Activating Transcription Factor 1 Directs Mhem
Atheroprotective Macrophages Through Coordinated Iron Handling and Foam Cell Protection

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Rationale: Intraplaque hemorrhage (IPH) drives atherosclerosis through the dual metabolic stresses of cholesterol-enriched erythrocyte membranes and pro-oxidant heme/iron. When clearing tissue hemorrhage, macrophages are typically seen storing either iron or lipid. We have recently defined hemorrhage-associated macrophages (HA-mac) as a plaque macrophage population that responds adaptively to IPH.

Objective: This study aimed to define the key transcription factor(s) involved in HO-1 induction by heme.

Methods and Results: To address this question, we used microarray analysis and transfection with siRNA and plasmids. To maintain physiological relevance, we focused on human blood-derived monocytes. We found that heme stimulates monocytes through induction of activating transcription factor 1 (ATF-1). ATF-1 coinduces heme oxygenase-1 (HO-1) and Liver X receptor beta (LXR-β). Heme-induced HO-1 and LXR-β were suppressed by knockdown of ATF-1, and HO-1 and LXR-β were induced by ATF-1 transfection. ATF-1 required phosphorylation for full functional activity. Expression of LXR-β in turn led to induction of other genes central to cholesterol efflux, such as LXR-α and ABCA1. This heme-directed state was distinct from known macrophage states (M1, M2, Mox) and, following the same format, we have designated them Mhem.

Conclusions: These results show that ATF-1 mediates HO-1 induction by heme and drives macrophage adaptation to intraplaque hemorrhage. Our definition of an ATF-1–mediated pathway for linked protection from foam cell formation and oxidant stress may have therapeutic potential. (Circ Res. 2012;110:00-00.)

Key Words: macrophages ■ atherosclerosis ■ heme oxygenase-1 ■ lipids ■ activating transcription factor-1

Atherosclerosis is an inflammatory disease of large artery walls, driven mainly by lipid peroxidant stress from oxidized low-density lipoprotein (OxLDL).1 Intraplaque hemorrhage (IPH) is particularly important in promoting both atherosclerotic lesion progression and destabilization.2 Thus, erythrocytes provide a potent combination of cholesterol-enriched membrane lipids and heme-iron, which together pose a serious metabolic challenge in a pathology largely driven by oxidized cholesterol.2 Along with intracranial hemorrhage, IPH is one of the most important examples of tissue damage due to extravasated blood.2 Heme-iron is an effective peroxidant catalyst, through hydrogen peroxide coordination and Fenton chemistry;3 cholesterol is modified by peroxidation to potent cytotoxic and inflammatory oxysterols (5', 6'-epoxycholesterol, 7'-ketocholesterol, and 21'-keto-cholesterol),4 and macrophages are abundant in atherosclerosis and generate hydrogen peroxide when activated. The combination of cholesterol loading, heme/iron loading, and macrophage activation would therefore promote lipid peroxidation.5 How monocytes entering plaques differentiate adaptively to clear hemorrhage-related iron and lipid may therefore be a key transcriptional decision in atherosclerosis.

Heme oxygenase-1 (HO-1) is a vital enzyme for iron homeostasis and protection from oxidant stress. It catalyzes pro-oxidant heme and generates biliverdin, free iron and carbon monoxide as reaction products.6 HO-1 activity also stimulates upregulation of ferritin genes, leading to the safe chelation of iron.6 Biliverdin is processed further to bilirubin, a direct antioxidant.6 Like nitric oxide, low levels of carbon monoxide activate soluble guanylate cyclase to signal vascular protective responses.8 For all these reasons, induction of HO-1 by heme provides a major pathophysiological homeostatic response in hemorrhage.

We have recently identified a unique state of macrophages in areas of hemorrhage within human atherosclerotic plaques...
that we originally designated HA-mac but have now renamed Mhem by analogy to M1, M2, and Mox states. These cells have increased intracellular iron but are likely to be atheroprotective, as they show increased HO-1 expression, reduced oxidative stress, suppressed inflammatory activation, and increased release of the anti-inflammatory cytokine IL-10. Furthermore, they do not show lipid accumulation of the type that defines foam cells. We have shown that the same state can be induced in human blood-derived monocytes in vitro by culturing in the presence of hemoglobin-haptoglobin complexes over 4–8 days. The in vitro generation of Mhem was found to be dependent on an autocrine loop involving release of the activating transcription factor 1 (ATF-1) as a key transcription factor mediating heme-induced HO-1 expression. ATF-1 is a close but relatively little studied homolog of cyclic-3′-5′-adenosine monophosphate (cyclic-AMP, cAMP) binding protein (CREB1), and both mediate transcriptional activation by cyclic-AMP by binding to the cyclic-AMP response element (CRE). Importantly, we have found that ATF-1 also induces a program of gene expression responsible for lipid export, providing a mechanism explaining the reciprocal nature of iron and lipid handling by the cell. Together, these mechanisms may underlie homeostatic adaptation to IPH in macrophages.

Methods

A full version of the Methods is at http://www.circres.ahajournals.org.

Cell Isolation and Culture

Peripheral blood-derived monocytes were purified and cultured from normal human volunteers as before, with informed consent and ethical clearance.

Microarray and Quantitative PCR

Standard molecular biology methods were used. Macroarrays were lysed, RNA-purified, and quality controlled, and then parallel aliquots were analyzed by both Agilent 4×44k and Affymetrix 1.0ST microarrays, following respective manufacturer’s instructions. Results were validated by reverse-transcription quantitative polymerase chain reaction (qPCR). Microarrays were analyzed with Agilent GeneSpring GX10 and genelists exported to specialized bioinformatics programs. For selected genes, qPCR was done over a full-time course in a further 5 donors.

Western Analysis

Westerns blots were by amendment of our previous methods. Cells were lysed and run by SDS-PAGE and blotted to PVDF using a commercial kit (Novex, Invitrogen). Blots were probed with multiple antibodies as indicated and visualized with ECL+ and Hyperfilm (GE Healthcare).

Quantitative Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChiP) was performed following a minor amendment of published protocols. Macroarrays were fixed in 1% formaldehyde at 4°C for 5 minutes, stopped with excess glycerine, lysed in nuclear lysis buffer AM-1, sheared by a 28-G needle 3 times (giving 1-kb fragments), and ChiP was carried out with the magnetic nonenzymatic kit (Active Motif) using Protein G beads (Invitrogen). Immunoprecipitates were DNA–purified, and quantitative (q)PCR was as above.

Quantification of ATF-1/DNA Binding

The 96-well plate assay for ATF-1 binding to promoter sequences was reverse-engineered from TransAm kit (Active Motif) for CREB1 activation (CREB1 and ATF-1 bind to a common motif). Oligos corresponding to human HO-1 and LXR-β enhancers or their ΔATF-1 mutants were bound to ELISA plates, macrophage lysates added, and ATF-1/DNA binding detected with anti–p-ATF-1 (Abcam).

Plasmids and siRNA

Plasmids (kind gifts, Y. Tsuji, North Carolina State University; A. Agarwal, University of Alabama at Birmingham; or purchased from Clontech) were amplified and purified by standard molecular biology
methods. Methods for transfection of volunteer blood-derived macrophages with plasmid DNA or siRNA were optimized and validated. Oligos for siRNA were from Dharmacon (Abgene) as indicated.

**Functional Assays**
Oxidative stress assays were carried out as before,7,8 using amino-phenyl fluorescein a selective dye assay for hypochlorite, hydroxyl radicals, and peroxynitrite (Invitrogen). Cholesterol export was measured by a minor modification of established methods.10 Macrophages were incubated with human recombinant ApoA1, an acceptor for cholesterol export, and cholesterol assayed with a specific high-sensitivity fluorescent kit (Invitrogen) as described.11 Luciferase analysis was a Dual-Luciferase kit (Promega) and a 96-well luminometer (BioTek Synergy HT). For the foam cell assays, macrophages were cultured on tissue culture glass slides, challenged with Ox-LDL, stained with 10 μmol/L Nile red (Sigma-Aldrich), and imaged by confocal microscopy (Zeiss LSM510), based on previous methods.7

**Results**

**Heme Induces HO-1 Through Activating Transcription Factor 1**
Macrophages in atherosclerotic plaques tend to store either iron (siderophages) or lipid (foam cells) (Figure 1A and 1B). To investigate the mechanisms underlying this distinction further, we stimulated freshly isolated human blood-derived monocytes with heme and measured gene expression after 1 and 4 hours using microarray technology. Genes induced at 1 hour were generally only modestly upregulated and consisted mainly of signaling molecules and transcription factors (Figure 1C and Online Figures I through V). In contrast, those upregulated at 4 hours included a cluster of highly expressed effector genes such as HMOX1, the gene for HO-1 (Figure 1D). When genes induced at one hour were explored in more detail using Gene Ontology, we found the most upregulated transcription factor to be activating transcription factor 1 (ATF-1). ATF-1 was also the only such regulator in the top 10 that was annotated as a validated transcription factor (http://www.ncbi.nlm.nih.gov/gene/466).
The most upregulated effector genes are shown in Figure 1F and Online Table I. In contrast, macrophage proinflammatory activation genes were consistently repressed (Online Figure VI).

