Hematoma Resolution In Vivo Is Directed by Activating Transcription Factor 1 (ATF1)

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ABSTRACT

Rationale. The efficient resolution of tissue hemorrhage is an important homeostatic function. In human macrophages in vitro, heme activates an adenosine monophosphate activated protein kinase / activating transcription factor 1 (AMPK/ATF1) pathway that directs Mhem macrophages through coregulation of heme oxygenase 1 (HMOX1, HO-1) and lipid homeostasis genes.

Objective. We asked whether this pathway had an in vivo role in mice.

Methods and Results. Perifemoral hematomas were used as a model of hematoma resolution. In mouse bone marrow derived macrophages (mBMM), heme induced HO-1, lipid regulatory genes including LXR, the growth factor IGF1, and the splenic red pulp macrophage gene Spic. This response was lost in mBMM from mice deficient in AMPK (Prkab1−/−) or ATF1 (Atf1−/−). In vivo, femoral hematomas resolved completely between day 8 and day 9 in littermate control mice (n=12), but were still present at day 9 in mice deficient in either AMPK (Prkab1−/−) or ATF1 (Atf1−/−) (n=6 each). Residual hematomas were accompanied by increased macrophage infiltration, inflammatory activation and oxidative stress. We also found that fluorescent lipids and a fluorescent iron-analog were trafficked to lipid-laden and iron-laden macrophages respectively. Moreover erythrocyte iron and lipid abnormally colocalized in the same macrophages in Atf1−/− mice. Therefore, iron-lipid separation was Atf1-dependent.

Conclusions. Taken together, these data demonstrate that both AMPK and ATF1 are required for normal hematoma resolution.

Keywords: Macrophage, hematoma hemorrhage resolution, inflammation, heme oxygenase carbon monoxide, hemorrhage, transcription regulation atherosclerosis, transcriptional regulation.
Nonstandard Abbreviations and Acronyms:

AMPK - Adenosine Monophosphate Activated Protein Kinase
ATF1 – Activating Transcription Factor 1
Heme Oxygenase 1 - (HMOX1 (approved gene symbol) commonly known as HO-1)
mBMM – mouse bone marrow derived macrophages
LXR – Lipid X Receptor
IGF1 – Insulin like Growth Factor 1
SPIC – approved gene symbol, spleen focus forming virus proviral integration 1 homologue C
PPARγ – approved gene symbol PPARG, peroxisome proliferator activated receptor gamma
NFE2L2 – approved gene symbol, also known as Nrf2, Nuclear Factor of Erythroid cells 2 like 2
CRE – Cyclic adenosine monophosphate response element
ARE – antioxidant response element
FCS – Fetal Calf Serum
RLT – proprietary name RNA Lysis Treatment
OCT – proprietary name Oxford Genome Technology
FDR – False Discovery Rate
PASTAA – computer program name, Predicting Associated Transcription Factors from Annotated Affinities
OPOSSUM – proper name of computer program
LASAGNA2.0 - proper name of computer program, Length-Aware Site Alignment Guided by Nucleotide Association
MEME - proper name of computer program, Multiple Em for Motif Elicitation
DREME - proper name of computer program, Discriminative Regular Expression Motif Elicitation
TRANSFAC – Transcription Factor Database
OCT – Optimal Cutting Technology
LSM - Laser Scanning Microscope
DIC – Differential Interference Contrast
SPSS – Statistical Product and Service Solutions
ANOVA – Analysis of Variance
Prkab1 – approved gene name, Protein Kinase Adenosine monophosphate activated Beta subunit 1
SAA – Serum Amyloid A
CD68 – approved gene name, Cluster Differentiation 68
SOCS1 – approved gene name, Silencer of Cytokine Signaling 1
NR1H2 - approved gene name, Nuclear Receptor subfamily 1, group H member 2, also known as lipid X receptor beta (LXRβ)
NR1H3 - approved gene name, Nuclear Receptor subfamily 1, group H member 3, also known as lipid X receptor beta (LXRα)
ABCA1 - approved gene name, Adenosine triphosphate Binding Cassette family subfamily A member 1
APOE – approved gene name, Apolipoprotein E
RPM – Red Pulp Macrophages (of spleen)
KLF4 – approved gene name, Krüppel Like Factor 4
CREB1 Cyclic Adenosine Monophosphate Response element Binding protein isoform 1
TFBS – Transcription Factor Binding Site
RELA – approved gene name, Human Relish homologue isoform A
NfκB – Nuclear Factor kappa B
NFκB – Tumour Necrosis Factor alpha
IL-6 – Interleukin 6
MAPK – Mitogen Activated Protein Kinase
GO – Gene Ontology

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INTRODUCTION

The efficient resolution of tissue hemorrhage (and hematoma) is important in life-threatening human diseases, such as intracranial hemorrhage and intraplaque hemorrhage of atherosclerotic lesions. Hematomas in other sites, including skin, subcutaneous compartments and musculoskeletal injury, are also common and important in trauma. The recurrent hematomas in hereditary hemophilia cause severe disability via the tissue reaction they elicit. Therefore, hematoma resolution is likely to be an important homeostatic and disease-preventing process in clinical and veterinary medicine. When successful, this process recycles erythrocyte components whilst minimizing inflammatory and oxidative stress from tissue hemoglobin.

