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Hemoglobin Directs Macrophage Differentiation and Prevents Foam Cell Formation in Human Atherosclerotic Plaques

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Abstract

Objectives—To examine selective macrophage differentiation occurring in areas of intraplaque hemorrhage in human atherosclerosis.

Background—Macrophage subsets are recognized in atherosclerosis but the stimulus for and importance of differentiation programs remains unknown.

Methods—We used freshly isolated human monocytes, a rabbit model, and human atherosclerotic plaques to analyze macrophage differentiation in response to hemorrhage.

Results—Macrophages characterized by high expression of both mannose and CD163 receptors preferentially exist in atherosclerotic lesions at sites of intraplaque hemorrhage. These hemoglobin (Hb)-stimulated macrophages, M(Hb), are devoid of neutral lipids typical of foam cells. In vivo modeling of hemorrhage in the rabbit model demonstrated that sponges exposed to red cells showed an increase in mannose receptor positive macrophages only when these cells contained hemoglobin (Hb). Cultured human monocytes exposed to hemoglobin:haptoglobin complexes (Hb:Hp), but not IL-4, expressed the M(Hb) phenotype and were characterized by their resistance to cholesterol loading and upregulation of ABC transporters. M(Hb) demonstrated increased ferroportin (FPN) expression, reduced intracellular iron, and reactive oxygen species (ROS). Degradation of FPN using hepcidin increased ROS, inhibited ABCA1 expression, and cholesterol efflux to ApoAI, suggesting reduced ROS triggers these effects. Knockdown of liver x receptor alpha (LXRα) inhibited ABC transporter expression in M(Hb) and macrophages differentiated in the anti-oxidant superoxide dismutase. Lastly, liver X receptor α (LXR) luciferase reporter activity was increased in M(Hb) and significantly reduced by overnight treatment with hepcidin. Collectively, these data suggest reduced ROS triggers LXRα activation and macrophage reverse cholesterol transport (RCT).

Conclusions—Hb is a stimulus for macrophage differentiation in human atherosclerotic plaques. A reduction of macrophage intracellular iron plays an important role in this non-foam cell phenotype by reducing ROS, which drives transcription of ABC transporters through
activation of LXRα. Reduction of macrophage intracellular iron may be a promising avenue to increase macrophage RCT.

Keywords
ABC transporters; atherosclerosis; inflammation; hemoglobin; macrophages; reactive oxygen species

Introduction
Macrophages are the major inflammatory cells involved in the progression of atherosclerosis. Microenvironment directs these cells into morphologically and functionally distinct phenotypes, which suggests that cellular events within the complex milieu of atherosclerosis influence macrophage differentiation. Some macrophages ingest lipoprotein particles and become foam cells while the character and function of others is less clear. At least two distinct subtypes of macrophages (so called M1 and M2) have been shown to exist within atherosclerotic plaques. While the stimulus for alternative activation of macrophages within human atherosclerosis is thought to be mediated through the action of cytokine interleukin-4 (IL-4), the precise signals that trigger macrophage differentiation remain incompletely understood.

Intraplaque hemorrhage is regarded as a potentially important inflammatory stimulus capable of promoting the influx of macrophages into atherosclerotic lesions. The pro-oxidant environment within the plaque helps to promote erythrocyte lysis where free hemoglobin (Hb) is bound by the plasma protein haptoglobin (Hp), forming Hb:Hp complexes. The CD163 receptor, exclusively expressed in monocyte/macrophages, binds to Hb:Hp mediating its endocytosis, and the heme subunit of Hb is degraded by the heme oxygenase enzymes. Prior data suggest a critical role for binding of Hb:Hp to CD163 in directing the anti-inflammatory responses of macrophages including upregulation of IL-10. Boyle et al. recently described that Hb ingestion by monocytes drives a novel macrophage phenotype referred to as “HA-mac”, however, the underlying mechanisms supporting this finding and its functional significance remains unknown.

In the present study, we examined selective macrophage differentiation occurring in areas of intraplaque hemorrhage in human atherosclerosis relative to macrophage-derived foam cells, which contribute to necrotic core formation.

