotes atherosclerosis, whereas its ablation ameliorates hypercholesterolemia in obese or Apolipoprotein E-deficient (ApoE−/−) mice (10, 30). Two small molecules, STF-083010 and 4 µg, which selectively inhibit IRE1’s RNase function, have been used in...
Fig. 2. IRE1 regulates lipid-induced IL-1β secretion in mouse and human macrophages. IL-1β mRNA (A) and protein (B) levels measured from LPS-primed and PA-stimulated mouse BMDMs treated with 60μM STF-083010 or DMSO (control) by qRT-PCR and ELISA, respectively. IL-1β mRNA (Q) and protein (D) levels measured from LPS-primed and PA-stimulated mouse BMDMs transfected with siRNAs against IRE1α or XBP1 by qRT-PCR and ELISA, respectively. Secreted IL-1β from LPS-primed, PA-stimulated human PBMCs and treated with or without 100μM 4μBc measured by ELISA. (F) Same as E, but protein levels were measured by immunoblotting to show the immature and processed forms of the cytokine (representative image of three independent blots). Statistics as in Fig. 1.

Fig. 3. IRE1 inhibitors block lipid-induced mtROS release and inflammasome activation. (A-B) mtROS production was measured in LPS-primed, PA-stimulated mouse BMDMs (A) after 100μM 4μBc or DMSO (control) treatment or (B) transfection with scrambled or XBP1-specific siRNAs. (C-E) Immunoblot of the levels of the zymogen (p45) and mature (p10) forms of caspase-1 in LPS-primed mouse BMDMs pre-treated with 4μBc (at the indicated doses) or DMSO (control) and stimulated with (C-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 and described in detail in the methods section. CE: cell extract. SN: supernatant. Blots shown are representative of three independent experiments. Statistics as in Fig. 1.
which inhibits protein N-linked glycosylation in the ER lumen, macrophages using RNA-seq. ER poisons, such as tunicamycin, first analyzed the impact of its inhibition on the transcriptome of Pro-atherogenic genes are regulated by IRE1

**RESULTS**

Pro-atherogenic genes are regulated by IRE1

To understand the contributions of IRE1 in atherogenesis, we first analyzed the impact of its inhibition on the transcriptome of macrophages using RNA-seq. ER poisons, such as tunicamycin, which inhibits protein N-linked glycosylation in the ER lumen, or thapsigargin, which disrupts ER calcium reuptake, activate all UPR branches simultaneously, impeding the dissection of the signaling contributions of individual UPR branches. By refraining from using these pleiotropic drugs, we aimed to identify the specific IRE1-regulated gene expression changes. To this end, we probed the transcriptional response to acute inhibition of IRE1 using STF-083010 in primary mouse bone marrow-derived macrophages (BMDMs)(34, 35). We analyzed differentially regulated mRNAs at early time points (6 hours) after IRE1 inhibition to distinguish immediate-responsive genes from those whose expression may be altered as part of an adaptive response to chronic inflammation. Using an arbitrary cut-off of 1.5-fold, we observed increased expression of 169 genes and decreased expression of 135 genes upon IRE1 inhibition (Fig. 1A, S.T able 1 and S.T able 2; p<0.05). To categorize the affected genes functionally according to their association with disease processes, we employed the Ingenuity Pathway Analysis (IPA) tool (36). IPA identified the down-regulation of many important pro-atherogenic genes, including cytokines, chemokines, and chemokine receptors upon inhibition of the steady-state IRE1 activity (Fig. 1B).

