IRF2BP2 Reduces Macrophage Inflammation and Susceptibility to Atherosclerosis


Rationale: Inflammation impairs macrophage cholesterol clearance from vascular tissues and promotes atherosclerosis. Inflammatory macrophages suppress expression of the transcription cofactor interferon regulatory factor 2–binding protein 2 (IRF2BP2), and genetic variants near IRF2BP2 associate with ischemic heart disease progression in humans.

Objectives: To test whether IRF2BP2 in macrophages affects atherosclerosis in mice and humans.

Methods and Results: We generated mice that delete IRF2BP2 in macrophages. IRF2BP2-deficient macrophages worsened atherosclerosis in irradiated low-density lipoprotein receptor null-recipient mice and in apolipoprotein E null mice. IRF2BP2-deficient macrophages were inflammatory and had impaired cholesterol efflux because of their inability to activate the cholesterol transporter ABCA1 in response to cholesterol loading. Their expression of the anti-inflammatory transcription factor Krüppel-like factor 2 was markedly reduced. Promoter studies revealed that IRF2BP2 is required for MEF2-dependent activation of Krüppel-like factor 2. Importantly, restoring Krüppel-like factor 2 in IRF2BP2-deficient macrophages attenuated M1 inflammatory and rescued M2 anti-inflammatory gene activation and improved the cholesterol efflux deficit by restoring ABCA1 activation in response to cholesterol loading. In a cohort of 1066 angiographic cases and 1011 controls, homozygous carriers of a deletion polymorphism (rs3045215) in the 3‘ untranslated region sequence of human IRF2BP2 mRNA had a higher risk of coronary artery disease (recessive model, odds ratio [95% confidence interval]=1.560 [1.179–2.065], P=1.73E-03) and had lower IRF2BP2 (and Krüppel-like factor 2) protein levels in peripheral blood mononuclear cells. The effect of this deletion polymorphism to suppress protein expression was confirmed in luciferase reporter studies.

Conclusion: Ablation of IRF2BP2 in macrophages worsens atherosclerosis in mice, and a deletion variant that lowers IRF2BP2 expression predisposes to coronary artery disease in humans. (Circ Res. 2015;117:671-683. DOI: 10.1161/CIRCRESAHA.114.305777.)

Key Words: 3‘ untranslated region ■ atherosclerosis ■ cholesterol ■ coronary artery disease ■ genetics ■ macrophages

Macrophage inflammation is a key process of atherosclerosis. Macrophages ensure tissue cholesterol homeostasis by removing oxidized low-density lipoprotein cholesterol (oxLDL-C) from the intercellular space. Macrophages then degrade the damaged apolipoprotein (Apo) B of oxLDL-C, but cannot degrade the cholesterol. Instead, they release cholesterol to high-density lipoprotein that carries it in the circulation back to the liver where it is excreted in bile salts, a process known as reverse cholesterol transport. However, if cholesterol accumulates in macrophages because of excess uptake of oxLDL-C or because of impaired cholesterol efflux, macrophages become foam cells that constitute the main component of atherosclerotic plaques. Of note, oxLDL-C stimulates foam cell production of inflammatory cytokines and chemokines that further impair macrophage cholesterol efflux and recruit circulating monocytes to the atherosclerotic lesion, leading to further plaque formation and artery occlusion.
Genome-wide association studies identified genetic variants in the vicinity of interferon regulatory factor 2–binding protein 2 (IRF2BP2) associated with elevated plasma total cholesterol in European descendants (rs514230, risk allele frequency =48% and LDL-C in black African descendants (rs744487, risk allele frequency =72%). A third variant (rs482329, risk allele frequency =64%) was associated with life-threatening arrhythmias in patients of European descent who had ischemic heart disease. Together, these studies suggest that IRF2BP2 may affect the susceptibility to coronary artery disease (CAD).

IRF2BP2 is a cofactor modulating IRF2 and myocyte enhancer factor 2 (MEF2), transcription factors important for innate immunity and gene expression in macrophages.

Exposure of macrophages to bacterial lipopolysaccharide activates the M1 inflammatory phenotype, whereas exposure to interleukin (IL)-4 favors the M2 anti-inflammatory phenotype. In human monocyte–derived macrophages, IRF2BP2 transcription factor Krüppel-like factor 2 (KLF2). Restoring KLF2 expression in IRF2BP2-deficient macrophages attenuated their inflammatory phenotype and improved their ability to efflux cholesterol. Homozygous carriers of a deletion variant in KLF2 were found to nearly undetectable levels in the M1 state (Geoprofiles, GDS2430:224570_s_at), suggesting that IRF2BP2 may be involved in macrophage polarization.

Here we report that IRF2BP2-deficient macrophages display an inflammatory phenotype prone to foam cell formation. Transplantation of IRF2BP2-deficient bone marrow to an atherosclerotic mouse model (LDLR−/−) increased atherosclerotic lesion size. Atherogenic propensity of IRF2BP2-deficient macrophages was also observed in ApoE−/− mice fed a western diet. We found that IRF2BP2 regulates the expression of an anti-inflammatory transcription factor Krüppel-like factor 2 (KLF2). Restoring KLF2 expression in IRF2BP2-deficient macrophages attenuated their inflammatory phenotype and improved their ability to efflux cholesterol. Homozygous carriers of a deletion variant in the 3′ untranslated region (3′UTR) sequence of IRF2BP2 have increased risk of CAD and reduced expression of IRF2BP2 (and its target KLF2) in peripheral blood mononuclear cells. Together, our findings identify IRF2BP2 as an important modulator of macrophage polarization implicated in coronary atherosclerosis.

Methods

Study Participants
The Research Ethics Board of the University of Ottawa Heart Institute approved this study. Written informed consent was obtained from all study participants. Details of the phenotypic characteristics and inclusion and exclusion criteria are in Methods in the Online Data Supplement and as described previously.

Animal Models
We generated a floxed allele of IRF2BP2 (Online Figure 1) and mated these mice to LysMCre mice that express Cre-recombinase in the myeloid lineage. These mice were further bred with ApoE−/− mice to generate double knockout (DKO) mice. All mice were bred into the C57BL6 background. The animal care and use committee of the University of Ottawa approved all procedures performed in mice. Male mice were fed with chow or high-fat diet (60% fat, TD06414 Harlan) for metabolic studies or a western diet (0.2% cholesterol and 21% fat, TD86137 Harlan) for atherosclerosis studies.