Methods
Human Tissue Specimens
Human plaques were selected from the CVPath Institute Sudden Coronary Death registry (CVPI SCDr). All plaques were identified according to a modified AHA classification. (See supplemental methods for details).

Cell Culture, Flow Cytometry, and Quantitative PCR
Human monocytes collected from healthy volunteers (Astarte Biologics) were differentiated over 7-days into macrophages in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% human serum (Invitrogen). (See supplemental methods for details).

Statistical Analysis
The data are expressed as means ± SD. A p value <0.05 was considered significant. (See supplemental methods for more detail).
Results

M2 Macrophage Markers Are Present in Human Atherosclerotic Plaques at Regions of Prior Hemorrhage/Angiogenesis/Iron and Are Characterized by Expression of MR (CD206) and CD163

To determine the role of hemorrhage in macrophage differentiation, we stained coronary plaques (frozen sections) from individuals who had died suddenly and were referred to our institution. We compared staining intensity in regions of prior hemorrhage/angiogenesis/iron (identified by glycophorin A/CD31/iron deposition) to peri-necrotic core areas devoid of these markers (Ctrl). As expected hemorrhagic regions demonstrated significantly greater staining for glycophorin A, CD31, and ferric iron (as identified by Perl Prussian blue stain) as compared to Ctrl (Figure 1a). Although CD68 density was not different, markers of M2 macrophages, mannose receptor and CD163, were significantly more prevalent in areas of prior hemorrhage/angiogenesis/iron (Fig. 1–2). In Ctrl regions devoid of hemorrhage/angiogenesis/iron, a minority of macrophages stained for these markers. Macrophages in areas of hemorrhage/angiogenesis/iron also demonstrated minimal staining for the proinflammatory M1 markers TNFα (Fig. 1h,p,s, Fig. 2i) and iNOS (supplementary Fig. 1), which were heavily expressed by foam cells.

By immunostaining (data not shown) and quantitative pcr (QPCR) of RNA extracted from the intima of atherosclerotic plaques from cross sectional regions poor (i.e. control) and rich in hemorrhage/angiogenesis/iron (i.e. high mannose receptor areas), heme oxygenase-1 (HO-1) was significantly upregulated (QPCR: 1.34±0.6 versus 3.7±2.3, p=0.03). (As compared to RNA collected from control, areas of hemorrhage demonstrated increased staining for mannose receptor/CD163 by immunohistochemistry (supplementary Table 1).

For clarity, macrophages expressing high mannose receptor/CD163 will be referred to as M(Hb) to distinguish them from the previously described IL-4 induced M2 phenotype.

CD163/Mannose Receptor Positive Macrophages (M(Hb)) Are Distinct From Foam Cells

M(Hb) exhibit a tissue profile distinct from macrophage foam cells (which do not stain for either mannose receptor or CD163). Macrophage foam cells stained positively for oil red O (ORO). Surprisingly, M(Hb) demonstrated very little ORO positivity (Figure 1 d,l,s, and Fig. 2g). The scavenger receptor CD36, responsible for uptake of oxidized LDL (oxLDL), was also minimally expressed in these compared to foam cells (Ctrl) (Fig. 1 f,n,s, Fig. 2h).

Rabbit Model of Simulated Hemorrhage Results in Increased Expression of IL-10 and MR Upregulation in Macrophages

In order to test if hemorrhage promotes monocyte differentiation into mannose receptor expressing cells, we used an in vivo model of foam cell formation, harvesting macrophages from subcutaneous sponges exposed to saline versus autologous erythrocytes. Sponges implanted for 28 days yielded between 1–2 million macrophages which were >98% positive for the macrophage marker RAM11 and focally Oil Red O (ORO) positive (Fig. 3a).