We next validated our findings using quantitative reverse transcription polymerase chain reaction (qRT-PCR). In these experiments, we observed a significant reduction in the mRNA levels of IL-1β (interleukin-1β), CCL2 (C-C Motif Chemokine Ligand 2), S100A8 (calgranulin A) and MMP9 (ma...
Fig. 5. IRE1 inhibitors alter plaque composition and inflammation. Immunohistochemical and TUNEL assay analyses of proximal aorta cryosections from ApoE−/− mice (in Fig. 4) treated with an IRE1 inhibitor. In each case a representative image is shown on the left and the quantification of the data appears on the right. (A) Monocyte/macrophage marker-2 (MOMA-2). Scale bar: 100 µm. (B) TUNEL assay (apoptotic cells are shown with arrows). Scale bar: 50 µm. (C) IL-1β. Scale bar: 100 µm. Statistics as in Fig. 1.

trix metalloproteinase-9) (p<0.05, p<0.001, p<0.01, p<0.01, respectively), following IRE1 inhibition either with STF-083010 or 4µ8c (Fig. 1C-E, S.Fig. 1A-E). Both of these drugs were used at concentrations that do not compromise cell viability or induce any other toxicity (21, 34, 35, 37-40). Consistent with earlier reports, these IRE1 inhibitors had no effect on the kinase function of IRE1 (S.Fig.1F-1H), confirming that the identified pro-atherogetic genes are regulated by IRE1’s RNase activity.

Since IRE1 is rate-limiting for the production of XBP1s, we next assessed if XBP1s regulates the expression of these pro-atherogetic genes. To this end, we forced expressed XBP1s or restored IRE1 function in IRE1-deficient (IRE1−/−) mouse embryonic fibroblasts (MEF) (S.Fig.1I). Both experiments showed a marked induction of IL-1β, CCL2, and S100A8 mRNA levels (Fig. 1F-H). Together, our findings confirm that the IRE1-XBP1 signaling branch of the UPR maintains the expression of key pro-atherogentic cytokines and chemokines in macrophages.

Induction of IL-1β and CCL2 depends on IRE1 during lipotoxicity

Our finding that IRE1 maintains the expression of several important, pro-atherogenic genes in macrophages suggests that, when induced by metabolic stress, heightened IRE1 activity could drive the atherosclerotic process. One important activating signal for the UPR in macrophages is exposure to excessive amounts of lipids, which elicits toxicity (14, 28, 41). This lipotoxicity results in increased production of reactive oxygen species (ROS), ER stress, and inflammation, and can result in apoptosis (41).

Based on these observations, we next investigated if lipid-induced IRE1 activation plays a role in IL-1β induction. Inhibition of IRE1 with STF-083010 led to a significant block in lipid-induced IL-1β mRNA production and secretion from BMDMs (Fig. 2A-B, S.Fig.2A-B). As expected, inhibition of IRE1 activity with 4µ8c showed the same effects (S.Fig. 2C-F). To further delineate the role of IRE1 and XBP1s in the regulation of IL-1β, we transfected BMDMs with a specific small interfering RNAs (siRNAs). We found that expression of siRNAs against IRE1 resulted in a significant reduction in lipid-induced IL-1β mRNA production and secretion in BMDMs (Fig. 2C-D; p<0.05, p<0.05, p<0.001, p<0.001, respectively and S.Fig. 2G-H). Moreover, treatment of human peripheral blood monocytes (PBMC) with lipids induced IL-1β secretion, which was blocked...
Inflammasome activation depends on IRE1 during lipotoxicity

Because IRE1 inhibition leads to a strong suppression of IL-1β secretion, we reasoned that IRE1 may contribute to the lipid-induced activation of the NLRP3 (Nod-like receptor family, pyrin domain containing protein-3) inflammasome, a multicomponent platform that contains caspase-1 and induces the caspase-1-dependent secretion of the pro-inflammatory cytokines IL-1β 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 (mtROS) from damaged mitochondria (44). In earlier studies from our and other laboratories showed that treatment of macrophages with saturated fatty acids activate 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 4μg treatment as well as by XBP1 knock down (Fig. 3A-3B and S.Fig. 4A).