Bone Marrow–Derived Macrophages Culture and Cholesterol Homeostasis
Mouse bone marrow–derived macrophages (BMDM) were cultured as described in the Online Data Supplement. Neutral lipid accumulation was visualized by oil red O staining, whereas cholesterol uptake, and efflux, and contents in BMDMs were measured as detailed in Methods in the Online Data Supplement.

Serum Lipoprotein Profile
Fast protein liquid chromatography analysis was conducted using 75 μL pooled serum from mice fasted for 16 hours (n=3 per group), separated on a Superose column (Amersham) with a flow rate of 0.4 mL/min. From each 500 μL fraction, 50 μL was used to measure total cholesterol content using the Wako Cholesterol E kit (No. 439–17501) and 16 μL was loaded for immunoblot analysis of serum lipoproteins.

Quantitative Real-Time Polymerase Chain Reaction and Microarray Analysis
Total RNA from BMDMs was extracted using the Qiagen RNAasy Mini Kit (74104). RNA purity, integrity, and concentration were determined using an Agilent Bioanalyzer. mRNA was compared by Affymetrix Mouse Gene 1.0ST expression arrays. Quantitative real-time polymerase chain reaction (qPCR) was conducted as described previously, and the results were normalized to GAPDH or cyclophilin. qPCR primers and their annealing temperatures used are listed in Online Table V.

Antibodies for Immunoblot, Immunofluorescence, and Chromatin Immunoprecipitation
Protein extraction and Western blot analysis were performed as described. A custom rabbit antibody against IRF2BP2 was described. Cryostat sections (10 μm) were subjected to immunofluorescence, TUNEL (Promega, G3250), hematoxylin and eosin (PerkinElmer), and immunohistochemistry. Lesion areas were quantified in sections were harvested, and every third section was used for histology or immunohistochemistry. Lesion areas were quantified in 8 to 12 sections 10 μm thick and 30 μm apart stained with oil red O, hematoxylin and eosin, and Masson’s trichrome. Additional sections were stained with macrophage–specific antibodies or with TUNEL stain. Lesion areas were also quantified by en face oil red O staining of aortas dissected from additional ApoE−/− mice.
Plasmid Constructs, Lentiviral Vector, Transfections, and Luciferase Assays
IRF2BP2 3′ UTR and KLF2 promoter luciferase reporter constructs are detailed in Methods in the Online Data Supplemental. Lentivirus expressing mouse KLF2 (Lenti-GIII-CMV-Mouse-KLF2-HA) and control lentivirus were purchased from Applied Biological Materials (Richmond, BC). Expression vectors and shRNA (short hairpin RNA) for IRF2BP2 and transient transfections were as described.12

Statistical Analysis
All results are presented as mean±SEM unless specified. For between-group comparisons, a 2-tailed unpaired Student’s t test was applied. Where appropriate, data were analyzed by ANOVA followed by Fisher’s least significant difference post hoc test to compare means between groups. For genetic association, continuous variables are presented as mean±standard deviation, and categorical variables are presented as N (%). Continuous variables were compared between CAD cases and controls by Student’s t test, whereas categorical variables were compared by the χ2 test. All statistical analyses were 2-sided and performed with SAS Enterprise Guide (v.4.3; SAS Institute Inc.) and R (v.3.0.2; The R Foundation for Statistical Computing, Vienna, Austria) and were considered significant at a threshold of P<0.05.

Results
IRF2BP2 Plays an Essential Role in Macrophage Polarization
IRF2BP2 was ablated in the myeloid lineage (KO; Online Figure I). BMDM were cultured from KO or littermate...
control mice (HET or IRF2BP2$^{\text{flox/flox}}$ [WT]). In KO BMDM, IRF2BP2 mRNA and protein levels were reduced to <10% of WT by quantitative real-time PCR and immunoblot analysis (Figure 1A and 1B). IRF2BP2 is produced as 2 alternatively spliced isoforms,\textsuperscript{9,12} and both are present in macrophages. Trace levels of the upper band in the KO BMDM likely reflect the presence of the long isoform expressed from a minority of nonmyeloid cells in our cultures, where LysMCre is not expressed. Interestingly, similar protein levels were detected in HET and WT BMDM, indicating that IRF2BP2 is haplo-sufficient, that is the presence of one allele is sufficient for normal protein expression.

Whole genome expression analysis was performed using RNA isolated from independent primary BMDM cultures isolated from 3 male wild-type and 3 male KO mice analyzed on Affymetrix Mouse Gene 1.0ST arrays. Results are presented in Online Table III and as a heat map in Online Figure II. Pathways affected by IRF2BP2 ablation are ranked according to gene ontology terms (Online Table IV).

Lipopolysaccharide lowered and IL-4 elevated IRF2BP2 mRNA and protein levels in mouse BMDM (Figure 1C and 1D). Ablation of IRF2BP2 in KO BMDM was associated with elevated mRNA of inflammatory M1 markers (IL-1β, TNFα, CCL2, and iNOS) even under basal conditions, and these markers were further elevated by lipopolysaccharide treatment (Figure 1E). In contrast, IL-4 induction of the mRNAs for anti-inflammatory M2 markers Arginase1, Retnllb, Mgl1 (Clec10A), and MRC1 (CD206) was compromised in KO BMDMs compared with WT and HET BMDMs (Figure 1F). ELISA analysis showed that KO BMDMs released more IL-1β into the medium than HET BMDM even without lipopolysaccharide stimulation (Figure 1G). Immunoblot analysis confirmed skewed polarization of KO BMDM (Figures 1H and 1I). These studies indicate that IRF2BP2 suppresses the M1 inflammatory macrophage phenotype and promotes an anti-inflammatory M2 phenotype.

**High-Fat Diet Increases LDL Cholesterol in KO Mice**

Local inflammation of liver-resident macrophages can impair hepatic cholesterol homeostasis, leading to elevated serum LDL C,\textsuperscript{21} particularly in animals fed a high-fat diet. Of note, the LysMCre transgene is expressed in Kupffer cells.\textsuperscript{22} KO mice had higher serum total cholesterol levels (Figure 2A), and the fast protein liquid chromatography profile of serum showed that intermediate-density lipoprotein, LDL, and high-density lipoprotein levels were more elevated after high-fat diet (Figure 2B and 2C). This suggests that loss of IRF2BP2 in macrophages can disturb hepatic cholesterol homeostasis and elevate the risk for atherosclerosis.