24 hour supernatants from macrophage harvested from sponges exposed to autologous erythrocytes resulted in significantly greater levels of IL-10 by ELISA as compared to control sponges (Fig. 3b). There was also a significant increase in mean fluorescence intensity (MFI) for mannose receptor staining as determined by flow cytometry compared to controls (Fig. 3c). To determine which component of the erythrocyte is critical in stimulating mannose receptor upregulation, sponges were exposed to erythrocyte ghosts devoid of Hb and compared to control. Macrophages from sponges exposed to erythrocytes ghosts demonstrated similar IL-10 levels as well as mannose receptor expression compared...
to controls (Fig 3d–e). These data implicate exposure to Hb as causal in increased macrophage expression of IL-10 and mannose receptor in vivo. To explore whether foam cells could be converted to mannose receptor positive macrophages consistent with recent reports about macrophage plasticity, rabbit foam cells were exposed to Hb:Hp complexes (1mg/ml, a dose previously shown to increase IL-10 expression) for 1 day. This did not result in any appreciable change in the amount of secreted IL-10 (data not shown) or in mannose receptor MFI by flow cytometry (Fig 3f). Lastly, rabbit peripheral blood monocytes were collected using anti-CD14 antibody and fluorescent activated cell sorting (FACS). Cells were plated and exposed to Hb or Hb:Hp complexes in vitro for 7 days. Exposure of monocytes to Hb alone (1mg/ml) did not affect IL-10 levels (Fig 3g) but resulted in an increase in MFI for mannose receptor (Fig 3h) whereas Hb:Hp exposure resulted in a significant increase in both IL-10 (Fig 3i) and an increase in MFI for the mannose receptor (Fig 3j) by flow cytometry. Collectively, these results indicate that Hb:Hp exposure primes monocytes to increase their expression of IL-10 and mannose receptor but that exposure of macrophage foam cells to Hb:Hp does not.

**Human Monocytes Exposed to Hb:Hp Complexes Express Markers Consistent with M2 Macrophages but Are Distinct From IL-4 Induced M2 Macrophages**

To test whether Hb induces macrophage differentiation as in human atherosclerotic plaques, human monocytes were exposed to Hb:Hp complexes (1 mg/ml) for 7 days. [A dose of 1 mg/ml was established by conducting a dose response curve for IL-10 (supplementary Fig. 2). This corresponds to a concentration of 6.7 μM which is well below the >1M concentration of Hb found in human erythrocytes]. A comparator set of cells were differentiated in the presence of human IL-4 (15 ng/ml) given 4 hours after plating or LPS (100 ng/ml) overnight on day 6 to induce M2 and M1 phenotypes, respectively, while control cells were kept in normal serum. Cells were analyzed by FACS using anti-mannose and anti-CD163 receptor antibodies. As indicated by a change in MFI, LPS treatment reduced expression of MR compared to control whereas IL-4 robustly increased MR and to a much lesser extent CD163 expression, consistent with previous reports (Fig 4. a,b). Hb:Hp robustly increased expression of both receptors (Fig 4. a,b). Exposure to Hb alone increased expression of both mannose and CD163 receptors but not to the same extent as Hb:Hp (n=3/group (data not shown)). This is consistent with known low affinity binding of Hb to CD163. Dual immunostaining of in vitro Hb:Hp differentiated human monocytes demonstrated a distinct population of cells, which expressed increased levels of both receptors (Fig. 4c) in a select population of macrophages by flow cytometry. This was in contrast to IL-4 which upregulated mannose receptor expression in a distinct population of cells.

To confirm the relevance of our findings, we performed dual immunofluorescent staining in seven atherosclerotic plaques with evidence of hemorrhage. As shown in the representative confocal images in figure 4d, areas of hemorrhage demonstrated a population of CD163 and CD206 (MR) positive macrophages, which co-localized with each other. To further verify if our findings had applicability to human plaques, RNA was extracted from the intima of fibroatheromas with hemorrhage/angiogenesis/iron as described above. Expression of both mannose receptor and CD163 transcripts by quantitative PCR (QPCR) was significantly higher in sections staining for mannose receptor compared to those with low mannose receptor staining (i.e. Ctrl) (Fig. 4e). A significant correlation between mannose receptor and CD163 transcripts was found by multiple linear regression (Fig. 4f). Collectively, these results suggest that differentiating monocytes in the presence of Hb:Hp can reproduce the M(Hb) cell phenotype found in areas of plaque with prior hemorrhage/angiogenesis/iron.