The microarray data were corroborated with 2 microarray systems and with qPCR (Online Figure VII). We then investigated the relationship between ATF-1 and HO-1 by qPCR in heme-stimulated monocytes from 5 additional donors. ATF-1 expression was transient and succeeded by a wave in HO-1 expression (Figure 2A) with a concomitant high time-lagged correlation coefficient ($R=0.71$, $P<0.05$). Therefore, we tested the hypothesis that ATF-1 expression causally drives HO-1.

ATF-1 is known to be activated by phosphorylation at Ser63. By Western analysis, ATF-1 and p-Ser63-ATF-1 protein both peaked at about 4 hours after heme-stimulation, corresponding with emergence of HO-1 mRNA (Online Figure VIII, A and B). Of note, whereas total ATF-1 protein was principally cytoplasmic, p-ATF-1 was located in the nucleus, consistent with functional activity (Online Figure VIII, C and D). We then used siRNA perturbation analysis to test the dependency of HO-1 induction by heme on ATF-1 induction. Pooled ATF-1 siRNA, and 2 independent ATF-1 siRNA oligonucleotides each suppressed both ATF-1 and p-ATF-1 (Figure 2B through 2E and Online Figure IX), and prevented HO-1 mRNA and protein induction in response to heme (Figure 2B through 2E). This had a concomitant stimulatory effect on release of highly reactive oxygen species (hROS) by monocytes and led to reduced cell survival (Online Figure X). Conversely, transfection (validated in Figure 2F) of an ATF-1–expressing plasmid into monocytes led to an increase in p-ATF-1 and HO-1 mRNA and protein (Figure 2G, H). Finally, transfection of ATF-1, but not unrelated plasmids or a Ser63-Ala nonphosphorylatable mutant (designated DN-ATF-1), also suppressed hROS (Figure 2I). In contrast, transfection of a close homolog of ATF-1,
cAMP response element binding protein (CREB1), led to an increase in hROS (Figure 2I).

Heme Induces a Set of Genes Distinct From M1, M2, or Mox

Analysis of the microarray data using FDR-adjusted ANOVA showed that 2400 genes reached statistical significance. Therefore, further conservative cutoffs on fold-regulation (over 1.4-fold up or down) and intensity (above the level of known muscle-markers) were applied to exclude weakly regulated or poorly expressed genes. These more stringent criteria indicated that heme significantly modulated approximately 800 genes (Heatmap in Online Figure XI, A and B). To make sense of this large number of genes, we first used a Venn diagram to compare the Mhem transcriptome with published human M1 and M2 transcriptomes (GEO database, NCBI). This revealed that Mhem had approximately 700 unique genes and shared only 1 gene with M2, which was LHFPL2, a gene of essentially unknown function (Online Figure XI, C, and Online Table II). The 44 genes in the Mhem / M1 intersection were counterregulated in Mhem and M1 (Online Table II). Thus, Mhem cells appear to be distinct from canonical M1 or M2 macrophages. An equivalent Venn diagram for Mox and Mhem is shown in Online Figure XI, D. Approximately 800 genes were uniquely regulated in Mhem, 157 genes were uniquely regulated in Mox, and 7 were coregulated (namely HMOX1, GCLM, OSGIN1, HPLP2, DCK, NINJ1, TRAF5). The fraction (approximately 1%) of shared genes was no different to chance (P > 0.95). Thus, even allowing for experimental variation between laboratories and platforms, although Mhem and Mox share occasional key genes, such as HO-1, they are distinctly regulated.

We probed the relationship of the heme-response state to M1, M2, and Mox states experimentally (Online Figure XII, A through I). Heme suppressed HLA-DR as we have previously reported (Online Figure XII, A).7 Either IL-4 treatment (M2) or interferon-γ/LPS treatment (M1) increased HLA-DR (Online Figure XII, B). By itself, this indicated the heme-treated state is distinct to M1 or M2. Heme suppressed HLA-DR in either M1 or M2 macrophages (Online Figure XII, C). Heme increased surface CD163 as previously reported (Online Figure XII, D).7,8 We found that in either M1 or M2 macrophages, heme did not increase CD163 expression (Online Figure XII, E). As a control, we assessed the plasticity of M1 versus M2 commitment. We cultured human blood-derived macrophages with M1 and M2 stimuli for 3 days, swapped the stimuli, and carried out flow cytometry after another 3 days for a canonical M1-M2 marker macrophage mannose receptor (MMR) (Online Figure XII, F). This indicated that MMR expression was conditioned by the initial stimulus, that is, M1 and M2 are committed.
human blood-derived macrophages with OxLDL prevented heme from either suppressing HLA-DR or inducing CD163, also indicating commitment (Online Figure XII, G and H).

Interestingly, culture of macrophages with OxLDL, or with M1 conditions, caused macrophages to induce p-ATF-1 more strongly in response to heme (Online Figure XII, A). In contrast, culture with POVPC (to induce Mox) or with M2 conditions suppressed p-ATF-1 induction by heme (Online Figure XII, I and J). This indicates that Mhem are actually antagonized by Mox or M2 differentiation. Thus, whereas the responses are complex and a full analysis would require a dedicated paper, Mhem are clearly distinct to M1, M2, or Mox states, and M1, M2, or Mox differentiation prevents the full Mhem state.

When added to macrophages, heme immediately induces hROS, before a later (biologically mediated) suppression.8 We then examined whether heme-induced ATF-1 was mediated by heme-induced oxidative stress. We assessed a prototypical direct antioxidant (N-acetyl-cysteine, NAC; free radical scavenger) and prototypical indirect antioxidant (curcumin, anti-inflammatory and inducer of endogenous antioxidants). We first validated that NAC suppresses hROS (curcumin induced p-ATF-1 and suppressed additional heme-induced p-ATF-1 (Online Figure XIII, C and D). Because NAC suppresses hROS but induces p-ATF-1, it seems highly improbable that heme induces ATF-1 through a simple oxidant stress mechanism alone.

**Heme Stimulates an ATF-1–Dependent Gene Regulatory Network That Coordinates Iron Handling With Lipid Handling**

We then used standard cluster analysis methods to define secondary response genes induced by heme at 4 hours. When this cluster was analyzed by Gene Ontology (GO) classification to assign biological meaning, we unexpectedly found that it was significantly enriched in genes involved in lipid handling (Figure 3A, listed in Online Table III and Online Figure XIV). These included the transcription factor liver X receptor beta (LXR-β), a “master regulator” of lipid metabolism (Figure 3B).

We extracted the regulatory sequences of each gene between human and mouse from the ENSEMBL database, identified conserved motifs, and analyzed these for transcription factor binding sites. Other than LXR-β itself (the ATF-1 responsive initiating gene), all of the key heme-inducible lipid handling genes had predicted LXR response elements (Figure 3C). We thus postulated that ATF-1 coinduces LXR-β and HO-1 and thereby directs a gene network that integrates lipid and iron handling (Figure 3C) and went on to test this linkage.

In time-course studies, ATF-1 preceded the largest part of the LXR-β rise (Figure 4A). Involvement of ATF-1 in LXR-β induction by heme was confirmed using ATF-1 siRNA (Figure 4B and 4C), and, again conversely, ATF-1 transfection induced LXR-β mRNA. Monocytes were transfected with 1 μg/mL plasmid DNA, as indicated, and examined by qPCR for LXR-β. *P<0.05, Student paired t test.

**Heme and ATF-1 Transcriptionally Activate HO-1 and LXR Genes**

To further test causality in this transcriptional network, we next assessed luciferase constructs. We first used an easily transfectable HEK cell line (not shown), which yielded more light-signal but obtained qualitatively similar results with human blood-derived monocytes, which were more
physiologically relevant (Figure 5). These we transfected with a series of luciferase constructs covering sequentially larger fragments of the 5-kb enhancer-promoter of HO-1 (Figure 5A through 5C). Heme-stimulation (Figure 5B) or ATF-1 cotransfection (Figure 5C) induced robust luciferase activity only with the full-length construct (4.9 kb), but not with the shorter constructs (2.2 kb and 0.3 kb), indicating a requirement for the enhancer sequence between 2.2 kb and 4.9 kb distal to the transcription start site; C, cotransfection of blood-derived monocytes with HO-1-luciferase reporter plasmids in A and B with an expression plasmid for ATF-1 produces the same pattern as challenge with heme, consistent with ATF-1–mediating HO-1-luc induction by heme, n = 3 donors. D, In the 4.9 kb HO-1 luciferase plasmid, the indicated inactivating mutations of the ATF-1 response site at −4 kb (4ΔATF-1) prevented HO-1-luc induction by 10 μmol/L heme at 4 hours in n = 3 donors. E, Chromatin immunoprecipitation (ChIP) analysis of heme-stimulated blood-derived monocytes, using anti–p-ATF-1 as in the diagram. The figures show the specific fold enrichment at 1 and 4 hours of HO-1 and LXR-β relative to IgG control and relative to control sequence (3’ end of ORF). F, ATF-1 binding to oligonucleotides matching HO-1 or LXR-β regulatory sequences. Y-axis, specific immunodetection of p-ATF-1 bound to solid-phase oligonucleotides, as in diagram (absorbance units, tetramethylbenzidine peroxidase substrate). Cons ATF-1 indicates consensus ATF-1 response element (consensus CRE) from the rat soma-
The Heme/ATF-1 Pathway Drives Lipid Export

Thus far, our bioinformatic data had suggested a coregulatory mechanism in which ATF-1 contributes to induction of LXR-β as well as HO-1, with each in turn driving many other genes regulating iron handling and lipid transport (Figure 3). Consistent with the latter, time-course qPCR showed that ApoE expression followed LXR-β induction (Figure 6A). We also examined the predicted LXR-β-responsive genes LXR-α and ABCA1 and found strong late induction of both LXR-target genes LXR-α and ABCA1 at 48 hours (Figure 6B). Heme-induced ApoE was prevented by ATF-1 siRNA (Online Figure XVI). LXR-β-specific siRNA was found to suppress levels of mRNA for both LXR-α and ABCA1 at 48 hours after heme stimulation, with Western blotting confirming the results for the latter at protein level (Online Figure XVII). Furthermore, we found that heme and ATF-1 cotransfection both induced LXR-α-luciferase plasmids at 48 hours (Figure 6C).