The molecular understanding of the normal clearance of tissue hemorrhage is patchy. It is most studied in the context of intracerebral hemorrhage. Hemorrhage resolution is understood to be dependent on macrophages and promoted by agonists of peroxisome proliferator-activated nuclear receptor gamma (PPARγ).

One of the key effector enzymes in hemorrhage-resolution is heme oxygenase-1 (HO-1). HO-1 is encoded by the gene \(HMOX1\), and has a very highly characterized enzyme function. Iron protoporphyrin-IX (heme) is the main prosthetic moiety of hemoglobin. HO-1 has a pocket that accepts heme, opens (decyclizes) the porphyrin ring and removes the ferrous ion. This ultimately results in safe chelation of iron in ferritin and in generation of bilirubin. Classical bruise evolution (blue/black – brown – green – yellow) reflects HO-1 activity.

The precise mechanism regulating HO-1 induction by heme or tissue erythrocytes had not been fully elucidated. This is surprising, given the intensive investigation of HO-1, and the physiological importance of its regulation by heme. We have characterized Hhem in human macrophages in vitro and human atherosclerotic intraplaque-hemorrhage tissues. Hhem denotes a homeostatic macrophage phenotype distinct to M2, dependent on AMPK and transcription factors ATF1 and NFE2L2. These mediate heme-stimulated induction of \(HMOX1\) via a cyclic-AMP response element like / anti-oxidant response element like (CRE-like / ARE-like) co-site at -4200bp. We define here the causal role of AMPK and ATF1 in the regulation of the normal process of hematoma resolution in vivo.

In a parabiosis model, ischemia-reperfusion injury modulates intracerebral hemorrhage-resolution via AMPK, which was thought most likely to be mediated by transferred leukocytes. Here, we here define a direct and local intrinsic hemorrhage-resolution mechanism, driven via an AMPK-ATF1 pathway by the hematoma itself.

We show here that AMPK and ATF1 are required for normal hematoma resolution in vivo. Loss of either AMPK or ATF1 prolonged hematoma resolution, resulting in increased inflammation, protracted iron deposition, and increased oxidative tissue-injury. ATF1 and AMPK mediated the dynamic regulation of the normal process of hematoma resolution.
of gene expression by heme, including genes that coordinate erythrocyte disposal and tissue repair genes. Thus, ATF1 is a novel and important gene for macrophage-mediated tissue homeostasis.

**METHODS**

The paper adheres to TOP guidelines. The data that support the findings of this study are available from the corresponding author upon reasonable request and the transcriptomic data will be publicly available once formatted and accepted at GEO.

More detailed Methods are available in the Online Supplement.

**Cell culture.**
Bone marrow macrophages were cultured by a minor modification of previous methods. The mouse was sacrificed by Schedule 1 method, and long bones cleaned sufficiently and flushed with ice-cold PBS as promptly as possible. The bone marrow is then cultured in 10% FCS Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% L929-conditioned medium. At 6 days culture, the bone marrow macrophages were scraped and transferred to tissue culture plates optimized for experiments, typically 24-well plates.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).**
RNA was purified by a proprietary kit (Qiagen). Cell culture supernatant was decanted and stored at -80°C. Then 200μL guanidinium-based buffer (RLT) was added, the lysates stored at -80°C until purification (up to several weeks). Then the Qiagen mini column system was used for purification according to manufacturer’s instructions.

**Microarray and bioinformatics.**
This was by minor modification of previous methods, detailed in the Supplement16. RNA was quality controlled, labelled and measured using the Agilent 4x44k mouse system, with a commercial service (OGT). The data were analyzed with GeneSpring, with significance decided at FDR=0.05 and Storey bootstrapping adjustment for multiple simultaneous comparisons. Gene Ontology was examined within GeneSpring and exported with the genelists. Genelists were analyzed outwith GeneSpring using standard packages, including Venn diagrams, GeneSet Enrichment Analysis. Gene names of characterized human and mouse genes are identical except for case. For human / mouse comparison, the cases were matched using Notepad++ and then submitted to Venn. Transcription Factor binding analysis was primarily using X2K (Ma’ayan laboratory), which used ChIP-validated data for matching. Transcription factor motif searches with other methods broadly corroborated (named PASTAA, OPOSSUM, LASAGNA, MEME, DREME, and TRANSFAC). There was no time-course on which to use time-series related methods of network inference, in contrast to our previous publications 16. STRING (https://www.expasy.org/, Swiss Institute for Bioinformatics), which incorporates largely protein-protein binding but also other interactions, was used to construct networks and the most highly connected nodes were identified.

**Femoral hematoma.**
Knockout and littermate control mice22, 23 were lightly anesthetized with isoflurane to maintain humane-ness and precision. Then autologous erythrocytes as 50μL anticoagulated blood were injected into the femoral canal region (Figure 1). A preliminary time course indicated that there were residual hematomas at day 8 but these had cleared at day 9. Subsequent experiments focused on events at day 8 and day 9.
Microscopy and confocal microscopy and image analysis.
Cryosectioning was at 5μm using a Bright cryostat and specimens frozen in Optimal Cutting Technology (OCT) on cooled isopentane. Fluorescence-labeled sections were mounted in Glycerol/PBS or in commercial anti-fade mountant and imaged within a few days on a Zeiss LSM750 (Imperial College FILM Facility). Standard ultraviolet / blue, 488, 560 and 750 channels were used, with additional differential interference contrast (DIC) on the 488 channel. This instrument was also used for the fluorescence emission scans. Paraffin sectioning was at 5μm and histochemistry and immunohistochemistry is described in the Supplemental.