Next, we compared cytokine release from Hb:Hp differentiated macrophages versus those treated with IL-4 or LPS using markers of alternative and classical activation. Culture media
from Hb:Hp differentiated macrophages expressed high levels of both IL-10 and IL-1 receptor antagonist (IL-1Ra) similar to macrophages differentiated in IL-4 (Fig. 5a). Hb:Hp differentiated macrophages expressed significantly higher levels of MCP-1 versus control and IL-4 differentiated macrophages but significantly less than LPS treated macrophages. Similar expression of the same transcripts was seen in RNA generated from human plaques (supplementary table 1 and Fig. 3a).

Differentiation of Monocytes in Hb:Hp Prevents Foam Cell Formation Through Downregulation of Scavenger Receptors and Upregulation of ABC Transporters

To examine the effects of Hb:Hp exposure on macrophage lipid uptake, human monocytes were differentiated as previously described. 7 day control, IL-4 differentiated and M(Hb) macrophages were loaded with oxidized LDL (oxLDL, 30ug/ml) for 48 hours. M(Hb) loaded with oxLDL demonstrated minimal ORO positive cells as compared to both IL-4 and control cells (Fig. 5b–c). A similar experiment using both control and Hb:Hp differentiated macrophages was conducted and cholesterol accumulation quantitated by enzymatic determination. M(Hb) demonstrated significantly lower levels of total and free cholesterol versus control cells and were significant more resistant to oxLDL loading (Fig. 5d). Similar results were obtained using acLDL (50ug/ml) (data not shown). Although fold increases in total and free cholesterol were greater in M(Hb) than in control cells, this was not the case for esterified cholesterol in which fold increases were lower (i.e. ≥50%) as compared to control cells. These data indicate that while some uptake mechanisms still seem to be active in M(Hb), those involving production of esterified cholesterol, are not as active in M(Hb).

We examined transcription by QPCR of scavenger receptors (SR) responsible for the uptake of oxLDL in Hb:Hp differentiated versus control macrophages. M(Hb) demonstrated downregulation of both class A (I and II) and class B SRs as compared to control (Table 1). Macrophage cholesterol efflux protects cells from free cholesterol and oxysterol induced toxicity and is mediated via the ABC transporters ABCA1 and ABCG1. Both were significantly upregulated in M(Hb) compared to control (Table 1). Liver X receptor-α (LXRα) is involved in cholesterol homeostasis through regulation ABC transporters. LXRα was unchanged compared to control (Table 1).

To confirm our observation that monocytes differentiated in the presence of Hb:Hp upregulate ABC transporters, we examined the correlation between mannose and CD163 receptors and ABCA1 and ABCG1 transcripts by QPCR in human plaques. There was a highly significant correlation between MR/CD163 and both ABC transporter transcripts (Supplementary Fig. 4a–b). Given other cell types also present in the intima are capable of expressing ABC transporters, an approach using laser capture microdissection (LCM) was performed to isolate macrophage rich areas. In 6 separate human atherosclerotic plaques with evidence of hemorrhage, LCM confirmed upregulation of ABC transporters in areas rich in CD163/CD206 positive macrophages (CD68) versus macrophage rich areas containing minimal expression of these receptors (Supplementary Fig. 4c). Further confirmation of changes in ABCA1 (as well as CD36) expression between M(Hb) and control areas was performed in 5 atherosclerotic plaques with evidence of hemorrhage using immunohistochemistry (supplementary Fig. 4d).