Because ABCA1 is a key cholesterol exporter to HDL, we next transfected ATF-1 or a control green fluorescent protein (GFP) construct into blood-derived monocytes to ask whether this pathway promoted cholesterol export, challenging the cells with OxLDL. We found that heme transfection suppressed cell-associated cholesterol (Figure 6D) and increased cholesterol efflux (Figure 6E), and both were prevented by ATF-1 siRNA (Figure 6D and 6E). Furthermore, ATF-1–transfected cells were resistant to foam cell formation when compared with controls (Figure 6F and 6G). These effects are similar to those induced by the LXR-agonist R-hydroxycholesterol and were inhibited by the LXR-antagonist S-hydroxycholesterol (not shown).

Mhem Macrophages in Human Intraplaque Hemorrhage Are Protected From Foam Cell Change and Coexpress p-ATF1, HO-1, and ABCA-1

Last, we went back to the human culprit plaques in which Mhem macrophages had first been described.7 Because Mhem macrophages and foam cells are found in distinct zones,7 we could easily reexamine the same cells in adjacent serial sections. We first compared the sizes of macrophages
Figure 7. Activated ATF-1 defines plaque Mhem macrophages. 

A. Size difference between Mhem and conventional inflammatory plaque macrophages (foam cells, FC). Sections were dual-stained with CD163 (blue) and HLA-DR (red) or stained with CD68 (brown). Scale bars—lengths indicated. The Mhem cells (CD163-high HLA-DR-low ie, blue) are obviously smaller and have smaller amounts of less foamy cytoplasm. The smaller size of Mhem is borne out on image analysis (right-hand side) *P<0.05, Student paired t test. 

B, Boyle et al ATF-1 Drives Atheroprotective Macrophages
identified as Mhem with those identified as conventional inflammatory plaque macrophages and found that Mhem cells were smaller, consistent with resistance to foam cell change (Figure 7A). We found p-ATF-1 in Mhem macrophages but not in control foam cell macrophages with expanded cytoplasm due to accumulated lipid (Figure 7B). Furthermore, p-ATF-1 was nuclear in the Mhem macrophages, consistent with transcriptional activation (Figure 7B). Relative to the foam cells, the Mhem macrophages expressed increased HO-1, ABCA1, ApoE, and LXR-β (Figure 7C and Online Figure XX to XXI). Using 4-channel confocal, we then also formally showed there was colocalization of p-ATF-1 with HO-1 and ABCA1 (Figure 7D). Thus, our mechanistic insights are likely to apply to macrophages within vulnerable atherosclerotic plaques.

Discussion
Atherosclerosis, the main pathology causing cardiovascular morbidity and mortality, largely comprises macrophage-mediated inflammation driven by oxidized lipids. The state of the infiltrating macrophages is a key pathophysiological determinant. IPH is pathogenic in advanced atherosclerosis through a combination of loading with iron, a lipid peroxidant, and erythrocyte membrane cholesterol. We have described a macrophage phenotypic adaptation to IPH that appears to partially counter its pathogenic effects.

Coordination of Lipid and Iron Homeostasis
We show that the hemorrhage-induced macrophage state is primarily directed by ATF-1–mediated coinduction of HO-1 and LXR-β. Together, these coordinate the safe handling of iron in parallel with lipid export from the cell (Figure 8). The segregation of lipid storage in foamy macrophages versus iron storage in hemosiderin-laden macrophages makes functional sense as a strategy to help prevent iron-mediated lipid peroxidation. Heme/ATF-1–mediated coinduction of HO-1 and LXR-β provides a molecular mechanism for this phenomenon. We defined this pathway by genetic modification of blood-derived monocytes from normal human volunteers to allow both pathophysiological relevance and specific molecular control.

Pathophysiological Relevance of the Model
The in vitro model of challenge with 10 μmol/L heme is physiologically relevant, being approximately 1/40th of that measured in subarachnoid hematoma in vivo. Furthermore, recent transcriptome analyses of plaque macrophages and pooled cell line microarray data indicate that gene expression in freshly isolated blood-derived monocytes is relevant to

Figure 7 (Continued). Phospho-ATF-1 is selectively found in nuclei of Mhem cells by confocal microscopy. Sections of hemorrhaged human atherosclerotic culprit lesions were incubated in anti-p-ATF-1 (red) followed by anti-rabbit alkaline phosphatase and Vector red, counterstained with macrophage lysosomal marker anti-CD68-FITC (green); C, expression of p-ATF-1 target proteins and relative quantification in Mhem cells relative to foam cells; D, coexpression of p-ATF-1 and key effectors HO-1 and ABCA-1 by Mhem cells but not foam cells, using multichannel confocal. Conditions for FC and Mhem images are equivalent; channels are as indicated. Staining for LXR-β (which was only transiently 2-fold regulated in culture) is shown in Online Figure XX. Corroborative quantification of HO-1 from images of bright-field immunohistochemistry with peroxidase/DAB is shown in Online Figure XXI.
hemorrhages (hemorrhage-associated macrophages, HA-mac).7 These had less oxidative stress than foamy macrophages—in the face of increased iron storage; higher HO-1 expression; less myeloperoxidase expression; and higher IL-10 expression. We modeled these in vitro and found that hemoglobin:haptoglobin complexes evoked cells with similar characteristics, switched by an autocrine IL-10/CD163 feedback loop.7 These cleared Hb more quickly than control macrophages, and we proposed that they were adaptively differentiatied to mitigate the otherwise accelerant effects of intraplaque hemorrhage on plaque development. We now show that the initiating transcriptional events involve induction and phosphorylation and ATF-1 and subsequent coinduction of an HO-1 cascade and an LXR/ABCA1/ApoE foam cell protection cascade. The HA-mac/Mhem cells in intraplaque hemorrhage indeed coexpress p-ATF-1, HO-1, and the canonical LXR targets ApoE and ABCA1 and have less foam cell change, a feature we did not actively comment on before in the absence of mechanistic evidence.

Cyclic-AMP Response Element–Mediated Regulation of Vascular Disease

ATF-1 is named for activating transcription in response to cyclic-AMP.20 Its nearest homolog, cAMP response element binding protein (CREB) has been much more extensively studied and is thought to be the major cAMP activated transcription factor, regulating key metabolic genes, for example, gluconeogenesis21–23 and lipoprotein lipase.24 Interestingly, Ruffell et al described dependency of wound-resolving macrophages on CREB, with a targeted inactivating mutation of its response element in a single target gene preventing in vivo healing.25 setting a precedent for a role of the CREB family in macrophage differentilaiton. Similarly, the cytoprotective role of ATF-1 that we describe has precendents in prosurvival and mitogenic effects in other lineages.18,26–29 In contrast to ATF-1–dependent cytoprotection in human blood monocytes, ATF-1 suppresses induction of ferritin heavy chain and the HO-1 gene through antioxidant response elements in HEK cells.30 Again in HEK cells, CREB-binding to the cyclic-AMP response element at \(\approx -4\) kb mediates the induction of HO-1 by the oxylipid nitroprilenoic acid.31 Here, we found an alternative use of the same response element (GTCA) in mediating heme-induced HO-1. As there has been little attention paid to determining how heme induces HO-1, the identification of the mechanism of this important physiological negative feedback loop is highly significant.

LXRs in Vascular Disease

Atherosclerosis is thought to largely hinge on phagocytosis of OxLDL by plaque macrophages through scavenger receptors,32 causing proinflammatory activation, endoplasmic reticulum stress, and oxidant stress, promoting death and necrotic core formation.33 However, macrophages also protect against inflammatory activation and lipid overload by lipid export to high-density lipoprotein (HDL) in reverse cholesterol transport,34 through adenosine-triphosphate-binding-cassette-transporter (ABC) proteins -A1 and -G1.34 ABCA1 and ABCG1 are upregulated by LXRs, former orphan nuclear receptors ligated by oxysterols and by cholesterol at “overload” concentrations.2,5,35–36 Thus LXRs sense cholesterol overload and cholesterol oxidation and negatively feedback to evoke cholesterol export and suppress inflammatory activation.37

Hemorrhage/heme-challenge feeds forward into this key defense through a novel heme→ATF-1→LXR-β→LXR-α cascade. Notably, in a landmark paper, peroxisome proliferation activation receptor γ (PPAR-γ) induced lipid export through transcriptional induction of LXR-α.35 The heme→ATF-1 pathway in our study induced greater fold-change in LXR-α mRNA (11-fold) than was previously observed through PPAR-γ (5-fold).35 Moreover, this appears to be the first documentation of LXR-β isoform induction by any stimulus. The magnitude of effect of heme→ATF-1 on cholesterol efflux is similar to that in the PPAR-γ→LXR→ABCA1 axis and comparable to differences between normal versus ABCG1-deficient mice.38,39 The heme→ATF-1→LXR-β cascade was devoid of induction of fatty acid synthesis genes, a major side-effect of some pharmacological LXR agonists that is through LXR-α activation.41–44 suggesting that heme→ATF-1→LXR-β mediated activation may be more beneficial.