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 whose increased levels 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 TXNIP in macrophages which was partially blocked by inhibiting IRE1 (S. Fig. 4B). Another mechanism by which ER stress can induce mitochondrial oxidative stress and activation of the NLRP3 inflammasome complex is through calcium mobilization (46). Indeed, we observed PA leads to a significant increase (3 fold) of calcium levels in the mitochondria, but this could not be prevented by IRE1 inhibition (S.Fig. 4C). Moreover, lipid-induced ER stress also induced pro-caspase-1 maturation (indicated by the appearance of the p10 fragment), an effect that was reduced by treatment with IRE1 inhibitors or siRNA-mediated silencing of IRE1α and XBP1 (Fig. 3C, S.Fig.4D-E). Taken together these results indicate that IRE1 plays a crucial role in perpetuating inflammasome activation and calcium mobilization.

We further investigated if IRE1 affects the activation of the NLRP3 inflammasome by other stimuli or the activation of different inflammasome complexes by specific inducers. While IRE1 inhibition blocked NLRP3 inflammasome activation, cleavage of caspase-1 and secretion of IL-1β by various different NLRP3 inflammasome inducers (including ATP, Alum and cholesterol crystals) (Fig. 3D), it did not alter the activation of other inflammasome complexes such as NLRC4 (NL family CARD domain containing protein 4) by flagellin or AIM2 (absent in melanoma 2) by poly (deoxyadenylc-deoxythymidylic acid) (poly (dA:dT)) as evident by the changes in caspase-1 cleavage (Fig.3E). Since IRE1 inhibition of BMDMs resulted in a dramatic elevation of XBP1 controls IL-1β mRNA production, we observed reduction in secreted IL-1β protein levels in all of the treatments (Fig. 3D-3E). Finally, together with our results showing IRE1 induces IL-1β, our data demonstrate that IRE1 plays a major role in controlling IL-1β signaling, both transcriptionally and by promotion of its maturation via the activation of the NLRP3 inflammasome.

Pharmacological inhibition of IRE1 combats atherosclerosis

The evidence presented above, in conjunction with previous results showing that restoring or improving ER function alleviates atherosclerosis (14, 17, 47), suggests that inhibiting IRE1 may impair atherosclerosis progression. Therefore, we postulated that administration of IRE1 modulators might have beneficial effects by limiting the inflammatory signaling associated with elevated ER stress in a mouse model of atherosclerosis. To test this notion, we challenged ApoE−/− mice with a Western diet (12 weeks) and then treated them daily with STF-083010 by intraperitoneal injection (6 weeks) (Fig. 4A). Even though limited pharmacodynamic data is available for the IRE1 inhibitors we employed, target engagement has been shown in cells and tissues (21, 39). We based our in vivo drug dosage and delivery on earlier in vivo studies that
successfully administered the IRE1 modulators without toxicity (21, 24, 31). Furthermore, we confirmed target engagement in tissues by assessing drug-induced suppression of XBP1 mRNA splicing and RIDD activities by observing a significant reduction in XBP1s mRNA (p<0.05) and a modest increase in canonical RIDD target mRNAs (p<0.05), respectively (S.Fig.5 A-C). Importantly, the IRE1 inhibitor we employed did not alter IRE1 phosphorylation in vivo (S.Fig.5D). Furthermore, we detected no differences in body weights, blood glucose levels, liver morphology, plasma alanine amino transferase (ALT) activity and cholesterol profiles between the STF-083010-treated and control groups (S.Table 3; S.Fig. 5E-F and 6). However, the analysis of en face aorta preparations showed that chronic administration of STF-083010 led to a significant decrease (35.8%; p<0.001) in atherosclerotic lesions when compared to the control 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 when compared to control mice (Fig. 4C).

Analogous experiments using 4μc in the same animal model (using previously published doses that showed no toxicity; (33, 37, 40, 48)) produced similar results (Figs. 4D-F): 4μc treatment led to a significant reduction (45.2%; p<0.01) in atherosclerotic lesion area in en face aorta preparations (Fig.4E), a significant reduction in XBP1s mRNA (S.Fig. 7A; p<0.05), but no change in IRE1 phosphorylation in the spleens (S.Fig.7B). Furthermore, 4μc treatment led to a reduced foam cell area (Fig. 4F), without overt differences in body weight, blood glucose levels (S.Table 4), liver morphology and plasma ALT activity between the inhibitor-treated and control mice (S.Fig.7C-D). These in vivo findings demonstrate that pharmacological inhibition of IRE1 can effectively mitigate plaque development in mice.