Intracellular Iron Affects ABC Transporter Expression and Cholesterol Efflux in M(Hb)

Since Hb:Hp treatment increases macrophage gene expression of ABCA1 and ABCG1, suggesting a potential consequence on cholesterol efflux, functional experiments were performed to determine the influence of Hp:Hp on cholesterol efflux to ApoAI. We incubated human monocyte/macrophages differentiated in Hb:Hp or control media with oxLDL (30ug/ml) for 48 hours to induce cholesterol ester accumulation. We subsequently
exposed them to ApoAI (100ug/ml) for 24 hours to induce cholesterol efflux and then determined cholesterol levels by enzymatic assay. ApoAI treatment significantly induced cholesterol efflux in M(Hb) as indicated by significantly decreased levels of total, free, and esterified cholesterol levels as compared to control cells (Fig. 6a).

Since iron loading may produce oxidant stress and subsequent modulation of gene expression programs\(^{15}\), we examined intracellular iron levels in M(Hb). Using calcein fluorescence, an assay based upon quenching calcein fluorescence by free iron\(^{16}\), we found higher levels of calcein fluorescence in M(Hb) compared to control, consistent with lower intracellular iron levels (Fig 6b). Ferroportin (FPN), the only known iron exporter in human cells, was increased in M(Hb) compared to controls as examined by western blotting (Fig. 6c). These results are consistent with previous data, which suggest that Hb loading of macrophages increases expression of FPN facilitating cellular export of iron and thus lowering intracellular iron\(^{17}\).

To examine whether low intracellular iron is causal in the changes in ABCA1 gene expression, we treated M(Hb) overnight (day 6) with hepcidin, a secreted hepatocyte peptide that controls iron homeostasis by negatively regulating expression of FPN and thus increasing intracellular iron\(^{18}\). (The efficiency of hepcidin in increasing intracellular iron and degrading FPN was confirmed in M(Hb) by calcein fluorescence and western blot, respectively (supplementary Fig. 5a–b.) Overnight treatment of M(Hb) with hepcidin (700nM) significantly decreased expression of ABCA1 by QPCR (Fig. 6d) but had no effect on its expression by control macrophages. Next, functional experiments were performed to determine the influence of increasing intracellular iron (using hepcidin) on cholesterol efflux to ApoAI in M(Hb). M(Hb) cells were treated overnight with hepcidin after 48 hours of cholesterol loading and subsequently exposed to ApoAI for to induce cholesterol efflux. Consistent with our earlier results, M(Hb) demonstrated decreases in total, free, and esterified cholesterol when exposed to ApoAI. These effects were completely abrogated by hepcidin treatment (Fig 6e). These data suggest a critical role for intracellular iron in modulating ABC transporter expression and cholesterol efflux activity in M(Hb).

**Reduction of ROS Mediates LXR\(\alpha\) Activation of ABC Transporters in M(Hb)**

Given the important role of iron in generating ROS, we next examined H\(_2\)O\(_2\) production in control and M(Hb) cells with and without overnight hepcidin treatment. H\(_2\)O\(_2\) as measured by 24 hour amplex red assay was significantly reduced in M(Hb) as compared to control cells consistent with previous reports about the anti-oxidant response of these cells to Hb:Hp loading\(^{7}\) (Fig. 7a). Overnight hepcidin treatment of M(Hb) cells resulted in a significant increase in H\(_2\)O\(_2\) production (similar to the level seen in control cells) (Fig 7a). These data demonstrate that iron efflux plays an important role in ROS modulation in M(Hb).

To characterize reactive oxygen species generation, especially the Fenton reaction product OH\(^{-}\) in M(Hb) in vivo, we used the redox sensitive dye dihydrodihoradamine123 (DHR) on cryosections of freshly isolated human carotid atherosclerotic lesions that contained M(Hb) as well as foam cells. M(Hb) areas demonstrated dull green fluorescence which was not nearly as intense as seen in foam cells, suggesting relatively reduced reactive oxygen species generation (supplementary Fig. 6).