**IRF2BP2-Deficient Macrophages Have an Increased Propensity for Foam Cell Formation**

KO BMDM accumulated more lipids after treatment with oxLDL than BMDM from their littermate controls.
IRF2BP2 Inhibits Macrophage Inflammation

(Figure 3A), in keeping with increased expression of the oxLDL-C receptor oxidized low-density lipoprotein (lectin-like) receptor 1 (OLR1) (Online Table III). KO BMDM had higher [3H]-cholesterol uptake (Figure 3B) and lower cholesterol efflux to ApoA1 and high-density lipoprotein (Figure 3C). Autocrine cholesterol efflux, a process dependent on macrophage ApoE production, was also lower in KO BMDM (Ctrl, Figure 3C) and associated with lower ApoE mRNA levels (Figure 3D). Immunoblot analysis revealed markedly impaired upregulation of the ABCA1 and ABCG1 cholesterol transporters in KO BMDM after cholesterol loading compared with HET BMDM (Figure 3E). This would account for reduced cholesterol efflux in KO BMDM.

As a result, total cholesterol was higher in cholesterol-loaded KO BMDM (Figure 3F). The ratio of esterified to free cholesterol was not different, indicating that cholesterol storage and mobilization are not impaired in KO BMDM (Figure 3G). However, the higher free cholesterol in KO BMDM (Figure 3F) would be toxic and proinflammatory.23 Together, these data indicate that IRF2BP2-deficient macrophages have an increased propensity for foam cell formation.
IRF2BP2-Deficient Macrophages Worsen Atherosclerosis

The atherogenic effect of IRF2BP2 ablation in macrophages was tested in 2 atherosclerosis-susceptible mouse backgrounds, LDLR−/− and ApoE−/− mice. LDLR−/− mice were irradiated and received bone marrow from KO or HET mice and were fed an atherogenic western diet for 12 weeks. We chose to use HET rather than WT macrophages to control for possible effects of the LysMCre transgene on macrophage function. The LysMCre transgene also allowed us to confirm
successful bone marrow engraftment by PCR (data not shown). Importantly, our data showed similar IRF2BP2 protein levels (Figure 1), M1 and M2 responses (Figure 1), and cholesterol handling (Figure 3) in WT and HET macrophages.

Atherosclerotic lesion areas were larger in LDLR−/− recipients of KO bone marrow cells (KO−>LDLR−/−, Figure 4A). The relative collagen deposition by Masson’s trichrome stain was similar in lesions of LDLR−/− mice receiving either HET or KO bone marrow, arguing that smooth muscle cells that produce collagen in lesions were affected similarly (Online Figure III). No difference was seen in fast protein liquid chromatography lipoprotein profiles between KO−>LDLR−/− and HET−>LDLR−/− mice after a 12-week atherogenic western diet (Figure 4B), likely because of the overriding effect of LDLR deficiency to elevate LDL-C. Macrophage proliferation within atherosclerotic lesions can increase progression of atherosclerosis.24 However, no difference in cell proliferation was observed between KO and control cultured BMDM, as assessed by Ki67 immunofluorescence (Online Figure IV). By contrast, more apoptotic cells were found at the lesions of KO−>LDLR−/− mice compared with HET−>LDLR−/− mice as detected by TUNEL (Figure 4C) and Caspase 3 staining.

Figure 5. Interferon regulatory factor–binding protein 2 (IRF2BP2) deficiency in macrophages increases atherosclerosis in the apolipoprotein (Apo)E−/− background. A, Compared with ApoE−/− bone marrow–derived macrophages (BMDM), double knockout (DKO, ApoE−/−: LysMCre/−/IRF2BP2flox/flox) have a more robust response to lipopolysaccharide (LPS)-induced M1 marker expression and an impaired response to IL-4. B, After 14 week’s western diet, aortic root sections stained with hematoxylin and eosin and oil red O revealed more extensive lesions in DKO (ApoE−/−: LysMCre/−/IRF2BP2flox/flox) compared with ApoE−/−: WT (IRF2BP2flox/flox) or ApoE−/−: HET (LysMCre/−/IRF2BP2flox/flox) mice. Quantification of lesion areas in ApoE−/−:WT, ApoE−/−:HET, and DKO mice. C, En face staining with oil red O showed more extensive atherosclerotic lesions in DKO mice, as quantified in n=8 male mice per group. Mean±SD. *P<0.05. Scale bar: 200 μm (B), 1 cm (C). D, Fast protein liquid chromatography (FPLC) serum cholesterol profile is similar in ApoE−/− and DKO mice after western diet.
Figure 6. Interferon regulatory factor–binding protein 2 (IRF2BP2) mediates myocyte enhancer factor 2 (MEF2)-dependent Krüppel-like factor 2 (KLF2) expression. A, IRF2BP2 LysMCre−/−/IRF2BP2flox/flox (KO) mouse bone marrow–derived macrophages (BMDM) expressed lower levels of KLF2 mRNA, as quantified by qPCR analysis. n=3 mice per genotype. B, Immunoblot analysis showed IRF2BP2 and KLF2 are induced by oxidized low-density lipoprotein cholesterol (oxLDL) exposure for 8 h in LysMCre−/−/IRF2BP2flox/wt (HET) mouse BMDM. KLF2 expression is reduced in KO BMDM. C, Chromatin immunoprecipitation (ChIP) with IRF2BP2 antibody pulls down DNA sequences of the mouse KLF2 promoter from wild-type (WT) BMDM. ChIP with H3K4me3 antibody shows open chromatin conformation at the KLF2 promoter in WT BMDM. D, Knockdown of IRF2BP2 in F11 cells decreased WT but not MEF2-mutant KLF2 promoter activity. Mean±SEM. n=4 each. E, IRF2BP2 overexpression increased WT but not MEF2-mutant KLF2 promoter activity. Mean±SEM. n=4 each. F, Lentivirus-expressing mouse KLF2 reduced M1 marker activation in response to lipopolysaccharide (LPS; 25 ng/mL). Immunoblot analysis confirmed reduced LPS-induced iNOS activation. G, Expressing KLF2 also restored M2 marker mRNA and protein activation in KO BMDM. H, KLF2 overexpression restored cholesterol efflux and uptake in IRF2BP2 KO BMDM, by normalizing the expression of ABCA1 and oxidized low-density lipoprotein (lectin-like) receptor 1 (OLR1), respectively. Mean±SEM, n=3, *P<0.05. acL indicates acetylated LDL; Ctrl, control; and oxL, oxLDL.
Increased apoptosis in IRF2BP2-deficient macrophages was also observed in cultured BMDM both under basal culture conditions and after oxLDL challenge (Figure 4E).