Macrophage Polarization

The Mhem anti-inflammatory state appears to be a polar opposite of the recently described M1-like iron-overload state that impairs wound healing.45 We suspect that this is what ATF-1 protects against, and therefore ATF-1-deficient mice may not cope with hemorrhage.46 However, this is an area of active investigation beyond the scope of the current report. Although plaque M2 macrophages may overexpress PPAR-γ, which also may induce LXR-α, our data indicate that Mhem is very distinct from an M2 state.47 Furthermore, although Leitinger’s group recently described a HO-1–expressing putatively protective mouse macrophage state Mox driven by oxidized phospholipids through Nrf2,13 transcriptome comparisons indicate that Mhem are not Mox. However, we have shown elsewhere that Nrf2 plays a role in heme-induced HO-1.8 It is possible therefore that ATF-1 and Nrf2 may be cooperative in HO-1 induction, as seen for NFκB/AP-1 cooperativity in macrophage responses to minimally modified LDL.48 The ATF-1 site immediately adjacent to a putative Bach/Nrf2 site (Online Figure XVIII), so cooperative binding would be plausible. Moreover, ATF-1 and Nrf2 are structurally related, basic zipper transcription factors, raising the more speculative hypothesis of ATF-1/Nrf2 heterodimerization. Extending the idea of transcription factor cooperativity more globally, we found by bioinformatic prediction from ChIP-Seq databases, overrepresentation of up to 10 transcription factor binding sites in the Mhem geneset (Online Figure XIX). This sets ATF-1 in a context of a more complex response comprising a network of several transcriptional factors that may modulate responses. As far as can be assessed without a very large (and largely repetitious) microarray experiment, Mhem appear to be distinct to recognized polarization patterns (M1, M2, Mox).
Both M1 and M2 were HLA-DR-high activation states distinct to the HLA-DR-low Mhem state and either M1 or M2 commitment prevented acquisition of a full Mhem state. However, both M1 and M2 cells committed relative to one another could be dynamically converted to HLA-DR-low by heme-treatment, suggesting that the activation-deactivation axis may show some plasticity. Interestingly, M1 or foam cell differentiation both enhanced heme-induced p-ATF-1 responses, whereas M2 or Mox differentiation suppressed both basal and heme-stimulated p-ATF-1. Furthermore, two relevant antioxidants, NAC (a prototypical direct antioxidant, ie, free radical scavenger) and curcumin (a prototypically indirect antioxidant and nutriceutical): both induced p-ATF-1 and prevented heme from inducing it any further. Although these data suggest that ATF-1 may also play a role in the mechanism of action of these important agents, testing this was beyond the scope of the current report. Venn diagrams identified a large number of apparently Mhem specific genes, the most fold regulated and highly expressed was HTRA3, which could be assessed as a useful Mhem marker in future.

What Kinases Mediate ATF-1 Regulation by Heme?

Although a study of heme-regulated ATF-1 kinases was beyond the scope of the present report, heme-induced ATF-1 could be activated by several serine kinases: protein kinase A, several stress kinases including adenosine monophosphate-activated kinase (AMPK) in skeletal muscle during exercise, p38 mitogen-activated protein kinase in leptin signaling, sale-inducible kinase/mitogen stress-activated kinase (SIK/MSK) downstream of ultraviolet light, and by protein kinase D. Tyrosine phosphorylation of ATF-1 by flavonoid and NAC (established treatment for paracetamol overdose and interstitial lung disease) is interesting. Our finding that ATF-1 coregulates HO-1 and LXR suggests significant therapeutic interest in each of the mediators alone, beyond the scope of the current report. Venn diagrams identified a large number of apparently Mhem specific genes, the most fold regulated and highly expressed was HTRA3, which could be assessed as a useful Mhem marker in future.

Therapeutic Potential

LXR agonists are being actively developed to initiate reverse cholesterol transport, and HO-1 is a target of interest for a variety of existing therapies. Considering that there is significant therapeutic interest in each of the mediators alone, our finding that ATF-1 coregulates HO-1 and LXR suggests that therapeutic manipulation of p-ATF-1 may provide a strong benefit/risk ratio in atherosclerosis. In this light, our findings that p-ATF-1 is induced by curcumin (a dietary flavonoid) and NAC (established treatment for paracetamol overdose and interstitial lung disease) is interesting.

Conclusions

In conclusion, Mhem is an atheroprotective macrophage state, distinct from M1, M2, and Mox, that develops in specific adaptation to IPH. Induction of ATF-1 coincides HO-1 and LXR-β, interlinking lipid and iron handling for the first time. By acting as a novel transcriptional integrator of iron and lipid homeostasis, ATF-1 is a key driver of human plaque monocytes to acquire the atheroprotective Mhem macrophage state.

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Disclosures

None.

References

ATF1-Drives Atheroprotective Macrophages

Boyle et al


Heme induces HO-1, which removes excess heme as part of normal homeostasis. Induction of HO-1 by external stimuli is vasoprotective. Although iron is potentially proinflammatory, macrophages in the vicinity of intraplaque hemorrhage show atheroprotective features even in the face of iron loading. We show for the first time that heme induces HO-1 through activating transcription factor 1 (ATF-1). Moreover, ATF-1 couples the induction of HO-1 to that of Liver X receptors (LXRs), thereby preventing foam cell formation and promoting the export of cholesterol to high density lipoproteins (HDL). This represents the first known example of coordination of iron and lipid metabolism. Through ATF-1/HO-1/LXR, heme induces a novel phenotypic state in macrophages, which is distinct from their alternatively activated phenotype but is specialized for atheroprotective/anti-inflammatory activity, and hemorrhage clearance. Patients with fatal coronary artery disease have macrophages with coordinated expression of ATF-1 and its response genes, indicating that the novel ATF-1-(HO-1/LXR) network is likely to have key pathophysiological significance. Hence, therapeutic control of ATF-1, could effectively combine the atheroprotective activities of HO-1 and LXR by converting macrophages to an atheroprotective phenotype.
Activating Transcription Factor 1 Directs Mhem Atheroprotective Macrophages Through Coordinated Iron Handling and Foam Cell Protection

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Supplemental Material

Methods

Cell isolation and culture.

Human peripheral blood was obtained with informed consent and favorable ethical opinion from Hammersmith Research Ethics Committee. PBMCs were prepared by density-gradient centrifugation and monocytes purified by adhesion as before. Except where indicated, heme stimulation was for 4h with 10μM heme (hemin, Sigma-Aldrich, Poole, UK), after 18 hours resting of the monocytes. Heme was made up to stock as 25mM in hybridoma-culture grade dimethylsulfoxide (DMSO), tested endotoxin-negative, diluted to 100μM in Iscove’s Modified Eagle’s Medium (IMDM) and then diluted to 10μM final in 10% serum IMDM. 1’-palmitoyl, 2’-oxo-valeryl phosphatidyl-choline (POVPC) was from Calbiochem (Merck, Beeston, UK), and is a purified chemically defined oxidised phospholipid found in oxidised LDL\(^1\). It is similar to the oxidised phospholipid used in the paper defining Mox by Kadl \textit{et.al.}\(^2\)[atmospherically-oxidised 1’-palmitoyl, 2’-arachidonyl phosphatidyl-choline (OxPAPC(s)], but more purified, without the confounding by (potentially bioactive) arachidonate acyl chain, and with a much better defined oxidative modification. POVPC was added to cultures at 50μg.mL\(^{-1}\) final concentration to match published concentrations\(^2\). Interleukin-4 (IL-4) and (IFN-\(\gamma\)) were from Preprotech (London), dissolved at 1mg.mL\(^{-1}\) and added to cultures at a final concentration of 10ng.mL\(^{-1}\). LPS was from Sigma-Aldrich and added to cultures at a final concentration of 10ng.mL\(^{-1}\).

Microarray and qPCR

Macrophages were lysed in guanidinium (RLT buffer, Qiagen, Crawley, UK) and RNA purified by silica-resin affinity (RINAEasy Mini, Qiagen, according to manufacturers
instruction). Sample quality was controlled with Nanodrop and Bioanalyzer micro-LC. Samples were prepared for Agilent 4x44k and Affymetrix 1.0ST microarrays following respective manufacturer’s instructions. The same RNA samples were used for both microarray platforms and both analyzed with GeneSpring GX10 Agilent. Initial validation was with quantitative polymerase chain reaction (qPCR) on the same RNA samples after reverse transcription (Invitrogen Superscript, manufacturers instructions) using BioRad iCycler real time PCR, MesaGreen mastermix (Sybr Green/Taq, Eurogentec, Southampton, UK) and 100fMol.µL⁻¹ custom primers (synthesized by MWG Biotechnology, Ebersberg, Germany). Primers were designed by Primer3 (www.ncbi.nlm.gov). Additional validation was on a further five donors over a longer time course, using qPCR by the same methods.