H\(_2\)O\(_2\) was the primary responsible for the signal in M(Hb) as co-incubation of the sections with the H\(_2\)O\(_2\) scavenger catalase (Cat) quenched most of the fluorescence activity. Co-incubation with DMSO, the scavenger for OH\(^{-}\), only very mildly attenuated the signal (supplementary Fig. 6). Identical results were found in all carotid plaques studied. Collectively, these results suggest that in M(Hb) lower levels of free intracellular (Fe\(^{2+}\)) iron results in less production of the highly toxic Fenton product OH\(^{-}\).
OH\(^{\circ}\) and other reactive oxygen species such as peroxynitrite (ONOO\(^{\circ}\)) can severely damage DNA and also lead to protein nitration. We assessed oxidant stress induced DNA damage in areas of plaques with and without the M(Hb) phenotype using an antibody specific for oxygen-adducted guanosine (8-oxo-G). As seen in supplementary figure 7, while peri-core foam cells stained heavily for 8-oxo-G, M(Hb) demonstrated minimal staining indicating reduced oxidant stress induced DNA damage. Similarly, an antibody against 3-nitro-tyrosine (3-NT), a selective marker for protein nitration due to ONOO\(^{\circ}\), demonstrated minimal staining in M(Hb) areas (supplementary Fig. 1).

To further investigate whether a reduction of oxidant stress plays an important role in upregulation of ABC transporters, we differentiated human monocytes in the anti-oxidant superoxide dismutase (SOD) (75U/ml) for 7 days. RNA from control and SOD treated macrophages was analyzed by QPCR for both ABCA1 and ABCG1. As seen in Fig. 7b, SOD treatment significantly increased transcription of both genes. Next, in order to demonstrate the mechanism by which Hb:Hp treatment increases ABCA1/ABCG1 transcription, genetic knockdown of LXR\(\alpha\), an inducible transcription factor known to be important in human macrophage ABC transporter transcription\(^{14}\), was conducted. The upregulation of ABCA1 and ABCG1 transcripts as detected by QPCR in 7 day Hb:Hp differentiated macrophages was significantly inhibited by treatment with LXR\(\alpha\) siRNA but had no effect on ABCA1/ABCG1 expression in control treated cells (Fig. 7c). These data indicate a critical role of LXR\(\alpha\) in mediating induction of ABC transporter expression in M(Hb).

Similar experiments were conducted in SOD treated monocyte/macrophages to examine whether LXR\(\alpha\) was also required for upregulation of ABC transporters in these cells. The upregulation of ABCA1 and ABCG1 transcripts as detected by QPCR in 7 day SOD differentiated macrophages was significantly inhibited by treatment with LXR\(\alpha\) siRNA. These data suggest that in SOD treated macrophages LXR\(\alpha\) is required for upregulation of ABC transporter expression.

Next, we examined LXR\(\alpha\) transcriptional activity in control (Ctrl), SOD and Hb:Hp treated monocyte/macrophages by transfecting differentiating cells with a lentivirus encoding an inducible LXR\(\alpha\)-responsive firefly luciferase reporter. Luciferase activity at 96 hours after transfection was significantly higher in both Hb:Hp and SOD treated monocyte/macrophages as compared to control (Fig. 7e). In order to demonstrate a critical role for intracellular iron in induction of LXR\(\alpha\) activity, we treated Hb:Hp differentiating cells transfected with the LXR\(\alpha\) reporter as described above. Cells were then treated with hepcidin (700nM) 18 hours prior to harvest to prevent iron efflux and thus increase reactive oxygen species. This resulted in significant reduction in luciferase activity as compared to M(Hb) cells not treated with hepcidin (Fig. 7e). Collectively, these data indicate an important role for intracellular free iron and reactive oxygen species in activation of LXR\(\alpha\) mediated transcription of ABC transporters in human macrophages.

**Discussion**

Recently, it has been recognized that different populations of macrophages reside within human plaques and that microenvironment plays a pivotal role in their differentiation programs\(^1\). Our work significantly expands and integrates previous observations to demonstrate that M2 macrophages first described by Bouhlel et al. in carotid atherosclerotic plaques are identified in areas of hemorrhage/angiogenesis/iron, are distinct from IL-4 induced M2, and result from Hb ingestion by monocytes (Fig. 8). These cells were first reported by Boyle\(^7\) in areas of hemorrhage but are now further characterized by us as having high surface expression of M2 markers mannose receptor and CD163. We choose to use the
term M(Hb) to refer to this subset instead of the previously used terms M2 or “HA-Mac” since this subset is induced by Hb not IL-4 or hemorrhage (i.e., not platelet or other white cell derived), respectively.