The most striking difference between HET and KO recipient mice was the high prevalence of inflammatory iNOS+ M1 macrophages within atherosclerotic lesions in KO recipients (Figure 4C) and the virtual absence of CD206+ anti-inflammatory M2 macrophages (Figure 4D). This inflammatory phenotype was accompanied by increased T-cell infiltration (Online Figure V), an effect that can exacerbate atherosclerosis. Together, these results indicate that loss of IRF2BP2 in bone marrow cells increases apoptotic and inflammatory macrophages within lesions and worsens atherosclerosis.

Incidentally, we observed hypertrophy of the ventricular myocardium and the interventricular septum in KO->LDLR−/− mice.
In another atherosclerosis model, IRF2BP2 was ablated in the myeloid lineage in mice deficient for ApoE (ie, DKO). An inflammatory phenotype was also observed in BMDM of DKO mice (Figure 5A). More extensive atherosclerosis was observed in DKO mice after 14 weeks of a western diet as revealed by oil red O staining of sections at the aortic root (Figure 5B) and by en face staining of dissected aortas (Figure 5C). No difference in lesion size was observed between ApoE−/−:WT and ApoE−/−:HET mice (Figure 5B and 5C). Fast protein liquid chromatography chromotologer profiles were not different between the 3 groups (Figure 5D). It is also noteworthy that myocardial hypertrophy was not observed in the DKO mice (data not shown).

IRF2BP2 Mediates MEF2-Dependent KLF2 Expression

Microarray analysis identified potential IRF2BP2-dependent genes in KO BMDM (Online Tables III and IV). Of particular interest, the anti-inflammatory KLF228 was significantly reduced, and this was confirmed at the mRNA (Figure 6A) and protein (Figure 6B) levels in KO BMDM isolated from a different cohort of mice. In addition, KLF2 protein was nearly undetectable in macrophages at atherosclerotic lesions of ApoE−/− mice (Online Figure VI). Expression of KLF2 in BMDM (Figure 6C). Chromatin immunoprecipitation with an IRF2BP2 antibody revealed the presence of IRF2BP2 at the KLF2 promoter in BMDM (Figure 6C). Chromatin immunoprecipitation with an antibody to trimethylated lysine 4 of histone 3 (H3K4me3), an indicator of open chromatin structure, revealed that the KLF2 promoter was less active in KO BMDM (Figure 6C). KLF2 expression is dependent on the transcription factor MEF2,29 and IRF2BP2 interacts with a Vgl4/TEAD4/MEF2 complex.512126 To test whether IRF2BP2 works through MEF2 to control KLF2, we generated KLF2 promoter luciferase reporters bearing a wild-type or a mutated MEF2-binding site. We found that IRF2BP2 knockdown decreased, whereas overexpression increased the activity of the wild-type KLF2 promoter, but had no effect on the MEF2 mutant (Figure 6D and 6E). These studies demonstrate that IRF2BP2 promotes KLF2 expression in an MEF2-dependent manner.

Next, we tested the functional requirement of KLF2 in IRF2BP2-dependent macrophage polarization. Lentiviral overexpression of KLF2 in KO BMDM attenuated inflammatory M1 marker activation and rescued anti-inflammatory M2 marker activation (Figure 6F and 6G). It should be noted that at a higher dose of lipopolysaccharide, the effect of KLF2 to suppress M1 markers was lost (Online Figure VII). KLF2 restoration also improved cholesterol handling in IRF2BP2 KO BMDM (Figure 6H and 6I) by increasing ABCA1 and lowering OLR1 protein levels in cholesterol-loaded KO BMDM (Figure 6J). Together, these results argue that loss of KLF2 expression because of IRF2BP2 ablation accounts for much of the inflammatory and impaired cholesterol handling phenotypes of macrophages.

Deletion Polymorphism (rs3045215) in the 3′UTR of IRF2BP2 Increases CAD Risk

We identified a 9-nucleotide deletion (rs3045215) that disrupts a conserved sequence in the 3′UTR of the human IRF2BP2 mRNA (Figure 7A). Because conventional single nucleotide polymorphism microarrays cannot detect small deletions, this variant has not been genotyped in prior genome-wide association studies. We exploited a BsrI restriction site that is interrupted by the 9-nucleotide deletion to genotype a random sample of 1066 angiographically documented CAD cases and 1011 controls from the Ottawa Heart Genomics Study.15 Homozygous carriers of this deletion variant had a higher incidence of CAD, odds ratio (95% confidence interval) = 1.560 (1.179–2.065), P=1.73E-03 in a recessive model (Figure 7B). By comparison, the 9p21.3 locus (tagged by rs133049) was associated with CAD risk with an odds ratio 1.45 (1.28–1.64), P=3.00E-09. Patient characteristics are provided in Online Table I. The rs3045215 deletion allele remains significantly associated with CAD after adjusting for known risk factors (smoking, hypertension, body mass index, male sex, LDL-C, high-density lipoprotein cholesterol, and triglycerides) by logistic regression analysis (Online Table II).

rs3045215 Deletion Variant Lowers IRF2BP2 Protein Expression

We next examined peripheral blood mononuclear cells from CAD patients genotyped for the rs3045215 polymorphism and found reduced levels of IRF2BP2 protein in homozygous carriers of the rs3045215 deletion polymorphism (Figure 7C). KLF2 protein levels were also reduced in homozygous carriers of the risk (deletion) allele of rs3045215 (Figure 7D). Individuals were matched for age of consent and other clinical characteristics (Online Table V). Of note, IRF2BP2 and KLF2 protein levels were not different between homozygous nonrisk and heterozygous individuals.