**Bioinformatics**

Gene Ontology analysis was with GeneSpring and DAVID³, and manual curation of GO-terms, then counting in Excel.

The ENCODE project annotated regulatory features to the human genome based on biochemical validation by ChIP and DNAse-I hypersensitivity ⁴. For Transcription factor (TF) binding site analysis, we started with regulatory sequences from the ENCODE database, which are displayed by a functional genomics track in Ensembl (http://www.ensembl.org/index.html). Supplemental Figure XXII shows typical Ensembl screenshot images with the functional genomics enabled, showing regulatory sequences in the *hmox1* genes for human and mouse. Raw FASTA-formatted regulatory sequence data were downloaded for human and mouse, and conserved motifs identified with Promoterwise (http://www.ebi.ac.uk/Tools/Wise2/promoterwise.html), a modified BLAST algorithm optimised for promoter/enhancer sequences⁵.
The following software and databases were used for identification of TF-binding sites within the conserved sequences: Matinspector\textsuperscript{6}, TESS\textsuperscript{7}, OPOSSUM\textsuperscript{8}, ChEA\textsuperscript{9}, JASPAR\textsuperscript{10}, ConSite\textsuperscript{11}, available at:

http://jaspar.binf.ku.dk/cgi-bin/jaspar_db.pl

http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/

http://fantom.gsc.riken.jp/4/consite/

http://www.cbrc.jp/research/db/TFSEARCH.html

http://tfbind.hgc.jp/

http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/

http://www.genomatix.de/cgi-bin/sessions/login

http://www.cbil.upenn.edu/cgi-bin/tess/tess

http://natural.salk.edu/CREB/

http://fantom.gsc.riken.jp/4/edgeexpress/view/#5558263

http://dbd.mrc-lmb.cam.ac.uk/DBD/index.cgi?Home

http://amp.pharm.mssm.edu/lib/chea.jsp

We also accessed the FANTOM database, which is based in cap analysis of gene expression in the THP-1 monocyte cell line\textsuperscript{12,13}. The most useful additional information was that ATF-1 has an ATF-1 response element upstream of itself, the exact site of which was defined by application of the methods above to ATF-1 (Supplemental Figure XXIII).

The PCR-validated data was entered into BANJO, a downloadable program for dynamic Bayesian network (DBN) inference\textsuperscript{14}. Several alternative gene networks were postulated on its basis. These were then curated by identifying transcription factor response elements, and by accessing peer-reviewed biochemically validated data on PubMed, to form the inferred network in Figure 3C.
**Western analysis**

Western blotting was by amendment of our previous methods. Cells were lysed in SDS-lysis buffer as before, run on Novex electrophoresis system using 4-12% precast gels, transferred to PVDF using Novex blot kit, and probed with anti-ATF-1 1:1000 (Abcam); anti-phospho-ATF-1 1:1000 (Abcam, Cambridge, UK); anti-LXR-β 1:1000 (Abcam); anti-HO-1 (Stressgen, Exeter, UK); or β-actin loading control 1:10,000 (Abcam) for 18h at 4°C, washed three times for 30 seconds and once for 5 minutes, then incubated with respective anti-mouse or anti-rabbit peroxidase secondary antibodies (Dako), and visualized with ECL+ and Hyperfilm (GE Healthcare, Chalfont, UK).

**Chromatin immunoprecipitation (ChIP)**

ChIP was performed following a minor amendment of published protocols. Macrophages were fixed in 1% formaldehyde at 4°C for 5mins, stopped with excess glycine, lysed in nuclear lysis buffer AM-1 (Active Motif, Roxensart, Belgium), sheared by 28G needle three times (giving a single 1 kb band on agarose gel electrophoresis indicating uniform fragment size (not shown)) and ChIP carried out with the magnetic non-enzymatic kit (Active Motif) using ChIP-grade anti-p-ATF1 (Abcam) and Invitrogen magnetic Protein G beads. Binding was done at 4°C for 16h on a rotator. The beads were magnetically separated and washed 3 times in ChIP wash buffer (Active Motif). Immunoprecipitates were DNA–purified (Qiagen DNA-purification columns, Invitrogen), and qPCR was as above.
Quantitative transcription-factor / solid phase oligodeoxynucleotide binding

The TransAm kit (Active Motif) for CREB1 binding to the rat somatostatin CRE was reverse engineered. ELISA plates were incubated with neutravidin 4°C for 16h, incubated with oligos corresponding to human HO-1 and LXR-β enhancers or their DCRE mutants. Then lysates were added in AM-1 buffer (Active Motif), washed once and probed with anti-p-ATF-1 (Abcam), with detection with anti-rabbit immunoglobulin (Dako).

Plasmids and siRNA

Plasmids (kind gift, S. Gordon, Oxford, UK; Y. Tsuji, Raleigh, North Carolina, USA.; A. Agarwal, Birmingham, Alabama, USA, or purchased from Clontech, BD Biosciences, Abingdon, UJ) were amplified in E.Coli DH5α and purified by Qiagen kit. Plasmid DNA 1μg was complexed with 1μL mannosylated polyethyleneimine for 20 mins (JetPEI-mac; which allows mannose receptor dependent entry; Polysciences). Complexes were then added to macrophages on the second day of culture in 10% serum IMDM for 24h before analysis.

SiRNA was from Dharmacon and were sequence 1, sequence 2 or pool where indicated. SiRNA was made into complexes with Interferin (PolyPlus, Strasbourg, France) by incubation of 100 pmol siRNA in 1μL with 1μL Interferin for 10 min, prior to addition to the cells in 1 mL medium with 10% serum for 18 hours. Where indicated, 10 μL AMS-liposomes were substituted for 1 μL Interferin and added to cells in IMDM without serum for 18 hours.

Confocal microscopy

This was by minor modification of previous methods\textsuperscript{15, 16}. We used 2-step immunofluorescence staining, AlexFluor-labelled goat secondary antibodies (2h, room temperature) as indicated (Invitrogen); and primary antibodies as follows: anti-p-ATF-1 (rabbit monoclonal, Cell Signaling Technology); anti-ABCA1 (rabbit IHC-grade polyclonal, Abcam); anti-ApoE (rabbit IHC-grade polyclonal, Abcam); anti-HO-1 (goat IHC-grade
polyclonal, Santa Cruz, Santa Cruz, CA). Nuclear counterstaining was with TOPRO (Invitrogen). Imaging was with a Zeiss LSM510Meta, with settings essentially as before\textsuperscript{15}.

**Image quantification**

Image J (NIH, Bethesda) was used for both western densitometry and confocal channel quantification as before\textsuperscript{15}. In both cases, images were measured by selecting a region of interest, applying the histogram function and exporting to Excel. Within Excel, the background was subtracted (based on histogram of a blank area of the image). Then intensity x number of pixels was calculated for each intensity level and summed. This gives an integral capturing intensity and positive area that gives semi-quantification for purposes of comparison, consistent with standard practice with western densitometry and confocal\textsuperscript{16}.

**Other assays**

Oxidative stress assays were carried out as before\textsuperscript{15, 16} using 1:1000 (1\textmu M final) aminophenyl fluorescein (Invitrogen), in PBS for 18 hours, stimulated with 30\textmu g.mL\textsuperscript{-1} hypochlorite-modified LDL (OCl-LDL) (LDL from Calbiochem, incubated with 1mM NaOCl (Sigma-Aldrich for 24h)). The 18h time point was after reaction completion and was chosen for consistency and convenience: kinetic studies indicated that the bulk of the fluorescence increase was over 1-4h. Cell fluorescence was read using a 96-well fluorimeter BioTek Synergy HT, at 488nm excitation and 535nnm emission, using automated sensitivity adjustment to blank wells. XTT (Invitrogen) was used to measure viable cell number and the fluorescence signal normalised to the number of cells.

For the cholesterol export assays, methods were adapted from papers from a leading laboratory \textsuperscript{18-20}. Macrophages were cultured for 4 days, then medium changed to IMDM supplemented with delipidated fetal calf serum (Sigma-Aldrich). Human recombinant ApoA1 was from Sigma-Aldrich and added at 10\textmu g.mL\textsuperscript{-1} for 24 hours. Cells and supernatant were then separated and cholesterol measured in each. The cholesterol assay was with Cholesterol
Oxidase / Amplex Red method using a kit (Invitrogen) using cholesterol standards as published\textsuperscript{21}. Luciferase analysis was a Dual-Luciferase kit (Promega) and a 96-well luminometer (BioTek Synergy HT). Renilla transfection was used as a control to calculate DLR, and the DLR ratios were then normalised to the empty Photinus vector pGL3. For the foam cell assays, monocytes were cultured on tissue culture glass slides (chamber slides, Nunc) challenged with 30\,µg.mL\textsuperscript{-1} OCl\textsuperscript{-} modified LDL in 50\% serum for 4 days, then fixed in 1\% formaldehyde at 4°C for 5 mins, stained with 10\,µM Nile Red (Sigma-Aldrich), and imaged by confocal microscopy (Zeiss LSM510) by minor modification of previous methods\textsuperscript{15}.