Optical quantification.
A Tecan Spectrafluor 96-well reader was used to acquire Ultraviolet-Visible absorption spectra, and for the multiwavelength fluorescence excitation / emission combinations.

Statistics.
More detailed statistical methods are given in the Supplement. Data were analysed with SigmaStat, GraphPad Prism, and SPSS. Data were tested for normality by Shapiro-Wilk where n≥5. Where appropriate, data are given as mean±SE. Where appropriate, Student’s t-test, Mann-Whitney, Repeated Measures ANOVA or Kolmogorov-Smirnov were used where specified. Only in-test corrections were made. Where p-values are not given the testing was not performed.

RESULTS
A subcutaneous hematoma model was developed as an experimentally reproducible and practical simulation of clinical hematoma (Figure 1A). Littermate autologous blood (50 μl) was injected into peri-femoral adipose tissue (Figure 1A). Then, mice were sacrificed at set intervals (Figure 1B). The injected region was excised post-mortem and examined by H&E or immunocytochemistry (Figure 1C). Hematomas in genetic control mice consistently resolved between day 8 and day 9 (Prkab1+/+ or Atf1+/+ littermate controls in Figure 2A, B). In contrast, mice deficient in AMPK (Prkab1−/−; Figure 2A) or ATF1 (Atf1−/−; Figure 2B) showed delayed hematoma resolution, as evidenced by the continued presence of blood at day 9. Specifically, residual erythrocytes were identified in both Prkab1−/− and Atf1−/− mice following staining with H&E or anti-spectrin antibodies (Figure 2A, B). Next, hematoma resolution in LysMCre x Prkab1fl/fl mice was examined. This strain has AMPK selectively deleted from their macrophages and exhibited the same effect. That is, they retained hematomas at day 9, whereas Prkab1fl/fl controls did not (Figure 2C). This indicated that it was macrophage AMPK that was required. To test further that the AMPK-ATF1 pathway was located in myeloid cells, a bone marrow transplant experiment was performed. In this, Atf1+/+ mice were reconstituted with marrow from Atf1−/− or Atf1+/+ mice using standard bone marrow transplant procedures. Atf1+/+ → Atf1+/+ cleared hematomas at day 9, and Atf1−/− → Atf1+/+ had residual hematomas at day 9 (Supplemental Figure 1). This indicated that the deficiency in clearing hematomas was transferred with Atf1-deficiency in the hematopoietic lineage.

A number of validation assessments were also made. It was necessary to reversibly anti-coagulate the blood in order to reproducibly transfer it to the recipients. This was done with a limiting concentration of citrate, which allows calcification and resumption of coagulation on injection to tissue. The effect of this was determined by comparing hematomas with untreated and anticoagulated blood. Anticoagulation caused a slowing of the resolution (Supplemental Figure 2B). A fuller time course was carried with the anticoagulated protocol, as it was more reproducible. This showed that hematomas appeared to be consistently maintained up day 8, but were lost between day 8 and day 9 (Supplemental Figure 2B). Measurement of the serum inflammatory marker serum amyloid A (SAA) indicated that the hematomas did not alter systemic inflammation (Supplemental Figure 2C). Extending the experimental time to 21 days...
showed that in Atf1/-/- mice, hematoma clearances was simply delayed and the tissue returned to normal at day 21 (Supplemental Figure 2D).

No statistically significant difference was observed between hematocrits in Atf1+-/ and Atf1++/ littermates (Supplemental Figure 3A). Spleen weights and spleen macrophage HO-1 expression were assessed in Atf1+-/ and Atf1++/ littermate controls. Spleens of Atf1+-/ mice had a slightly lower mass than Atf1++/ littermate controls and Atf1/-/- mice (Supplemental Figure 3B). Atf1+-/ mice and Atf1++/ littermate controls had an equivalent CD68+HO-1+ macrophage population in the splenic red pulp (Supplemental Figure 3C-D). Atf1+-/ mice and Atf1++/ littermate controls had indistinguishable patterns and levels of splenic red pulp iron, which was located in splenic red pulp macrophages (Supplemental Figure 3E-F).

Immunolabeling of day 8 hematomas from AMPK-deficient mice (Prkab1/-/-) for phosphorylated ATF1 (pATF1) showed significantly less pATF1 expression than hematomas from genetic control mice (Figure 3A). This indicated pATF1 was dependent on AMPK. The null-labeling in Atf1+-/ mice confirmed the specificity of the antibody (Figure 3A). Image quantification showed that lesonal p-ATF1 was consistently profoundly suppressed in Prkab1/-/- mice and absent in Atf1/-/- mice. (Figure 3B). As shown in Figure 3C, induction of the ATF1 gene by heme in cultured mouse BM-derived macrophages (mBMM) was profoundly reduced by AMPK deficiency. This indicated that ATF1 transcriptional activation is AMPK-dependent. These observations, combined with our previous data on human macrophages in vitro 17, indicate that ATF1 expression and phosphorylation due to heme are dependent upon AMPK.