Sequences within the 3′UTR are known to affect mRNA stability and translation efficiency. To test whether the 9-nucleotide deletion in the 3′UTR of IRF2BP2 mRNA directly lowers IRF2BP2 protein expression, we synthesized luciferase reporters bearing the complete IRF2BP2 3′UTR sequence (2877 nucleotides) differing only by the 9-nucleotide deletion (rs3045215). The reporter construct bearing the deletion allele showed markedly reduced luciferase activity (Figure 7E), indicating post-transcriptional regulation of IRF2BP2 expression. To test whether the deletion affects mRNA stability, cells transfected with the 2 reporter plasmids were treated with actinomycin D to block de novo transcription 24 hours after transfection. The insertion and deletion showed similar rates of decay (Figure 7F), indicating that the deletion does not affect mRNA stability.

Discussion

Inflammatory macrophages worsen atherosclerosis in animal models, yet the genetic program that controls macrophage
inflammation remains only partially understood. Here we discovered that macrophages deficient in IRF2BP2 are inflammatory, have impaired cholesterol handling, and worsen atherosclerosis in mice. We showed that IRF2BP2 is required for the expression of the anti-inflammatory transcription factor KLF2 and that, by restoring KLF2 expression, we can attenuate the inflammatory phenotype and improve cholesterol handling by IRF2BP2-deficient macrophages. In humans, homozygous carriers of a 9-nucleotide deletion in the 3′UTR of IRF2BP2 have lower IRF2BP2 (and KLF2) protein expression and have an increased risk of CAD.

Our findings that IRF2BP2 is required for MEF2-dependent expression of KLF2 and that loss of IRF2BP2 affects open chromatin structure at the KLF2 promoter suggested that loss of KLF2 might account for the inflammatory phenotype of IRF2BP2-deficient macrophages. Lentiviral overexpression of KLF2 in IRF2BP2-deficient macrophages suppressed inflammatory M1 marker activation by lipopolysaccharide and restored anti-inflammatory M2 markers in response to IL-4. The anti-inflammatory function of KLF2 has been reported for human monocytes, and patients with CAD have lower KLF2 expression in circulating monocytes.

The inflammatory M1 phenotype of IRF2BP2-deficient macrophages would predict their propensity for foam cell formation because IL-1β impairs ApoE secretion and ApoE-mediated autocrine cholesterol efflux from macrophages. IL-1β also suppresses the expression of the cholesterol transporter ABCA1. Indeed, we found that IRF2BP2-deficient macrophages produce and secrete more IL-1β, had reduced ApoE expression, and failed to sufficiently activate ABCA1 in response to cholesterol loading. Moreover, in vivo, inflammatory IRF2BP2-deficient macrophages worsened atherosclerosis in 2 different atherosclerosis-susceptible mouse backgrounds fed an atherogenic western diet: (1) irradiated LDLR−/− mice transplanted with IRF2BP2-deficient bone marrow and (2) ApoE and myeloid-specific IRF2BP2 DKO mice. In addition, few M2 macrophages were detected in lesions of KO−/−LDLR−/− mice. Because M2 macrophages help to resolve inflammation, the lack of M2 macrophages likely also contributes to increased atherosclerosis.

M2 macrophages are important to remove apoptotic cells in atherosclerotic lesions. The reduced presence of M2 macrophages with IRF2BP2 ablation would prevent the clearance of apoptotic cells, and this might account for the elevated apoptotic index we observed. Alternatively, loss of KLF2 might also account for the increased susceptibility of IRF2BP2-deficient cells to apoptosis because others have reported that KLF2 has an anti-apoptotic function in B lymphocytes. Ablation of IRF2BP2 has been reported to augment apoptosis in cultured cells.

We also observed an effect of IRF2BP2 on human CAD risk. Humans who carry 2 copies of a deletion polymorphism (homozygotes) have lower IRF2BP2 protein levels (and its target protein KLF2) in peripheral blood mononuclear cells and increased risk of CAD. It is important to note that the deletion variant associates with CAD risk in a recessive model (i.e., in homozygous carriers). Consistent with a recessive phenotype of this allele, heterozygote carriers did not have reduced protein levels of IRF2BP2 in peripheral blood mononuclear cells nor did they have an increased risk of CAD. The recessive phenotype of the human deletion variant is reminiscent of the phenotype we observed in mice, where hemizygous mice (HET, lacking 1 copy) are not different from wild-type mice. We observed similar IRF2BP2 protein levels in hemizygous and wild-type BMDM and saw no difference in the atherogenic phenotype on an ApoE−/− background.

The effect of the 9-nucleotide deletion to suppress IRF2BP2 protein expression was supported by luciferase reporter assays. Because we saw no difference in the decay of the IRF2BP2 mRNA with or without the deletion, the deletion is unlikely to affect mRNA stability. Instead, our luciferase reporter study suggests that the deletion polymorphism that occurs in a highly conserved region of the 3′UTR of human IRF2BP2 may affect mRNA conformation and translation. The RNA-binding protein Staufen 1 was reported to bind human IRF2BP2 mRNA. Staufen 1 is known to promote translation of highly structured mRNAs. Whether the 9-nucleotide deletion affects Staufen 1 binding and regulation of IRF2BP2 translation remains to be tested. Understanding how the deletion variant reduces IRF2BP2 protein expression may reveal a novel therapeutic target for atherosclerosis.

Our study has several limitations. First, the association of the deletion variant with CAD risk was determined in a single case/control cohort. Because the linkage of the deletion variant to single nucleotide polymorphisms genotyped on commercial arrays is not known and it has not yet been elucidated in the 1000 genomes (http://www.1000genomes.org/), we cannot identify surrogate single nucleotide polymorphisms to confirm our discovery in other genome-wide association studies. Genotyping the deletion variant by the restriction fragment method used here is labor-intensive and too costly to test for replication in other cohorts. Future fine mapping studies defining the haplotype structure of the deletion variant to identify surrogate markers will enable large-scale genotyping to confirm association of this variant with CAD. Although our luciferase reporter studies indicate that the deletion polymorphism is functional, we cannot exclude that a yet-to-be-identified single nucleotide polymorphism linked to this polymorphism might also contribute to increased CAD risk. A second limitation is that no association of the rs3045215 deletion allele was detected with total or LDL-C levels in our cohort of 2087 individuals. This may reflect higher use of statins in our cases, masking the association with cholesterol. Our study may also be inadequately powered to detect such an association. Of note, a study with over 100000 individuals was able to identify an association of total cholesterol with another IRF2BP2 variant (rs514230).