For dual iron-lipid staining, we made a minor adaptation of well established clinical histochemical methods for stored fat and iron. Macrophages were fixed in 1\% paraformaldehyde 24h, incubated in Oil Red O in isopropanol 24h, then incubated in Perl’s reagent for 24, then washed and imaged. Perl’s reagent is a 1:1 mix of 2\% potassium ferricyanide and 2\% hydrochloric acid and forms a vivid blue complex coordinate (Prussian blue) with cellular ferric ions. Oil Red O is red lipid dye that stains neutral lipid by simple partition. Both can be used together since the isopropanol does not dissolve iron deposits, and the aqueous phase coordination chemistry is unaffected by the Oil Red O, which exclusively partitions to the lipid compartment. The timings needed to be longer, since the cells were fixed and unpermeabilised.
Supplemental Tables

Supplemental Table I
Genes over 2-fold induced at 4h, with official gene abbreviation, description, NCBI accession number, and induction at 1h and 4h.

Supplemental Table II
Genes in intersections in Venn Diagram in Supplemental Figure XI. Official gene abbreviations are given. Columns are (left to right): column 1, genes regulated in Mhem and in M1 (counter-regulation: the fold changes were in opposite directions); column 2, genes regulated in M1, M2 and Mhem; column 3, the single gene coregulated in M2 & Mhem.

Supplemental Table III
Lipid metabolism genes induced at 4h, with official gene abbreviation, description, NCBI accession number, and fold-change at 1h and 4h. Genes were extracted as (i) statistically significant induction (ANOVA with Benjamini-Hochberg FDR correction, Agilent GeneSpring); and (ii) lipid metabolism mentioned in gene description, gene ontology categorization, or by manual curation of PubMed or NCBI Gene. Fold change was calculated by taking exponential to the power of 2 of the difference between log normalised values.
**Supplemental Figure Legends**

**Supplemental Figure I** Panel (A) Shows a bar graph of numbers of heme-regulated genes involved in main signalling pathways, by signalling pathway. Panel (B) shows essentially the same data as (A), but displayed as a pie-chart.

**Supplemental Figure II**

Genes involved in cAMP and AMP signalling, X-axis, log fold regulation (up or down) at 1h and 4h as indicated.

**Supplemental Figure III**

Genes involved in insulin-signalling, X-axis, log fold regulation (up or down) at 1h and 4h as indicated.

**Supplemental Figure IV**

Genes involved in cytokine-signalling, X-axis, log fold regulation (up or down) at 1h and 4h as indicated.

**Supplemental Figure V**

Genes involved in nuclear factor kappa b (NFkB)-signalling, X-axis, log fold regulation (up or down) at 1h.

**Supplemental Figure VI**

Macrophage activation-associated genes (inflammatory marker genes, based on GO terms); all of which were repressed. Genes graphed ordered by log fold repression (X-axis).
**Supplemental Figure VII Validation of microarray**

In (A), fold induction of indicated genes at indicated time-points was validated by qPCR. Data normalized to vehicle (=1). Data are mean ± SE. In (B), fold changes in gene expression by qPCR were compared to fold changes measured by Agilent 4x44k (open circles) and Affymetrix 1.0ST (filled triangles) platforms as indicated. Respective correlation coefficients are shown. Table of qPCR data with exact mean values is in (C).

**Supplemental Figure VIII Time course of protein expression**

Western blots for indicated times (hours) (A) 1, 2, 4, 8h or (B) 8, 24, 48, 96h; Human monocytes after overnight resting were treated with either vehicle or heme (10μM) as indicated. Bands were of appropriate Mr as shown, when immunblotted for indicated proteins. Actin-β was the loading control; (C) human monocytes after overnight resting. Heme = heme 10μM for 4h. Immunolocalization of ATF-1 and p-ATF-1 (green) as indicated, with TOPRO nuclear dye (red). Scalebars = indicated distances; (D) higher magnification of cell in experiment in (C), colors as indicated; scalebars = 20μm (C) and 5μm (D).

**Supplemental Figure IX siRNA knockdown shows ATF-1 mediates heme-induced HO-1 and LXR-β**

Monocytes were purified from normal human volunteers as before, in the presence of siRNA complexes (Methods) with the oligos and protocols indicated, and then cultured in 10% autologous serum. The qPCR assays for gene expression are as detailed in Methods. Y-axes, qPCR measurements normalised by the ∆∆Ct method, for indicated gene. X-axes, siRNA and heme treatment or controls as indicated. Experiments with siRNA for ATF-1 and measurements of RNA levels for genes ATF-1, HO-1 and LXR-β. * p<0.05, Student’s t-test, for indicated comparison, n=4 donors.
Supplemental Figure X ATF-1 mediates heme-induced antioxidant protection and survival

(A) uptake of FITC-labeled siRNA into donor monocytes; X-axis, uptake, Y-axis, cell number, histograms as indicated. PEI = addition of liposomes alone, siRNA-FITC = addition of labeled RNA duplexes alone; PEI + siRNA-FITC; (B, C) ATF-1 siRNA (protocols correspond to Supplemental Figures IX, X) renders heme toxic. Oxidative stress was measured as OxLDL-evoked hROS (APF –fluorescence, Methods) in (B), and survival was measured in (C) in monocytes (Methods) after 24h siRNA, then 24h heme (10 μM) stimulation as indicated. *p<0.05, Student’s paired t-test. Data are mean ± SE of five donors.

Supplemental Figure XI Heatmap and Venn diagrams of microarray data

Heatmap (A) showing hierarchical clustering pattern of approximately 800 genes significantly regulated by heme. Red = increased expression. Green = decreased expression. (B) High power view of blue box in (A) with superimposed examples of early and late heme-induced genes. Box shows groups of early and late heme-induced genes as indicated; (C) Venn diagram comparing M-hem genes with published experimental microarray data for canonical M1 and M2 phenotypes. Numbers in white at intersections indicating genes regulated in both M1 and M2 (803, regulated in the same direction), M-hem and M2 (1), M-hem and M1 (44, regulated in opposite directions, not shown) and M1, M2 and M-hem (40, apparently activation markers, coregulated in M1-M2 but counterregulated in M-hem). (D) Venn diagram comparing heme-regulated geneset with the reported Mox geneset. 872 Heme-regulated genes were unique, 157 Mox genes were unique, and Mhem and Mox had 7 coregulated genes (HO-1, GCLM, OSG1N1, IHPLP2, DCK, NINJ1, TRAF5). Although some of these genes are obviously biological important, the genesets had no more genes in common than would be expected by chance ($\chi^2$, p>0.95).
Supplemental Figure XII M1, M2 or Mox differentiation prevents full commitment to Mhem

(A-F) In this set of flow cytometric histograms, macrophages were incubated under one stimulus for 3 days and then swapped to a different stimulus for 3 days and then assessed by flow cytometry for HLA-DR and CD163 at culture day 6, as before \textsuperscript{15}; (A-C) Expression of HLA-DR, surface activation marker. X-axes, HLA-DR, Y-axes, cell number. Conditions as indicated on each histogram. (A) untreated macrophages are normally HLA-DR\text{high} but culture with heme for 6 days suppresses HLA-DR representative of n=25 donors; (B) culture with 10ng.mL\textsuperscript{-1} IL-4 (M2) for 6 days or with combined 10ng.mL\textsuperscript{-1} LPS and 10ng.mL\textsuperscript{-1} IFN-\gamma (M1) for 6 days increases HLA-DR as expected \textsuperscript{22}; (C) Swap experiment. Cells were incubated under M1 or M2 conditions for 6 days (M1 and M2) or under M1 or M2 conditions for 3 days and then swapped to Mhem (10\textmu M heme) for 3 days where indicated (M1 → Mhem, M2 → Mhem). Heme (3 days) suppresses HLA-DR to below control levels even with 3 days of preconditioning with M1 or M2; (D-F) show CD163 flow cytometry on the same cells. (D) is a typical experiment in which CD163 is normally weakly expressed but induced by 10\textmu M after 6 days of heme. We have previously shown the induction happens by 3 days \textsuperscript{15}, representative of n=25 donors; (E) Pretreatment with M1 or M2 stimuli prevents heme from inducing CD163 (treatments as indicated), representative of n=3 donors; (F) surface flow cytometry for M2 marker macrophage mannose receptor (MMR). Treatments as indicated, representative of n=3 donors. M1→M1, IFN-\gamma 10ng.mL\textsuperscript{-1} + LPS 10ng.mL\textsuperscript{-1} for 6 days; M2→M2, IL-4 10ng.mL\textsuperscript{-1} for 6 days; M1→M2, IFN-\gamma 10ng.mL\textsuperscript{-1} + LPS 10ng.mL\textsuperscript{-1} for 3 days, then IL-4 10ng.mL\textsuperscript{-1} for 3 days; M2→M1, IL-4 10ng.mL\textsuperscript{-1} for 3 days then IFN-\gamma 10ng.mL\textsuperscript{-1} + LPS 10ng.mL\textsuperscript{-1} for 3 days; (G-H) macrophages were incubated with OxLDL and then with heme, and then analysed by flow cytometry for HLA-DR (G) and CD163 (H) at culture day 6. Individual treatments are as indicated; (I) human blood-derived monocytes
were incubated for 24 under the conditions specified the stimulated with 10\(\mu\)M heme (H) or vehicle (V) for 4h, lysed and westerns run. Control, 10% AHS / IMDM; OxLDL, 100\(\mu\)g.mL\(^{-1}\) OCl-modified LDL; POVPC, 50\(\mu\)g.mL\(^{-1}\) POVPC (Calbiochem); M1 combined IFN-\(\gamma\) 10ng.mL\(^{-1}\) and LPS 10ng.mL\(^{-1}\); M2, IL-4 10ng.mL\(^{-1}\); (J) is the western densitometry on (I) *\(p<0.05\), Student’s paired t-test, n=3.