We have previously reported that HMOX1 is an ATF1 target gene in human macrophages exposed to heme 16. This was also true of mice. We found impaired expression of HO-1 in Day 8 hematomas (HO-1) from Atf1+-/ mice (P=0.001) (Figure 4A,B) and significantly reduced Hmox1 gene-expression in heme-stimulated Atf1+-/ (P=4x10^-6) (Figure 4C). Consistent with the dependency of ATF1 on AMPK, similar data were obtained with hematomas from Prkab1/-/- mice in vivo and with Prkab1/-/- mBMM in vivo (Figure 4 A-C). The time-course of gene expression in response to 10μM heme was also assessed for a number of other genes potentially affected by AMPK or ATF1 deficiency (Figure 5). We found that AMPK and ATF1 deficiency had very similar effects on expression of heme-responsive genes, including Hmox1, Socs1, Nr1h2 (LXRβ), Nr1h3 (LXRα), Abca1, Apoe, and Igf1 (Figure 5A). The gene Spic was also Atf1-dependent (Figure 5A). Heme-dependent commitment to splenic red pulp macrophages (RPM) is mediated by Spic 24, indicating that Atf1 may also be above Spic in the hierarchy of heme-dependent regulation more generally. Taken together with the in vivo data presented above, these results support an AMPK/ATF1 pathway regulating heme-induced homeostatic genes involved in iron metabolism, lipid-metabolism and inflammation (Figure 5B).

We extended the assessment of gene regulation by using microarrays in an unbiased manner across the entire transcriptome. This allowed us to compare human and mouse heme-induced responses, and to evaluate the role of ATF1 and other transcription factors. Based on the qPCR data above, we chose the 8 h time-point to compare responses to heme versus vehicle in Atf1+-/ and Atf1++/ WT macrophages. Approximately one half of the heme-regulated genes (458 out of 930) were regulated by heme in an Atf1-dependent manner (Figure 6A). Identification of over-represented transcription factor binding sites (TFBS) in genesets in each category showed increased sites for Krüppel Like Factor 4 (KLF4), ATF1/CREB1 and Nuclear Factor of Erythroid Cells Like 2 (NFE2L2) (NRF2) in the heme- and Atf1-regulated genes (Figure 6A). The enrichment in TFBS for basic Zipper (bZIP) members - specifically CREB/ATF1, AP-1 and NFE2L2/Bach/MafG - was similar to humans 16. The enrichment for KLF4 sites was intriguing since KLF4 has been linked to other pro-resolution pro-repair homeostatic differentiation patterns in macrophages 25,26.

Next, taking the heme- and Atf1-regulated genes, and examining their interactions in the public protein-protein binding database STRING revealed that highly connected regulators were known key inflammatory genes including nuclear factor kappa B (NFκB) (Rela), Interleukin 6 (IL-6), Tumour Necrosis factor alpha (TNF-α), and members of the Mitogen Activated Protein Kinase (MAPK) family (Figure 6B). This was consistent with heme modulating inflammation via Atf1.
Next, the genes in the 3 areas of the Venn diagram (Heme-only, \textit{Atf1}-only, and heme-\textit{Atf1} coregulated intersection) were compared by Gene Ontology classification (http://www.pantherdb.org/). This revealed similarities and also substantial differences between the three sets of genes (Figure 6C). Broadly, they modulated cell stress, cell death, division and differentiation. Many of the genes were involved in metabolic processes (lipid, nitrogen, protein, carbohydrate, aromatic compound). At a more detailed level, there were substantial differences between the 3 pathways. Thus, the heme-\textit{Atf1} coregulated intersection corresponded to fewer stress genes; the \textit{Atf1}-specific section to more cell-cycle genes and to regulation of signaling; and heme-specific genes more to cell differentiation (Figure 6C).

Of note, the next most frequent group of GO terms was the relatively small number corresponding to immune regulation (not shown). Whilst all 3 pathways modulated immune regulatory genes, they modulated distinct categories. Thus, genes modulated by heme and \textit{Atf1} had 13% of genes corresponding to GO term “lymphocyte activation”; 2.3% of heme-specific genes to “regulation of lymphocyte-mediated immunity” and 0.61% of genes modulated by \textit{Atf1}-only corresponded to “immunoglobulin-mediated response”, with 0 value for the remainder (not shown). This indicates that although the 3 pathways modulated inflammation / immunity, they did so in patterns with alternative biological fine-tuning.

On the basis of these results, we next explored whether delayed blood clearance in \textit{Prkab1}^−/− or \textit{Atf1}^−/− mice was associated with disrupted homeostasis, oxidative / nitrosative stress and tissue injury. As expected from the continued presence of erythrocytes, deficiency in either AMPK or ATF1 led to the persistence of Berlin Blue - stainable iron at Day 9 (Figure 7A). We also found an increase in macrophage number, as evaluated by anti-CD68 immunolabeling (Figure 7B). This was associated with increased macrophage inflammatory activation, as reflected by increased NF\kappa B \textit{p65 Rela} cytoplasmic-to-nuclear translocation (Figure 7C), as well as increased expression of inducible Nitrile Oxide Synthase (\textit{iNOS, Nos2}) (Figure 7D). Protein and DNA modifications in the presence of iron and activated macrophages was evidenced by increased immunolabeling for nitrotyrosine (Figure 7E) and 8-oxo-deoxyguanosine (Figure 7F), respectively indicating nitrosative and oxidative stress.