In summary, we have shown that the transcription cofactor IRF2BP2 limits macrophage inflammation and atherosclerosis in mice, largely through its effect on the anti-inflammatory transcription factor KLF2 expression. In humans, homozygous carriers of a deletion variant that lowers IRF2BP2 protein expression, as well as its downstream target KLF2, had increased risk of CAD. Maintaining macrophages in an anti-inflammatory state by targeting a translational mechanism to sustain IRF2BP2 levels may help to reverse atherosclerosis.

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Disclosures

None.

References

What Is Known?

- Inflammation impairs macrophage cholesterol handling and worsens atherosclerosis.
- Macrophage inflammation is controlled by anti-inflammatory transcription factors.
- Interferon regulatory factor–binding protein 2 (IRF2BP2) is a transcription cofactor whose expression is suppressed in inflammatory macrophages.
- IRF2BP2 locus has been associated with plasma cholesterol levels in genome-wide association studies.

What New Information Does This Article Contribute?

- Mice lacking IRF2BP2 in macrophages are more susceptible to atherosclerosis.
- IRF2BP2 is required for the expression of the anti-inflammatory transcription factor Krüppel-like factor 2.
- Restoring Krüppel-like factor 2 in IRF2BP2-deficient macrophages attenuates inflammation and improves their cholesterol handling.
- A deletion polymorphism in the 3′ untranslated region of the human IRF2BP2 mRNA is associated with reduced protein expression of IRF2BP2 and its target gene Krüppel-like factor 2 and increased risk of coronary artery disease.

Genetic studies identified variants near the IRF2BP2 locus that associated with plasma levels of total cholesterol and low-density lipoprotein cholesterol. Given the key function of macrophages in the formation of atherosclerotic lesions, we investigated the role of IRF2BP2 in macrophages. We found that IRF2BP2 suppresses macrophage inflammation and promotes expression of the anti-inflammatory genes. Mice that lacked IRF2BP2 in their macrophages had impaired cholesterol efflux and more severe atherosclerotic lesions. We showed that IRF2BP2 controls expression of an anti-inflammatory transcription factor Krüppel-like factor 2 that mediates some of the effects of IRF2BP2 deficiency in macrophages. In humans, we found that a deletion polymorphism in the 3′ untranslated region of IRF2BP2 lowered protein expression and is associated with increased risk of coronary artery disease. Thus, our study identifies IRF2BP2 as a novel modulator of macrophage inflammation and a potential target for the treatment of coronary artery disease.
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Supplemental Methods

The OHGS
By design, the OHGS excluded individuals with diabetes mellitus, as described previously \(^1\). CAD cases were defined by angiography as having a stenosis of greater than 50% in a major coronary artery. Controls were asymptomatic elderly individuals recruited from the community, or individuals who underwent coronary angiography prior to heart valve surgery, or symptomatic individuals but with minimal (<30%) stenosis. Individuals with intermediate stenoses between 30% and 50% were excluded. Information regarding medication to treat hypertension, and baseline plasma total cholesterol, LDL-C, HDL-C, and triglycerides were measured by colorimetric assays on a Roche Modular Analytics P Module clinical chemistry analyzer (Roche Diagnostics Inc., Laval, Quebec, Canada).

BMDM culture
Femurs and tibias from each mouse were flushed with DMEM (GE Hyclone) and cells were centrifuged at 1400 g for 5 min, re-suspended and cultured in DMEM containing 20% FBS, 15% L929 conditioned medium, penicillin/streptomycin, l-glutamine on non-tissue culture treated plates (BD Falcon) for 6 days. Conditioned medium was collected from L929 cells cultured for 7 days, centrifuged at 2000g for 5 min and filtered through a 0.45µm low protein binding filter. Medium was changed at DIV4. BMDM were then stimulated with LPS (25 or 100 ng/ml, Sigma) or IL-4 (5 ng/ml, Sigma) for 8 hours and harvested for mRNA and protein analysis. IL-1β secretion into the culture medium was measured by ELISA assay (R&D Biosystems) according to the manufacturer’s instructions.

Cholesterol accumulation, uptake, efflux and content
To visualize cholesterol accumulation, BMDMs were grown on cover slips and treated with oxLDL (30 µg/ml, Biomedical Technologies, Inc) for 24 hours, then washed fixed with 4% paraformaldehyde in PBS, washed once with PBS, once with 60% isopropanol and incubated with 0.3% Oil Red O in 60% isopropanol for one hour, washed twice with PBS and once with 60% isopropanol. BMDMs were stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted for imaging purposes.

Cholesterol uptake was determined \(^2\). Briefly, acetylated LDL (AcLDL) 50 µg/ml was pre-incubated with \(^3\)H]-cholesterol (PerkinElmer) 1 µCi/ml for 16 hours at 37°C before and used to treat BMDMs for 30 minutes. The cells were then washed, lysed with 0.5 N NaOH for two hours at room temperature. Radioactivity of aliquots of medium from each condition (200 µl) was measured and values were normalized to the protein amount of BMDMs cultured simultaneously in separate dishes.

Efflux was quantified as described \(^3\). BMDMs were treated with \(^3\)H]-cholesterol 1 µCi/ml for 48 hours, washed with PBS and incubated with serum-free medium or medium containing HDL 50µg/ml (Biomedical Technologies, Inc) or ApoA1 30 µg/ml (Biomedical Technologies, Inc). After 4 hours, radioactivity in 150 µl aliquots of medium was measured and normalized to the radioactivity of total cell lysates (lysed with 0.5N NaOH overnight).

Cholesterol content in BMDM was measured using “Cholesterol/cholestereryl ester quantification colorimetric kit” (Biovision, Catalog # K603-100) after treated with AcLDL 50µg/ml for 24 hours. Cholestereryl ester was calculated by subtracting free cholesterol from total cholesterol.
IRF2BP2 3’UTR luciferase reporters
A CMV enhancer-driven pGL3 luciferase vector was constructed with the complete 2,877 nucleotide long 3’UTR of human IRF2BP2 with or without the 9 nucleotide deletion of rs3045215. PCR primers containing an Xbal site (underlined) (forward: 5’-CCT CTA GAG CAC ACA TGC AGA AAT GCA GAG TC-3’, reverse: 5’-CCT CTA GAC ATA CAG ATA GCT ACC ACA AAT TAG GTC-3’) were used to amplify human IRF2BP2 genomic DNA. Insert orientations were determined by restriction enzyme digestion and constructs were fully sequenced.