Importantly, our data also expand upon previous observations to demonstrate conclusively the non-foamy nature of these cells and reveal the mechanisms by which modulation of intracellular iron and ROS regulate cholesterol efflux proteins. Consistent with data from human plaques, M(Hb) were resistant to foam cell formation through downregulation of scavenger receptors and upregulation of ABC transporters by in vitro assays. Hb loading of macrophages increased ABC transporter expression and cholesterol efflux to ApoAI by reducing intracellular iron and reactive oxygen species generation through upregulation of FPN. These effects were reversed by increasing intracellular iron levels with hepcidin and emphasize the importance of iron homeostasis in directing cholesterol handling by macrophages.

Bouhlel et al. were the first to report that cells within atherosclerotic carotid plaques demonstrate expression of M2 markers. Although IL-4 has been classically described as the prototypical stimulus for such conversion, it remains controversial whether such a paradigm exists in human atherosclerosis. Recently, Chinetti-Gbaguidi et al. described the presence of CD68+MR+ macrophages in human plaques that contained smaller lipid droplets. Although this is consistent with our findings, the authors attribute the existence of these cells to IL-4 induced differentiation. In contrast to our findings, they report decreased levels of ABCA1 by QPCR and immunostaining in human plaques compared to 68+MR- cells, suggesting lower cholesterol efflux capacity. It should first be noted that this data contradicts the findings of others who have reported increased ABCA1 expression in IL-4 differentiated M2 mouse macrophages, although it remains unclear to what extent these data can be applied to the behavior of human macrophages. Although it is beyond the scope of this manuscript to discuss every discrepancy that exists with our findings, one major weakness that deserves attention is lack of clear identification for the source of IL-4 responsible for M2 differentiation in human plaques. Previous work has found very little expression of IL-4 in human plaques. Here we demonstrate conclusively that the location at which CD68+MR+ macrophages are found is in areas of prior hemorrhage. Moreover, we were able to reproduce using Hb:Hp complexes both in an animal model and in vitro not only the surface markers (MR and CD163) that characterize this macrophage subtype but also the profile of cytokine release as well as the mechanism by which they resist lipid deposition. Human macrophages expressing these surface markers could only be generated by exposure of human monocytes to Hb:Hp and not by IL-4. While lipid loading as assessed by ORO staining was attenuated by IL-4 differentiation, the effect of Hb:Hp was more striking in regards to this effect. Our data are further supported by the observations of Boyle et al. who also reported that macrophages in areas of hemorrhage are characterized by surface expression of CD163 and display a non-foam cell phenotype. Collectively, these data emphasize the importance of Hb in macrophage differentiation programs within atherosclerotic plaques.

Although it has long been suspected that iron depletion might play a favorable role in risk for cardiovascular disease, whether such an effect really exists and the mechanisms underlying it remain uncertain. While our data cannot answer this question completely, they do support a role for how alterations in macrophage iron levels are important in controlling cholesterol efflux proteins. We demonstrate that lowering of intracellular iron within M(Hb) is causal in increasing both ABCA1 and ABCG1 expression through its effect on lowering ROS. Knockdown of LXRα in M(Hb) significantly reduced the effects of Hb:Hp on upregulation of ABCA1 and ABCG1, suggesting these effects are mediated in part by LXRα transcriptional activation of these genes. These findings were further confirmed by

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luciferase reporter assays. The exact mechanism by which lowering ROS activates LXRα transcription deserves further attention and is beyond the scope of the present study.

We would be remiss if we did not discuss briefly the limitations of this study. Autopsy examination involves certain selection bias in that these findings may not be representative of humans with intraplaque hemorrhage who survive this event. It is possible that the selection of plaques types may have biased our findings but this is unlikely because we found a similar phenotype of macrophages in all atheromas with evidence of prior hemorrhage/angiogenesis/iron.