**Pharmacological inhibition of IRE1 alters plaque composition**

Endothelial cells (ECs), 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 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 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 (S.Fig. 8A). During plaque formation, VSMCs migrate from adventitia to intima, secreting collagen and seal the fibrous cap of the plaque. The analysis of the lesions in STF-083010-treated mice (with Mason’s Trichrome staining) showed 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 (S.Fig. SB-SD). However, we did not observe significant differences in the fibrous cap thickness between the treatment and control groups (S.Fig.8C). Finally, STF-083010 treatment did not alter CD3+ T cell numbers in the adventitia/lesions (S.Fig. 8E).

Taken together, these results indicate that the major consequences of IRE1 inhibition include a reduction in macrophages in 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 demonstrate the anti-atherogenic 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 while macrophages bridge innate and adaptive immune processes that contribute to lesion development. T-helper (Th) cells form the majority of lymphocytes in the atherosclerotic plaques. Th-1 cells are pro-inflammatory, 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-1β play an important role in the polarization of Th-1 and Th-17 responses (52). Since inhibition of IRE1 suppressed inflammasome activation (Fig.3C and S.Fig. 4D-E) and IL-1β production in lipid-challenged macrophages (Fig.2), as well as in lesions and tissues (Fig. 5C, S.Fig. 9A-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 of IL-4 or IL-17— from splenocytes (Fig. 6B; p<0.01; Fig. 6C-D, S.Fig.9C-E). We did not observe changes in the overall T cell counts in atherosclerotic lesions after STF-083010 treatment (S.Fig. 8E), 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-1β and IL-18 levels in Fig.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, pharmacological 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 necrotic core of lesions while allowing apoptosis. We infer that this effect is likely to stem from reduction in CCL2, a strong macrophage chemo-attractant, 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 upon 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 matrix metalloproteases (MMPs). In fact, early in our study we observed that MMP9 is regulated by IRE1, while another study reported RIDD-dependent 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 pro-atherogenic cytokines, chemokines, and their receptors including IL-1β, CCL2 and chemokine receptor 2 (CCR2). Indeed, IRE1, through XBP1s, regulates IL-1β and CCL2 mRNA induction in lipid stressed macrophages. Moreover, oxidative stress can activate NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) 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 activating the NLRP3 inflammasome and subsequent IL-1β and IL-18 production (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 and activation of the NLRP3 inflammasome, and subsequent IL-1β 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 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-1β and IL-18 production in vivo, in plaques and in 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 adventia/lesions between the IRE1 inhibitor treatment and control groups, and previous studies have shown the IRE1 inhibitor used in this study does not affect T regulatory cells (33, 37). Collectively, these findings show that prevention of inflammasome-associated cytokine production by IRE1 inhibitors in vivo has dramatic effects on countering atherosclerotic disease progression. Our findings correlating suppression IL-1β with atheroprotection are timely in view of a major ongoing clinical trial on the effect of an anti-IL1β trial in coronary artery disease (by Cantos). While 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 anti-atherogenic 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 of IRE1 inhibitor-treated ApoE−/− mice. This notion contrasts with results of a previous study, in which mice bearing a liver-specific deletion of XBP1 displayed a profound reduction in plasma cholesterol and triglyceride levels (57). However, it is important to note that this apparent disparity may exclusively result from feedback activation of IRE1 upon genetic loss of XBP1 (57). Notably, additional siRNA-mediated IRE1 depletion partially reverted the hypolipidemic phenotype in vivo, hinting at a RIDD-dependent function that controls lipid metabolism (30). Furthermore, recent studies have shown feedback activation of IRE1 or any signs of liver toxicity with the IRE1 modulators. Since XBP1s promotes the transcriptional upregulation of a lipogenic gene program, these findings could be interpreted to mean that the splicing and RIDD outputs of IRE1 have opposing effects on lipid metabolism (30). While most of the direct targets of XBP1 participating in triglyceride and sterol lipogenic programs were also identified as RIDD substrates in this study (Ref 30, Fig. 3), IRE1 (through its RIDD modality) seems to be able to reduce the steady-state expression of a larger number of lipogenic genes independent of XBP1, hinting at a complex lipogenic regulatory program that depends on the interplay between XBP1 and IRE1 signaling (30, 57). Thus, the reduction in plaque size we observed in our study is likely to result from IRE1 inhibitor-mediated anti-inflammatory changes and not the product of changes in lipid metabolism.