Macrophages engaged in hematoma clearance normally become hemosiderin-laden macrophages (siderophages) or lipid-laden macrophages (foamy macrophages, foam cells). Consistent with this, dual-histochemistry with Oil-Red-O (ORO) and Berlin Blue (Prussian Blue, Perls' stain, a well-characterised iron histochemistry reaction) demonstrated that iron was contained in small macrophages and lipid in large macrophages (Figure 8A). This size separation, measured as a bimodal size distribution, was disrupted in \textit{Prkab1}^−/− and \textit{Atf1}^−/− mice (Figure 8B). When this was addressed in more detail with ORO-Berlin Blue dual-histochemistry, there were separate iron-positive and lipid-positive macrophages in \textit{Atf1}^+/+ littermate controls, but iron and lipid were colocalised in the same macrophages in \textit{Atf1}^−/− mice (Figure 8C). That is, loss of \textit{Atf1} resulted in accumulation of lipid and iron in the same macrophages. The same pattern was seen in paraffin sections using the foam cell marker Perilipin-1 and Berlin Blue (Figure 8D).

To probe causality, we next examined active tracking of metal and lipid metabolites from erythrocytes into the siderophages and lipid-laden macrophages, in the presence and absence of \textit{Atf1}. The ATF1-mediated co-regulation of lipid and iron homeostatic genes in response to heme provides a mechanism for separate differentiation into iron-laden macrophages and lipid-laden macrophages. Whether AMPK and ATF1 were required for separation into iron-laden macrophages and lipid-laden macrophages by directing active trafficking of metabolites was next tested using fluorescnetly-labelled lipid and iron analogs. BODIPY-PC and NBD-Cholesterol served as tracking reagents respectively for phospholipid and cholesterol. In preliminary experiments we validated ruthenium (Ru) as a fluorescent analog for iron tracking (Supplemental Figures 4-56). Ru and Fe are in the same group in the periodic table, have approximately the same ionic radius and Ru becomes fluorescent on complexing with the pyrrole nitrogen in porphyrins or histidine (in ferritin). Ru is not severely toxic. Erythrocytes containing Ru-PPIX or fluorescently-labelled lipids were injected as above to create labelled hematomas. Fluorescently-labelled lipids trafficked to large macrophages (Supplemental Figure 5). Fluorescence corresponding to Ru-ferritin...
was identified in small macrophages, indicating that the labelled erythrocyte-derived RuPPIX had been taken up, processed and stored (Figure 8E). Loss of Atf1 resulted in delayed clearance of Ru-associated fluorescence, with the appearance of Ru-associated fluorescence in cells that were large and lipid-laden, corresponding to foam cells (Figure 8F-H).

We next asked whether the deficiency in Atf1−/− for hematoma clearance was specific to erythrocytes or reflected a more generalized macrophage dysfunction. Clearance of apoptotic neutrophil leukocytes (efferocytosis) was therefore studied. Strain controls were sacrificed and bled. Neutrophils were purified from blood, and then killed with UV irradiation (20 minutes), labelled for tracking and then injected to the perifemoral area. After a time-course, mice were sacrificed and the femoral area examined by classical histology, as for the hematomas. Deficiency in Atf1 did not affect apoptotic leukocyte clearance (Supplemental Figure 7). Conversely, apoptotic leukocyte clearance in this model was severely impaired by si-Crebl knockdown (not shown). Crebl canonically mediates transcriptional responses to cyclic-AMP, the second messenger used by PGE2 and specialized pro-resolving mediators (SPMs) that play an important role in inflammation 30-32.

DISCUSSION

We show here that deletion of either ATF1 or AMPK impairs normal hematoma resolution in vivo, causing hematoma persistence with inflammation and oxidative stress. Heme modulated numerous genes via Atf1, including metabolic genes, and genes for nitrogen-metabolism, cell death, protein localisation and gene expression. Fluorescent erythrocyte components tracked into macrophages, with metal tracking into siderophages and lipid into foamy macrophages. This separation was disrupted by Atf1 gene deletion. Loss of Atf1 induced inflammation, oxidative stress and pathological colocalisation of lipid and iron. Atf1 is hierarchically upstream of Spic.

Heme modulated important clusters of genes. We found an Atf1-dependent and an AMPK-dependent induction of genes regulating growth, metabolism and inflammation. This corresponded with the broader microarray data in which heme and Atf1 modulated genes widely across metabolic regulation. We also established in vivo – in vitro and mouse-human correlations in which the previous in vitro data implicating AMPK in Atf1 activation were replicated in vivo. Hematoma p-ATF1 activation was prevented in AMPK-knockouts, resulting in loss of Hmox1 induction, with promotion of inflammation and oxidative stress.

We took care to establish that Atf1 actively separated iron and lipid. We established that Ru would track Fe on a cell by-cell basis, a necessary improvement on isotopic methods for lineage determination. We coupled this with cell size analysis, lipid tracing and iron/lipid dual-histochemistry to show that metal and lipid from erythrocytes are actively trafficked into different macrophages dependent on Atf1.