KLF2 promoter construct
Wild type and MEF2 mutant 224 bp fragments of the human KLF2 promoter (-193 to +31) were synthesized by Integrated DNA Technologies with flanking KpnI and Xho1 sites and cloned into the IDTSmart vector. KpnI/Xho1 fragments were released and sub-cloned into the pGL3-Basic luciferase reporter plasmid (Promega). The fragments contained the following sequences: hKLF2 224 WT Kpn1 to Xhol: 5’aaggtaccAAG GCCCAGGCCG CCGCAGTGGC CACGGCCGCT GCCGCCCGCCG GCCGCCCGCG CGCTTATATA CCGCGGCTATA CTAGTTAGGCTG CGCCCGAGCT CGTCCCATCGGGGACCGTTTATCCGCGCGTTTGC GCCCGGCCCG CGCCGCGCCG CCCGGCTATAA AGGGTGGGCGG GCCCGGCCGCG CGGCCCACACAG AGCCGTCCCC GCCCCCGCCGC GCCCCGGACCA GCCCCGGCGCTC Gagaa-3’, and hKLF2 224 MEF2 mutant Kpn1 to Xhol: aaggtaccAA GGCCCAGGCCGCCGCAGTGGG CGCCCGGCCG TGCCCGCGGC CGCCGTTATAT ACCGCCGCTG gATggAGGCT GCCCGGGAG CTGTCCTCCCC ATCCGGGACCG TCCTTCGCCCG CCGCCCGCTTT GGCCCGGGC CGCCGCCGCCG CGCCGCGCGG CGCCGCTATAA AGGGTGGGCGG GCCCGGCCGCG CGGCCCACACAG GAGCCGTCCCC GCCCCCGCCGC GCCCCGGACCA GCCCCGGCGCTC Gagaa-3’. The wild type MEF2 and mutant sequence are underlined. The start of transcription is double underlined.

Microarray analysis.
To identify differential gene expression between knockouts and controls, a Bayesian t-test analysis was performed using the Cyber-T analysis package for R 4. A Bayesian estimation of the standard deviation for microarray expression data values is more accurate than the standard estimation employed by the Student’s t-test, when dealing with a low (N<5) number of replicates 5,6. The Benjamini and Hochberg (BH) False Discovery Rate (FDR) method was used to correct for multiple testing 7. The significance threshold for the BH corrected p-values was set at 0.05, equal to an FDR of 5%. All microarray raw intensity values and intra-array differences were normalized through log base 2. Log base 2 transformation is essential as microarray data is heavily skewed with a high level of mean-dependent variance of the standard deviation.
References


Supplemental DATA

Online Figure I
Schematic diagrams showing the generation of IRF2BP2flox mice. LoxP sites were inserted into a genomic DNA fragment upstream of the promoter of IRF2BP2 and another site was inserted downstream of the IRF2BP2 3’UTR. A PGKneo selection cassette flanked by Frt sites was used for selection of recombinants. In the presence of Cre recombinase driven by the Lysozyme promoter, the sequence between the LoxP sites (the entire IRF2BP2 coding sequence and 3’UTR) was deleted in the myeloid lineage. Germline transmission and myeloid lineage-specific ablation (including macrophages and microglia) were confirmed by PCR.
Online Figure II
Heat Map of differentially expressed genes in IRF2BP2-deficient BMDM revealed by microarray analysis (also see Online Tables III and IV).
Online Figure III
Collagen deposition revealed by Masson’s trichrome (A) was not different between HET→LDLR−/− (n=9) and KO→LDLR−/− (n=8) mice after 12 weeks of western diet. (B) Lesion collagen area was measured using the Zeiss Axiovision software. Mean areas ± SEM. Scale bar = 200 µm.
**Online Figure IV**
No change in cell proliferation in IRF2BP2 KO BMDM. Proliferating BMDM was labeled by immunofluorescence with Ki67 antibody and counter stained with nuclear DAPI. The ratio of Ki67-immunopositive over DAPI-positive nuclei was similar between IRF2BP2 HET and KO BMDM under basal conditions or after 24 hours exposure to oxLDL (50 µg/ml). n=10 fields each from BMDM of 2 HET and 2 KO mice. Mean ± SEM. *, p<0.05. Scale bar, 100 µm.
Online Figure V.

T-cell infiltration is higher in atherosclerotic lesions of DKO mice. Immunofluorescence staining with CD3 antibody identified T-cells (red) at atherosclerotic lesions. Macrophages were labeled with CD68 (green). Right panel shows quantification, n=8 per group. *, p<0.05. Scale bar = 50 µm.
Online Figure VI.
KLF2 expression in macrophages is markedly reduced in atherosclerotic lesions of DKO mice. Immunofluorescent staining showed that KLF2 is readily detected in CD11b+ macrophages in atherosclerotic lesions of ApoE−/−:HET mice (arrows). In contrast, few KLF2+ macrophages were detected in atherosclerotic lesions of DKO mice. See right panels for quantification. N=8 per group, ns, not significant. The bottom panels are negative controls (no primary, only secondary antibody) for each of the panels shown. Nuclei were stained with DAPI. Scale bar = 50 µm.
Online Figure VII.
Lentiviral vector expressing KLF2 fails to attenuate M1 inflammatory marker expression in IRF2BP2-deficient BMDM when challenged with a high dose of LPS (100 ng/ml) for 8 hours. (A) qPCR and (B) immunoblot. Fold changes in protein expression relative to HET under basal conditions were quantified and normalized to actin. Mean ± SEM. n=3; ns, not significant.
Online Table I
Clinical characteristics of CAD cases and controls in the OHGS.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=1,066)</th>
<th>Missing (N)</th>
<th>Controls (n=1,011)</th>
<th>Missing (N)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, (years)</td>
<td>57 ± 10</td>
<td>169</td>
<td>74 ± 6</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male sex, N (%)</td>
<td>758 (71.1)</td>
<td>0</td>
<td>482 (47.7)</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.6 ± 5.4</td>
<td>1</td>
<td>26.6 ± 4.2</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Smoking, N (%)</td>
<td>824 (77.3)</td>
<td>0</td>
<td>538 (53.2)</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HTN, N (%)</td>
<td>596 (58.5)</td>
<td>47</td>
<td>479 (47.4)</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total-C, mmol/L</td>
<td>6.00 ± 1.47</td>
<td>279</td>
<td>5.65 ± 1.08</td>
<td>134</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>3.80 ± 1.17</td>
<td>385</td>
<td>3.52 ± 0.88</td>
<td>163</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.17 ± 0.46</td>
<td>342</td>
<td>1.48 ± 0.46</td>
<td>148</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>2.29 ± 1.60</td>
<td>305</td>
<td>1.43 ± 0.94</td>
<td>141</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Continuous variables are presented as mean ± SD. Abbreviations: BMI, body mass index; HTN, antihypertensive medication; Total-C, total-cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; TG, triglycerides. Differences between cases and controls were tested using the Chi-square test for categorical variables (of which rs3045215 was one) and the Student’s t-test for continuous variables.
### Online Table II
Logistic regression analysis of the association between the minor allele of rs34045215 and CAD in the OHGS.