While we have seen important gains in mitigating atherosclerosis by pharmacologically targeting IRE1 in our experimental models, it is important to note that the other UPR branches, particularly the PERK-CHOP branch, are also induced as atherosclerosis progresses and appear to be instrumental for macrophage apoptosis (5, 11). Previous studies focusing on the engagement of apoptotic pathways initiated by the UPR (such as those mediated by CHOP and JNK) showed that mice deficient for these apoptotic effectors are protected from atherosclerosis (5, 11-13). Independent of CHOP or JNK engagement, here we found that modulating IRE1 signaling in vivo with small molecule IRE1 inhibitors modifies a different branch of UPR signaling that impinges on metaflammation and alters the course of atherosclerosis. These results support the notion that it may be possible to uncouple metabolically induced ER stress from inflammation by calibrating UPR signaling, thereby improving the clinical outcome of atherosclerosis. With the advent of specific inhibitors that can target different UPR branches, exploring the efficacy of combinatorial UPR calibration in this chronic disease setting becomes a promising endeavor.

**EXPERIMENTAL PROCEDURES**

A list of primers used in the study can be found in the supplemental materials.

**Reagents and Plasmids**

IRE1−/− MEFs were provided by Gokhan Hotamisligil (Harvard School of Public Health). BMDMs were isolated from of C57BL/6 mice. Plasmids encoding XBP1s (21363), wt IRE1a (13009) were purchased from Adgen (LEadgene). Dulbecco’s modified Eagle’s medium (DMEM), phosphatase buffered saline (PBS), Hank’s balanced salt solution (HBSS), penicillin/streptomycin (PS), fetal bovine serum (FBS), and RPMI-1640 medium (Invitrogen) were purchased from Thermo Fisher Scientific. Trypsin, ampicillin, kanamycin, Bradford assay reagents, ultrapure lipopolysaccharides (LPS), palmitic acid (PA), phosphatase inhibitor cocktail, and 0.4% w/v calcium chloride (Sigma) were purchased from Sigma-Aldrich. Primary antibodies used for immunoblotting were purchased from the following suppliers: Anti-IRE1 phospho S724 antibody (Abcam ab48187), IL-1β (Abcam ab9722), IRE1a Rabbit mAb (3294, Cell Signaling), β-Actin (sc-80007, Santa Cruz Biotechnology), and IL-1β (R&D Systems AF-401-NA). Enhanced chemiluminescence Prime Western (Enzo Life Sciences). 100 μg/ml cycloheximide (CHX) was purchased from Sigma Chemical Co.

**Preparation of PA-bovine serum albumin complex**

PA was dissolved in absolute ethanol to yield a stock concentration of 500 μM and stored at −80°C. Stock PA was diluted to the working concentration and suspended with 1% fatty acid free BSA in RPMI-1640 medium (without serum) by mixing at 50°C for 30 minutes.

**Preparation of Cholesterol Crystal**

10 mg/ml cholesterol solution was prepared in ethanol and was heated to 60°C and brought to room temperature to allow for crystallization, washed with PBS, and resuspended in RPMI-1640 medium at 5 mg/ml. These cholesterol crystals were used for treating the cells (for 20 hours) as described (58).

**Cell Culture and Treatments**

**Isolation of BMDMs**: Bone marrow were collected from the tibia and femurs of mice into RPMI-1640 medium containing 1% penicillin/streptomycin
(PS). After filtering through a cell strainer (BD Biosciences, 352350), the cells were washed with PBS and resuspended in medium enriched with 15% L929 conditioned medium and 1% PS, followed by seeding for growth and differentiation into macrophages on petri dishes for 7 days.