Much research emphasis in recent decades has been on resolution of inflammation, notably mechanisms of leukocyte clearance, more particularly disposal of apoptotic leukocytes (efferocytosis). In preceding decades, there had been interest in the resolution of hematomas and tissue hemorrhage. For example, a time-course and eventual outcome of hematomas artificially injected in arterial walls was defined 33. Such studies allowed the definition of HO-1 as the enzyme activity that degrades heme and thereby mediates the classic color-sequence of bruise resolution 8, 11, 34, 35. The key inducible isoform of heme oxygenase was defined in the 1980s 36 and sequenced in the 1990s 37. This paper defines HO-1-regulation and co-regulation in its original physiological context. Our future work will focus on understanding gene regulation during erythrocyte-resolution relative to inflammation-resolution, focussing on the specificity mechanisms between ATF1-related gene regulation and CREB1-related gene regulation.
The subcutaneous femoral hematomas resolved between days 8 and 9, which indicated a relatively abrupt clearance event. This was unexpected, but facilitated robust model measurement and experimental data. This model, together with in vitro experiments, provided new insights into erythrocyte clearance. A more precise molecular understanding of the inter-regulation and specificity mechanisms of AMPK, CREB1, ATF1 and their target genes is a priority. A full follow-up of the dependency of Spic on Atf1 was also outwith the scope of the present paper. Spic mediates commitment to splenic RPM, apparently in response to heme. At face value, that would suggest that splenic RPM may fail to develop in the Atf1−/−. However, although the spleens in Atf1−/− were of slightly lower weight, no statistically significant difference was observed in iron content or in histology or macrophage content of HO-1-positive macrophages. Atf1 may therefore reflect a specifically dynamic system that adapts to transient high-level heme, whilst other signalling pathways to Spic mediate the constitutive differentiation of RPM.

Moreover, we restricted examination to one artificial model of hematoma. It may be that multiple diseases and disease models involving hemorrhage are modulated by the Hmox1-regulating function of Atf1. These may include advanced atherosclerotic plaques, intracranial hemorrhage, alveolar haemorrhage, ischemia-reperfusion injury and neurodegeneration. Indeed, in humans, ATF1 is a risk locus for sudden cardiac death in the context of coronary calcification. Unfortunately, there are no current good models for these and we are in the process of developing these with collaborators. However, this model has clinical relevance given the complications of femoral hematoma post-angiography and primary coronary intervention. These include prolonged in-patient stay and indeed mortality, so are not minor problems. Predisposing factors include strength of anticoagulation, comorbidities and age.

This specificity was mirrored in vivo, with deficient erythrocyte clearance in Atf1−/− mice (but not leukocyte clearance) whilst leukocyte clearance was deficient in si-Creb1 knockdown. The specificity mechanism is unknown, but potentially fascinating.

Conclusions.

AMPK and ATF1 are required for hematoma clearance in vivo. Their loss increases iron deposition, inflammation and nitrosative and oxidative stress. ATF1 coregulates genes for hematoma clearance, anti-inflammatory genes and Spic, the transcription factor that drives splenic RPM. Erythrocyte iron and lipid are systematically segregated into distinct macrophage populations dependent on Atf1. ATF1 therefore plays a specific and important role in the normal resolution of tissue hemorrhage, which may be its principal function.

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DISCLOSURES
None.
SUPPLEMENTAL MATERIALS
Expanded Materials & Methods
Online Figures I - VII
-omic dataset (Online Video I)
References 16-19, 22, 23, 46-49, 49-60

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FIGURE LEGENDS

Figure 1. Hematoma model. (A) is a diagram (left) and photograph of a representative dissection (right) of the site of subcutaneous injection of littermate autologous blood (50 μl) into peri-femoral adipose tissue; (B) shows the time-course of the model; and (C) shows histological micrographs taken immediately after injection, showing topography. Arrows point in the direction of skin and muscle; H - injected hematoma; a dashed line marks the edge of the hematoma. Scale bars are 100μm.

Figure 2. Loss of AMPK or ATF1 delays hematoma clearance. The photomicrographs are representative of images of hematomas obtained at day 8 or day 9 from mice with genetic deficiency of (A) AMPK (Prkab1−/−; n=9) or (B) ATF1 (Atf1−/−; n=6) or their littermate WT controls (Prkab1+/+; Atf1+/+; n=9 and 9 respectively). Upper image rows show H&E staining (scale bars 100μm) and lower image rows show immunostaining with anti-spectrin antibodies (ie anti-erythrocyte) labelled with Alexa-488 (Green) as indicated, arrowheads. Nuclei are stained blue with DAPI to give total number of cells as indicated. Hem – hematoma, SkM – skeletal muscle. Scale bars 100μm. H&E (cell number) and immunostaining data are shown in the left and right-hand graphs respectively. The data points represent individual mice. Exact p-values are as indicated for the indicated comparisons selected a priori (Mann-Whitney). Lineage-specificity of the deletion is shown in (C). The Prkab1-KOMP-Ko-first-conditional-possible allele was first crossed with Frt-mice to remove the STOP, yielding Prkab1-/+/+ . These were then crossed again to remove the Frt. The progeny with the crossed with LysMCre and the F1 double-het progeny intercrossed, yielding LysMCre x Prkab1-/+/+ double homozygotes. These were then selected and bred as homozygotes. These Mac-AMPK-KO mice were then studied in the same hematoma model as before. In the model, mice had hematomas as expected at the earlier time-point d8 (not shown, n=4). Left image, floxed controls without the Cre driver did not have hematomas at the later time-point (d9, n=9), indicating that these all cleared hematomas as quickly as the other wild-type controls. In contrast, mice lacking myeloid AMPK (LysMCre x Prkab1-/+/+ ) consistently had hematomas still present at day 9 (10/10 mice, n=10) (right image). This indicates that the lack of hematoma clearance in this model is specific to loss of AMPK in the macrophage lineage. The Scalebars, size indicated (100μm). Epi – epidermis. De – Dermis. SC – subcutis. SkM – Skeletal Muscle. Hem- hematoma. Graph, genotypes as indicated, each point represents one mouse. Exact P-value, Mann-Whitney.