**Supplemental Figure XIII direct and indirect antioxidants induce p-ATF-1.**

Validation of NAC as a direct antioxidant is in (A) NAC, H\(_2\)O\(_2\) and heme were added to a cell-free system and oxidative stress measured with hROS reporter dye APF and fluorimetry after 20 mins as before\(^\text{16}\); Heme = heme 10\(\mu\)M, H\(_2\)O\(_2\) = H\(_2\)O\(_2\) 10\(\mu\)M, NAC = N-acetyl-cysteine 10mM (a widely reported *in vitro* concentration) *\(P<0.05\), ANOVA, n=5. Heme and H\(_2\)O\(_2\) at 10\(\mu\)M synergistically induced hROS, and this was abrogated by NAC in a cell-free chemical system; (B) NAC was then validated as an effective antioxidant in macrophages. Human blood-derived macrophages were cultured for 18h, then either heme 10\(\mu\)M, NAC 10mM, or both were added for 1h and oxidative stress measured with APF reporter dye and fluorimetry as before \(^\text{16}\) *\(p<0.05\), Student’s test n=5 donors; (C) cells were maintained in control 10% AHS / IMDM for 24h (control), or supplemented with either 1mM N-acetyl-cysteine (NAC) or 100\(\mu\)M curcumin for 24h; then heme 10\(\mu\)M (H) or DMSO vehicle (V) was added for 4h, the cells were lysed and run on western; (D) densitometry on (C), *\(p<0.05\), Student’s test n=5.

**Supplemental Figure XIV heme-inducible lipid metabolism genes**

Graph of lipid metabolism genes induced by heme, filtered by lipid terms in GO, and ordered by fold-induction. X-axis, fold induction. Y-axis, NCBI-recognised gene abbreviations.
Supplemental Figure XV Genomic location, sequence and homology of ATF-1 sites in HO-1 and LXR-β enhancers in human and mouse

ATF-1 site is underlined and red bold as indicated. Human (upper), Mouse (lower) and homologous motif (middle) sequences are as indicated. The positions indicated are relative to TSS. Data were processed as in Supplemental Methods.

Supplemental Figure XVI ATF-1 is causally required for ApoE gene induction

Y-axis, ApoE gene levels measured by qPCR. X-axis, treatment with heme (10µM) or vehicle and ATF-1 or control siRNA as indicated. * p<0.05, paired Wilcoxon; Data are mean ± SE of n=4 experiments, n=4 donors.

Supplemental Figure XVII LXR-β mediates LXR-α induction

Both (A, B) show the effects of treatment with siRNA for LXR-β (oligo-1, Dharmacon) or control with measurement of RNA for LXR-α (A) and ABCA1 (B) by qPCR; n=4 donors, mean ± SE; (C) shows western analysis to corroborate the effects on ABCA1 at the protein level, using siRNA as in (A, B); immunoblots and Mr as indicated, with densitometry as indicated. * p<0.05, paired Wilcoxon, n=4 experiments, n=4 donors.

Supplemental Figure XVIII Predicted transcription factor binding sites in human and mouse hmox1 gene

Panel (A) shows the output from ConSite, a web-entry algorithm, which finds motifs conserved between any human and mouse and matches to TRANSFAC and JASPAR databases\(^{11}\); (B) shows sequences conserved between human and mouse, from Promoterwise, with TF-site identification by Matinspector\(^{6}\), which matches TRANSFAC sites; (C) shows output of Opossum\(^{23}\), identifying statistically increased predicted transcription factor binding sites in heme-regulated genes.
Supplemental Figure XIX ChIP-Seq validated transcription factor binding sites accounting for Mhem transcriptome

X-axis, number of transcription factor / DNA binding site pairs from validated ChIP-Seq data incorporated in assessment. Y-axis, % of M-hem genes explained by transcription factors. Red box, ChIP-Seq validated sites for CREB1 (which also bind ATF-1, see Figure 5) account for the single largest fraction of genes (39%, 181 genes out of 463). This was derived from the database ChEA of ChIP-Seq data for actual transcription factor binding locations ⁹.

Supplemental Figure XX LXR-β confocal of human lesions

LXR-β confocal in Mhem cells in lesions with intraplaque hemorrhage. Colours and image location are as indicated, and correspond to Figure 7. The Mhem cells, but not foam cells, are LXR-β positive at the settings used.

Supplemental Figure XXI HO-1 semi-quantitation in human lesions

Panel (A) shows brightfield microscopy for HO-1 immunostaining and peroxidase/DAB detection as before¹⁵, with quantification of the DAB intensity in (B).

Supplemental Figure XXII Example regulatory sequences used for bioinformatics

Ensembl screenshots showing syntenic (equivalent) regions for Human and Mouse as indicated, with regulatory sequences (red boxes) in ENCODE, annotated on the basis of ChIP and DNAse-I hypersensitivity⁴, upstream of the RNA-coding sequences (green boxes). Sequences in red boxes were copied from human and mouse, conserved motifs identified with Promoterwise, and assessed by response element identification.
Supplemental Figure XXIII ATF-1 response element upstream of ATF-1 gene

Sequence is from ENSEMBL. Distances are relative to TSS, assuming 5’→3’ is positive. The gene is on the (+) strand. Human and mouse regulatory sequence was identified, and conserved motifs defined as before. Matinspector was used to identify the ATF-1 motif. The match is so strong that simple text matching for the canonical motif also identifies it. This autoregulatory interaction is also suggested by the Fantom database.
List of oligonucleotides used

Expression qPCR
ABCA1; product length = 157
Forward primer 1 AACAGTTTGTGGCCCTTTTG 20
Reverse primer 1 AGTTCCAGGCTGGGTACTT 20

ACADS; product length = 149
Forward primer 1 CCGAGAAGGAGTTGTTTCCC 20
Reverse primer 1 GTAGGCCAGTAAACGAGGC 20

ACOT8; product length = 199
Forward primer 1 ACTGTGAGACCCTATTCGCAGT 24
Reverse primer 1 CGAGAGCCACCAATATAGCCCCG 23

ApoE; product length = 131
Forward primer 1 TGGGTCTGCTTTTGGGATTAC 20
Reverse primer 1 GGCCTTCAACTCCTTCATGG 20

ATF-1; product length = 146
Forward primer 1 GAAGATACACCGGGCAGAAA 20
Reverse primer 1 CTTCGAGACTGTATGCTCC 20

BRCA2; product length = 177
Forward primer 1 GGAGGCCCAACAAAAGAGAC 20
Reverse primer 1 GGGTCTGCTGCATTCTCCAC 20

CD36; product length = 190
Forward primer 1 GGTCTCTACATCTCCGAAAGCAAGC 24
Reverse primer 1 GCACCTGTTTTCTTTGAAACTCCTGG 25

CD163; product length = 88
Forward primer 1 ACATCTGGCTTGACAGCGTT 20
Reverse primer 1 TTGCAATAATGCTTTCCCA 20

CLCF1; product length = 120
Forward primer 1 CTCCGGGAGAGGAGCAGGCAC 20
Reverse primer 1 GGCACTGCAGGGGAGGCAGCC 20

CRTC2; product length = 145
Forward primer 1 TTCACTGTCAGTCTCCAGGT 20
Reverse primer 1 GCTGAACCTGCTCCAGATTC 20

FASN; product length = 111
Forward primer 1 GCAGGCTGCTGCTGGAAGTCA 20
Reverse primer 1 GTCTCAGAGCCGCTCACGC 20

GCLM; product length = 176
Forward primer 1 GCTGAGCTGGATGCCCACCCAG 20
Reverse primer 1 CGAATACCGCAGTAGCCACA 20

HMGCR; product length = 101
Forward primer 1 ACAGTGGGAGGTGGGACCAA 20
Reverse primer 1 TGCCGGGCATTTTCCCCAGGC 20

HO-1; product length = 158
Forward primer 1 CTTACACCTTCCCCCAACATTG 20
Reverse primer 1 CCTGCAACTCCTCAAGAGGC 20

HPRT; product length = 133
Forward primer 1 TTGGTCAGGCAGTATAACCC 20
Reverse primer 1 GGGCATTACCTACAACAAAC 20

HTRA3; product length = 97
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P2RY12; product length = 135
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SREBF1; product length = 158
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ChIP-qPCR

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HO-1 -2500; product length = 113bp
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Reverse primer 1 TGCCATGAAAAGCACAAGG 20
HO-1-4258; product length = 165bp
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Transcription Factor binding

**HO-1CRE-5Kb-Biotin**
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Rev_5’-AACATGACGACAGAATGCAGCTGCAC-3’

**HO-1deltaCRE-5Kb-Biotin**
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**hLXRbCREv2-Biotin**
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**hLXRdeltaCRE-Biotin**
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Rev_5’-TTCTTCCGGCAACCCCGCCCTTTGATGATTGGCGTCC-3’

**Canonical_CREB (EMSA)-biotin**
For_5’-GGAGAGATTGCCTGACGTCAGAGCTAG-3’_biotin
Rev_5’-CTAGCTCTCTGAGCTGACGACGATACTGTC-3’

Reference List


(7) Schug J. Using TESS to predict transcription factor binding sites in DNA sequence. *Curr Protoc Bioinformatics* 2008; Chapter 2: Unit.