Transgene induction: BMDMs were pre-treated (1 hour) with 100 µM STF-083010 or with the indicated concentrations of 4µc, followed by stimulation with ultrapure LPS (200 ng/ml) for 3 hours, then treated with Poly (A)- dt (200 µM) (5%) (w/v) dry milk or BSA and visualized by enhanced choliniumcence in a Bio-Rad imager.

Transfection

IRE1β and XBP1 siRNAs (Qiagen; 1027281). 24 hr after transfection, the cells were harvested and used for the respective cytokines in mouse plasma or from conditioned medium, as indicated, according to manufacturer’s instructions.

Plasma Measurements

Bio-Optica). Fibrous cap, mouse IL-18 ELISA Kit (Medical & Biological Laboratories) and a mouse CCL2 Elisa Kit (Abcam) was used for determining the interleukin-1β and erythrocytes were removed using red blood cell lysis buffer as described earlier (43). Cells were stimulated for 4 h with phorbol-myristate-acetate (PMA) (50 ng/ml, Abcam) and ionomycin (1 µM, Abcam) in the presence of Golgi stop (BD Biosciences). Live cells were discriminated from dead ones by using Zombie Green (BioLegend). Cell surfaces were stained with PerCP-Cy5.5-conjugated anti-CD4 antibody (BD Biosciences) followed by incubation in Cytofix/Cytoperm solubilization buffer and surface staining. The percentage of gated populations were calculated using CytometerScan v2.1 software. Data were analyzed on BD LSRFortessa.

Measurement of Secreted IL-1β and IL-18 and CCL2 Cytokines

ApoE knockout (ApoE−/−) according to the manufacturer’s instructions. All quantifications were performed using ImageJ (National Institutes of Health). Percentage of average cross-sectional stained area per lef开封 was calculated from all three values.

Atherosclerotic Lesion Analysis

Quantification of mtROS

mtROS measurement was performed with MitoSOX™ Red mitochondrial superoxide indicator (M36008, Thermo Scientific) and analyzed on a Zeiss LSM510 confocal microscope. Mitochondria specific red fluorescence was quantified using MitoTracker Red (M25-200, Life Technologies) as a mitochondrial marker and stained lesions (as per the manufacturer’s protocol). Mitofluor, mitochondrial calcium measurement according to previously published protocols (60). Heart tissue sections were stained with Oil Red O stain for plaque area quantification and hematoxylin-eosin for necrotic core quantification in accord with previously published protocols (43). All quantifications were performed using ImageJ.

Westerndiet (21). The other ApoE−/− mice were injected with STF-083010 (10 mg/kg) both given in 1% DMSO, both given in 16% Cremophor EL (Sigma) saline solution via intraperitoneal injection, as described previously, for 6 more weeks while mice were continued on the Western diet. The other ApoE−/− mice continued on the Western diet for another 2 weeks. All atherosclerosis experiments were fed a Western diet for 8 weeks. Then they were injected with 4µc (10 mg/kg) or DMSO, both given in 16% Cremophor EL saline solution via intraperitoneal injection every other day, while 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 and frozen immediately into liquid nitrogen and stored at -80 °C. Perfusion was performed with ice-cold PBS and heparin (1000 U/ml), followed by 10% formalin solution. After fixation, the aorta was dissected intact, immersed immediately in 10% formalin and stored at 4 °C.

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until analysis. The heart was removed at the proximal aorta and placed into a tissue dish, covered with OCT (Tissue-Tek), frozen in cold isobutene solution and stored in -80 °C. All animal experiments were performed according to approved protocols by the experimental animal care committee at Bilkent University.

**Statistical Analysis** Values are expressed as mean ± SEM. Some samples were treated as outliers and left out of analysis. Statistical significance was evaluated using the Student’s t test or Mann Whitney test (for in vivo analysis, as indicated in the figures). P<0.05 was considered as significant.


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**Footnote Author**