Figure 3. AMPK deficiency prevents ATF1 induction. 
(A) Representative Images of Hematomas obtained at day 8 or day 9 after injection, after immunostaining green for phosphorylated ATF1 (pATF1) as indicated. The upper row of images shows Prkab1+/+ (n=9) versus Prkab1−/− (n=9) mice, and the lower image row shows Atf1+/+ (n=9) versus Atf1−/− (n=6). Scale bars are 100μm. Nuclei are stained blue with DAPI as indicated. (B) Image quantification of the effects of AMPK and ATF1 deficiency are shown in the left and right-hand graphs respectively. Data points are individual mice. Exact p-values are indicated for indicated comparisons selected a priori (Mann-Whitney); (C) shows the failure of Atf1 mRNA induction in Prkab1−/− macrophages in vitro. mBMM from Prkab1−/− or WT littermate controls were stimulated by 10μM heme and cells harvested after varying durations for assay of mRNA transcripts by RT-qPCR. The y-axis is fold induction relative to vehicle, calculated using the −ΔΔCt method. The x-axis is time using a log scale to facilitate plotting of both rapidly changing and slowly-changing expression. Exact p-values given for Repeated Measures One Way ANOVA. Data points are mean ± SE, n=6, values in Supplemental.

Figure 4. AMPK or ATF1 deficiency prevents HO1 expression in hematomas. 
Hematomas obtained at day 8 or day 9 after injection were immunostained green for HO1 as indicated. (A) Representative images. The upper image row shows Prkab1+/+ (n=9) versus Prkab1−/− (n=9) mice, and the lower image row shows Atf1+/+ (n=9) versus Atf1−/− (n=6). Scale bars are 100μm. Nuclei are stained blue with DAPI as indicated. (B) Image quantification of the effects of AMPK and ATF1 deficiency are shown
in the left and right-hand graphs respectively, based on images in (A). Data points are individual mice. Exact p-values are shown for the indicated comparisons selected a priori (Mann-Whitney); (C) shows the impact of AMPK or ATF1 deficiency on transcriptional activation of Hmox1 in mBMM stimulated with heme (10μM). Exact p-values given for Repeated Measures One Way ANOVA. See Figure 3 legend for more methodological detail. Data points are mean ± SE, n=6, values in Supplemental.

**Figure 5.** Heme-regulated gene expression in mBMM and effects of AMPK or ATF1 deficiency. (A) mBMM from Prkab1-/-, Atf1-/- or WT littermate controls were stimulated in vitro by 10μM heme and cells harvested after varying durations for assay of mRNA transcripts by RT-qPCR, as in Figure 3B. Data points are mean ± SE, n=6; Exact p-values given for repeated measures ANOVA. (B) shows a summary of the impact of AMPK and ATF1 deficiency, which had very similar effects.

**Figure 6.** Genomic responses to heme in ATF1 deficient compared to WT mice. Microarrays were used to interrogate gene expression in mBMM stimulated by heme for 8 h, comparing cells from Atf1-/- and litter-mate Atf1+/+ mice. (A) Venn diagram showing numbers of genes regulated by heme and/or ATF1. Over-represented TFBS are shown for each section; (B) Network analysis of mouse genes regulated by both heme and Atf1. The most connected nodes are shown, and point to immune and stress response drivers as key hubs for the Atf1-dependent response to heme; (C) Gene ontology analysis of genes from (A), colored by whether they reflect genesets in the heme-specific (red) Atf1-specific (green) or heme- and Atf1-coregulated (yellow) sections of the Venn diagram in (A).

**Figure 7.** Prolonged inflammatory activation and tissue injury in the absence of AMPK or ATF1. Hematomas were obtained at day 8 or day 9 after injection from Prkab1-/- (left hand graphs, n=9) or Atf1-/- (right hand graphs, n=6), together with Prkab1+/+ (n=9) or Atf1+/+ (n=9) littermate WT controls. Sections were stained (A) with Perl’s iron stain (Prussian blue), with nuclear counterstaining by nuclear fast red; or immunostained (green) for (B) macrophages (CD68); (C) p65 Rela (NFκB), with quantification of % nuclear translocation; (D) iNOS (Nos2), (E) nitrotyrosine and (F) 8-oxo-deoxyguanosine. Data points are individual mice. Exact p-values are as indicated for the indicated comparisons (Mann-Whitney).