# Genes over 2-fold induced at 4h (Agilent, GeneSpring and Resolver Processing)

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### Lipid metabolism genes induced at 4h

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<td>Homo sapiens thioredoxin-like 4A (TXNL4A)</td>
<td>1.468731</td>
<td>1.488559</td>
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<td>ACOT7</td>
<td>Homo sapiens acyl-CoA thioesterase 7</td>
<td>1.369419</td>
<td>1.480431</td>
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<td>RDH5</td>
<td>Homo sapiens retinol dehydrogenase 5 (11-cis/9-cis) (RDH5)</td>
<td>1.569863</td>
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<tr>
<td>DGAT1</td>
<td>Homo sapiens diacylglycerol O-acetyltransferase homolog 1 (mouse) (DGAT1)</td>
<td>1.388949</td>
<td>1.471024</td>
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<tr>
<td>FABP3</td>
<td>Homo sapiens fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)</td>
<td>1.58115</td>
<td>1.467389</td>
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<tr>
<td>APOBEC3B</td>
<td>Homo sapiens apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B (APOBEC3B)</td>
<td>1.474532</td>
<td>1.452127</td>
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<td>PCSK7</td>
<td>Homo sapiens apolipoprotein convertase subtilisin/kexin type 7 (PCSK7)</td>
<td>1.195403</td>
<td>1.433817</td>
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<td>GPX4</td>
<td>Homo sapiens glutathione peroxidase 4 (phospholipid hydroperoxidase) (GPX4)</td>
<td>1.270999</td>
<td>1.432227</td>
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<td>TALDO1</td>
<td>Homo sapiens transaldolase 1 (TALDO1)</td>
<td>1.337651</td>
<td>1.411819</td>
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<td>SRCRB4D</td>
<td>Homo sapiens scavenger receptor cysteine rich domain containing, group B (4 domains)</td>
<td>1.384378</td>
<td>1.409793</td>
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<td>SOC2</td>
<td>Homo sapiens SOC cytochrome oxidase deficient homolog 2 (yeast) (SOC2)</td>
<td>1.366721</td>
<td>1.402916</td>
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<td>ECHS1</td>
<td>Homo sapiens enoyl Coenzyme A hydratase, short chain, 1, mitochondrial (ECHS1)</td>
<td>1.47008</td>
<td>1.40109</td>
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<td>ECH1</td>
<td>Homo sapiens enoyl Coenzyme A hydratase 1, peroxisomal (ECH1)</td>
<td>1.415137</td>
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<td>PEX16</td>
<td>Homo sapiens peroxisomal biogenesis factor 16 (PEX16)</td>
<td>1.281948</td>
<td>1.380401</td>
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<td>COX8A</td>
<td>Homo sapiens cytochrome c oxidase subunit 8A (ubiquitous) (COX8A)</td>
<td>1.417099</td>
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<td>PEX11B</td>
<td>Homo sapiens peroxisomal biogenesis factor 11B (PEX11B)</td>
<td>1.346822</td>
<td>1.370249</td>
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<td>ACADS</td>
<td>Homo sapiens acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain (ACADS)</td>
<td>1.205227</td>
<td>1.360952</td>
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<td>SLC27A4</td>
<td>Homo sapiens solute carrier family 27 (fatty acid transporter), member 4 (SLC27A4)</td>
<td>1.215553</td>
<td>1.34463</td>
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<td>PIGV</td>
<td>Homo sapiens phosphatidylinositol glycan anchor biosynthesis, class V (PIGV)</td>
<td>1.340283</td>
<td>1.342279</td>
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<td>PITPNA</td>
<td>Homo sapiens phosphatidylinositol transfer protein, alpha (PITPNA)</td>
<td>1.282136</td>
<td>1.340402</td>
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<td>DCI</td>
<td>Homo sapiens dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoeyl-Coenzyme A isomerase)</td>
<td>1.293352</td>
<td>1.340022</td>
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<td>PI4KBII</td>
<td>Homo sapiens phosphatidylinositol 4-kinase type II (PI4KBII)</td>
<td>1.13716</td>
<td>1.327816</td>
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<td>APOC4</td>
<td>Homo sapiens apolipoprotein C-IV (APOC4)</td>
<td>1.41013</td>
<td>1.325248</td>
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</table>

**Supplemental Table III**
Supplemental Figure I

Genes for signaling cascades induced or repressed at 1h

A

Signaling Cascade

Notch
Ion channel
Insulin
Calmodulin
Redox
Jak/Stat
AMP / cAMP
Cytokine
GTPase activator
G-Protein
NF-kappa-B
Zinc Finger

Number of genes regulated

B

Supplemental Figure I
Genes for cAMP and AMP signaling induced or repressed at 1h

Supplemental Figure II
Genes for insulin signaling induced or repressed at 1h

Supplemental Figure III
Genes for cytokine signaling induced or repressed at 1h

Supplemental Figure IV
Genes for NFkB signaling induced or repressed at 1h

Log₂ change

Supplemental Figure V
Genes marking macrophage inflammatory activation (all repressed)

Supplemental Figure VI
A

Supplemental Figure VII

B

C

ID | 1h | 4h
--- | --- | ---
SIRT2 | 1.056 | 2.009
CD36 | 0.44 | 0.31
LXR-α | 1.07 | 2.79
LXR-β | 2.6 | 3.26
ATF-1 | 6.07 | 1.42
HO-1 | 1.35 | 11.7
TFRC | 0.5 | 0.44
OSGIN1 | 0.77 | 2.48
SLC48A11 | 1.38 | 4.47
FHC | 1.25 | 1.46
P2Y12 | 5.66 | 0.76
GCLM | 1.05 | 5.37
ApoE | 2.07 | 8
HTRA3 | 1 | 11.8
Supplemental Figure VIII
Supplemental Figure IX
A Untreated PEI siRNA-FITC PEI + siRNA-FITC

Oxidative stress (hROS arbitrary units)

Control siRNA ATF-1 siRNA
Veh Heme Veh Heme

Supplemental Figure X
Supplemental Figure XI
Supplemental Figure XIII

A

B

C

D

Supplemental Figure XIII
Fold induction at 4h

Lipid Metabolism Genes

Supplemental Figure XIV
Site: CREB/ATF-1

HuHmox1 -3978: TCTGCTGC\textcolor{red}{GTCA}TGTTTGGGAGGGGA\textcolor{red}{GCTCGC}--AACA\textcolor{red}{GGAAGG}
HO-1 Homol T TGCTG \textcolor{red}{GTCA}TG TTGGGAGGGG CT CGA A \textcolor{red}{GCAAGG}
MuHmox1 -3923: TATGCTGT\textcolor{red}{GTCA}TGTTTGGGAGGGGTGATTA GCAAGACAAGGG

HuLXRβ-1700: CTTCCGGACG\textcolor{red}{TGACG}C GCGGGGTGGCAGAAGTGGCAGAATTACTTTTG
LXR-β Homol CTTCCGGA \textcolor{red}{TGACG}C GCGGGGTGGCAGAAGTGGCAGAATTACTTTTG
MuLXRβ+83: CTTCCGGAAG\textcolor{red}{TGACG}C GCGGGGTGGCAGAAGTGGCAGAATTACTTTTG
Supplemental Figure XVI
Supplemental Figure XVII
**Supplemental Figure XVIII**

### Background gene hits vs. Target gene hits

<table>
<thead>
<tr>
<th>TF</th>
<th>Background gene hits</th>
<th>Background gene non-hits</th>
<th>Target gene hits</th>
<th>Target gene non-hits</th>
<th>Fisher score</th>
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<tr>
<td>CREB1</td>
<td>11650</td>
<td>3500</td>
<td>6</td>
<td>3</td>
<td>8.68E-01</td>
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<tr>
<td>RORA_1</td>
<td>12572</td>
<td>2578</td>
<td>8</td>
<td>1</td>
<td>5.31E-01</td>
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<tr>
<td>SP1</td>
<td>13755</td>
<td>1395</td>
<td>9</td>
<td>0</td>
<td>4.19E-01</td>
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<tr>
<td>MZF1_5-13</td>
<td>14393</td>
<td>757</td>
<td>9</td>
<td>0</td>
<td>6.31E-01</td>
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<tr>
<td>REL</td>
<td>13546</td>
<td>1604</td>
<td>9</td>
<td>0</td>
<td>3.65E-01</td>
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</table>
Supplemental Figure XIX
Supplemental Figure XX
Supplemental Figure XXI

A

HO-1 Intensity

FC Mhem

B

HO-1 Intensity (arbitrary density units x10^6)

FC Mhem
ATF-1 enhancer

Hu: -2332 CAAGATGGCGGC CGGTCA CGTGAGG CGC CGG GTGACG CAG GC ACG CGGC
Homol CAAGATGGCGGC GC TACG TG CA CGCGTGACGCAG ACG CGGC
Mu: -1206 CAAGATGGCGGC GC TACG TGACCA CGCGTGACGCAGCAC GCGC