<table>
<thead>
<tr>
<th>Variable</th>
<th>β or OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3045215</td>
<td>1.228 (1.029, 1.465)</td>
<td>0.023</td>
</tr>
<tr>
<td>Male sex</td>
<td>1.658 (1.280, 2.148)</td>
<td>1.30E-04</td>
</tr>
<tr>
<td>Smoking</td>
<td>2.389 (1.853, 3.080)</td>
<td>1.80E-11</td>
</tr>
<tr>
<td>HTN</td>
<td>1.577 (1.242, 2.003)</td>
<td>1.88E-04</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>0.033 (0.007, 0.058)</td>
<td>0.011</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>0.225 (0.104, 0.346)</td>
<td>2.56E-04</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>-0.937 (-1.295, -0.580)</td>
<td>2.74E-07</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>2.578 (1.945, 3.211)</td>
<td>1.48E-15</td>
</tr>
</tbody>
</table>

For continuous variables beta is given with the 95% confidence interval (CI). For the categorical variables the odds ratio (OR) is given with the 95% CI. Abbreviations: BMI, body mass index; Smoking was defined as smoking history including past and present smokers versus never-smoked. HTN, hypertension; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; TG, triglycerides. Only the individuals with data for all variables were included in the analysis (N=1,481).
Online Table III
Microarray analysis comparing IRF2BP2 KO to WT BMDM.

Online Table IV
Pathway analysis and list of GO Terms

Online Table V
Patient characteristics of CAD cases sampled for immunoblot analysis of PBMCs

<table>
<thead>
<tr>
<th></th>
<th>Male sex (n)</th>
<th>Age at CAD</th>
<th>Age at Consent</th>
<th>BMI</th>
<th>Tot Chol</th>
<th>TG</th>
<th>LDL-Chol</th>
<th>HDL-Chol</th>
<th>Chol meds</th>
<th>HTN meds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo Risk (n=10)</td>
<td>9.49±6</td>
<td>51±9</td>
<td>30±5</td>
<td>4.34±1.32</td>
<td>1.90±0.87</td>
<td>2.50±1.32</td>
<td>0.96±0.31</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Het (n=4)</td>
<td>4.44±3</td>
<td>47±9</td>
<td>26±4</td>
<td>3.74±1.44</td>
<td>1.73±1.03</td>
<td>2.21±1.15</td>
<td>0.82±0.18</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Homo Non-Risk (n=3)</td>
<td>3.44±3</td>
<td>49±2</td>
<td>25±7</td>
<td>4.47±0.64</td>
<td>1.84±0.33</td>
<td>2.86±0.35</td>
<td>0.83±0.10</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

None of the clinical characteristics were significantly different between groups.

Online Table VI
The primer sequences and annealing temperatures used for qPCR or ChIP analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginase1</td>
<td>GGGAAAGGCAATGAAGAGCTG</td>
<td>AGAAAGGACACAGGTTCG</td>
<td>55</td>
</tr>
<tr>
<td>CCL2</td>
<td>CACCTCACCTGCTGCTACTCTTC</td>
<td>JCTTTGGGACACCTGCTG</td>
<td>57</td>
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<tr>
<td>CD206</td>
<td>CCCAAGGGCTCTTCTAAAGCA</td>
<td>GGCAACCTATCACCA</td>
<td>60</td>
</tr>
<tr>
<td>KLF2</td>
<td>ACCAAGAGCTGCACTATAAA</td>
<td>GCACTGAAAGGGCTCTGT</td>
<td>58</td>
</tr>
<tr>
<td>INOS</td>
<td>AGCCCTCACCTACTTCTCG</td>
<td>JCTCTGCTATCCGTCTC</td>
<td>59</td>
</tr>
<tr>
<td>IL1-β</td>
<td>GCTTCAGGCAGGCAGTATC</td>
<td>ATGGGCTCTTCTTCAAAG</td>
<td>56</td>
</tr>
<tr>
<td>IRF2BP2</td>
<td>AGTTCTGTTCCTCCCTTTGCTCC</td>
<td>JACCTTACATCCCGG</td>
<td>54</td>
</tr>
<tr>
<td>Mgl-1</td>
<td>TGAGAAAGGCTTTAAGAACTGGG</td>
<td>CACCTGATGTATGTTGGG</td>
<td>60</td>
</tr>
<tr>
<td>Retnlb</td>
<td>TCCAGCTAAACTATCCCCTACTGT</td>
<td>CATCTGTTCATAGCTTGA</td>
<td>57</td>
</tr>
<tr>
<td>TNFα</td>
<td>GGTCTGTCCCTCCCACCTCAC</td>
<td>JCTCTGAGCTTCC</td>
<td>56</td>
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<tr>
<td>GAPDH</td>
<td>TCTCTGCGACTTTCACAGAC</td>
<td>FCCAGGTTTCTTACTTC</td>
<td>54-60</td>
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<td>Cyclophilin</td>
<td>TGAGGCAACGGACAGGACAGA</td>
<td>CGAGTGCTGACATGAT</td>
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<tr>
<td>Cre</td>
<td>CCCAGATAACGGATTTGACG</td>
<td>CTGGGGTCCGGAATTTTCTC</td>
<td>64</td>
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<td>Klf2 promoter</td>
<td>CTTTCTGCGTCTGTGACG</td>
<td>CCCAGGCTTATAGGC</td>
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