**Figure 8.** AMPK and ATF1 are required for divergent differentiation into iron-laden macrophages and lipid-laden macrophages, reflecting trafficking of iron and lipid from erythrocytes respectively into iron-laden macrophages or lipid-laden macrophages. (A) shows the relative sizes of foamy macrophages (lipid-laden, ORO stain) and hemosiderin-laden macrophages (iron-laden, Perl’s stain) in resolving mouse hematoma, from a normal mouse at day 8. The absolute cell areas are shown on the y-axis. Each point represents one mouse (n=9). *Exact p-value, Mann-Whitney; (B) size distribution of macrophages (Cd68+) and effect of knockout status, showing a bimodal distribution, indicating that there are 2 populations, that is small and large macrophages. *, Kolmogorov-Smirnov test, small macrophages are fewer in either Atf1-/- (P=0.00019) or Prkab1-/- (P<0.04). Data obtained by histological image cytometry; (C) representative micrographs, effect of loss of Atf1 on separation of lipid and iron. Dual-stained with Berlin Blue and Oil Red O (red - neutral lipid, blue- iron). Left-hand image, iron and fat are found in separate cells in Atf1-/-; Right-hand, iron and fat are found in the same cell in Atf1-/-. Scalebars, 50 microns. (D) Paraffin sections of hematomas at day 8, stained with Berlin Blue histochemistry combined with immunohistochemistry for Perilipin-1, an antigen induced in foam cells, stable in formalin-fixed-paraffin-embedded tissues. Representative images, scalebars 100 microns. Left image, large perlipin-positive foam cells largely without iron, with small iron-positive cells in distinct zones (not shown). Right image, foam cells are far more strongly positive for iron in Atf1-/- mice indicating increased colocalization of iron and lipid.(E) Representative images of hematomas with metabolite tracking. Erythrocytes were injected containing the indicated label (BODIPY-PC, NBD-Chol, Ru) and then imaged at 8 days, with immunostaining for Cd68 macrophage marker. Right-hand panel, overlay. Fluorescent markers are as indicated, colors approximate true color (green ~530-540nm; red ~540-560nm, blue ~460-480nm). Right hand column, overlay where yellow = green and red, indicating colocalisation.
between respective metabolite and macrophages. Representative n = 5 mice each. Scalebars = 100μm. **(F-H)** Quantification of tracking of fluorescent lipid and metal to lipid-laden and iron-laden macrophage, in the presence and absence of Atf1. Exact p-values are shown for the indicated comparisons (Mann-Whitney) selected a priori. The data passed Shapiro-Wilk but in view of economical mouse numbers and skew on visual inspection, a non-parametric analysis was used. Only two analyses were critical to the hypothesis. **(F)** Labelled cholesterol tracks into lipid-laden macrophages normally (*P=0.008, Mann-Whitney), but into an abnormal lipid/iron dual-positive macrophage population in Atf1−/− (P=0.004, Mann-Whitney). **(G)** Labelled-Phosphatidyl Choline (PC) (P=0.0002, Mann-Whitney), tracked into lipid-laden macrophages normally, but into iron/lipid double-loaded macrophages in Atf1−/−. **(H)** Ru, the fluorescent Fe tracker has the converse pattern, tracking into iron-laden macrophages normally (*P=0.002, Mann-Whitney), but into the abnormal lipid/iron dual-positive macrophages in Atf1−/− (P=0.014, Mann-Whitney).
NOVELTY AND SIGNIFICANCE

What Is Known?

• Hematomas occur during leakage of blood vessels and can contribute to cardiovascular morbidity or mortality in various settings such as stroke.

• Hematomas may also occur at sites of vascular access and be worsened in anticoagulated patients. Hematoma resolution occurs over time as the blood debris is removed.

• Heme is cleared via Heme Oxygenase 1.

What New Information Does This Manuscript Provide?

• We developed a novel and facile model of hematoma resolution.

• Hematoma resolution required the signalling enzyme adenosine monophosphate activated protein kinase (AMPK) and gene activation by Activating Transcription Factor 1 (ATF1).

• This pathway protected against inflammation and oxidative stress, suggesting therapies for resolution in the setting of hemorrhagic stroke.

The enzyme Heme Oxygenase 1 (HO-1) degrades redox-active hemin from haemoglobin into stored iron and protective metabolites. HO-1 plays a key role in vascular homeostasis and its clinical genetic deficiency causes a fatal pediatric disease. However, the regulatory mechanisms for this pathway are only partially understood. In this study, we show that hematoma clearance in vivo, and HO-1 induction by hemin, are mediated by Adenosine Monophosphate Activated Protein Kinase (AMPK) and by Activating Transcription Factor 1 (ATF1). Fluorescent tracking studies indicated that AMPK and ATF1 actively partition iron and lipid into different macrophages. Their loss causes colocalisation of these metabolites, oxidative stress and inflammation. Taken together, our findings provide insights into the mechanism of hematoma resolution and point to potential therapeutic pathways in hemorrhage-related diseases such as types of atherosclerosis and stroke.
Figure 1

A

Femoral artery and vein

Injection

Subcutaneous erythrocyte injection

B

Sedated with inhaled isoflurane

Injected 50μl autologous blood subcutaneously (superficial to right femoral artery)

C

Injected leg

Muscle

Skin

H
Figure 4

A

Day 8

Prkab1<sup>+/+</sup>  Prkab1<sup>-/-</sup>

Atf1<sup>+/+</sup>  Atf1<sup>-/-</sup>

Day 9

Prkab1<sup>+/+</sup>  Prkab1<sup>-/-</sup>

Atf1<sup>+/+</sup>  Atf1<sup>-/-</sup>

B

% Cells that are HO-1 positive

P<sub>0.005</sub>

P<sub>0.001</sub>

C

Hmox1 mRNA Fold

P<sub>0.00034</sub>

P<sub>4x10^-6</sub>
Figure 5

A  

Socs1  

ApoE  

Nr1h2  

Abca1  

Nr1h3  

Igf1  

Spic  

C  

|                  | WT(Prkab1++) v Prkab1−/− | WT(Atf1++) v Atf1−/− |
|------------------|--------------------------|----------------------|
| Iron homeostasis |                           |                      |
| Hm1              |                          |                      |
| Lipid homeostasis|                           |                      |
| Lrα1, Lrβ, ApoE, Abca1 |                     |                      |
| Growth factors   |                           |                      |
| Igf1, Pdgf       |                          |                      |
| Anti-inflammatory |                           |                      |
| Atf1, Socs1      |                          |                      |