Collectively, these data may help us understand better macrophage diversity within human atherosclerosis. Moreover, our findings suggest that therapies aimed at altering iron homeostasis within macrophages may represent a new avenue to prevent foam cell formation and atherosclerotic lesion progression.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**Abbreviations**

- **M(Hb)**: hemoglobin stimulated macrophages
- **Hb**: hemoglobin
- **Hp**: haptoglobin
- **Hb:Hp**: hemoglobin:haptoglobin complex
- **LXR**: Liver X receptor
- **MR**: mannose receptor
- **MFI**: mean fluorescence intensity
- **ORO**: oil red o stain
- **QPCR**: quantitative pcr
- **SR**: scavenger receptors

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Figure 1. M2 Macrophages Markers CD206 and CD163 Are Present in Human Coronary Atherosclerotic Lesions At Sites of Hemorrhage/Angiogenesis/Iron and Are Distinct From Foam Cells
(a) Plaque regions were identified by the presence of angiogenesis/hemorrhage/iron and compared to control (Ctrl) peri-core regions of macrophages devoid of angiogenesis/hemorrhage/iron. (b) Representative frozen section of human coronary fibroatheroma from a 48 year old male who died suddenly. High power image from control area (Ctrl, red box) shows foamy macrophages in the peri-necrotic core (NC) region. The blue box shows an area rich in angiogenesis and iron. (low power image = Movat stain, high power images = H&E stain). Photomicrographs of the boxed areas from control (Ctrl, red boxes) (c–j) and
area of hemorrhage/angiogenesis/iron (Angio/Hemo/Iron, blue boxes)(k–r). Note the control area shows lack of CD31 staining (brown) (c), abundant oil Red O (ORO) positivity (red) (d), macrophage infiltration (CD68, brown) (e), CD36 staining (brown) (f), and no iron staining (blue, Perl Prussian blue) (g). It also demonstrates abundant TNF-α positivity (brown) (h) but there is minimal mannose receptor (CD206, brown) (i) and CD163 (brown) (j) immunostaining. The bottom panels (k–r) are from an area of hemorrhage/angiogenesis/iron showing abundant CD31 staining (k, black arrows point to angiogenesis), rare positive cell for Oil red O (l), but abundant C68 staining (m). This area also demonstrates minimal CD36 staining (n), but abundance of iron (o), minimal TNF-α staining (p), but positive staining for CD206 and CD163 (q–r). Quantitative analysis (s) from Ctrl and hemorrhage/angiogenesis/iron areas from 14 plaques (see supplementary methods for more detail) demonstrated equivalent macrophage area density (CD68) but higher expression of CD163 and CD206 in regions of hemorrhage/angiogenesis/iron than in control regions. Scale bars: low power: 2mm, high power: 200 micron. (Non-normal distribution CD68 and CD163).
Figure 2. Identification of M(Hb) Macrophages in an Area of Old Hemorrhage with Iron Deposition in a Human Coronary Artery Exhibiting a Fibroatheroma with Calcification

A 40-year old male was found unresponsive at work. At autopsy he had severe narrowing of the right coronary artery and moderate narrowing of the left circumflex artery (LCX). (a) Cryosection shows a fibroatheroma (LCX) with a heavily calcified necrotic core (NC, arrows). Movat pentachrome staining. (b–i) represent the area within the black box in “a”. (b) Accumulation of inflammatory cells in an area of prior hemorrhage adjacent to the NC, H&E. (c) Demonstrates the accumulation of iron (Fe) in a dense cluster of cells near the periphery of the necrotic core. (d) Immunohistochemical identification of macrophages by CD68 shows strong reaction product within the cell cluster and adjacent necrotic core with select cells remaining null. (e) Intense staining for the mannose receptor (MR,CD206) is seen within the cell cluster; note however, the adjacent necrotic core shows negative staining. (f) The same MR positive macrophages within the cluster are also strongly positive for CD163, while the necrotic core remains essentially negative. (g) Shows that the same cell cluster of cells are essentially negative for lipid (ORO) while the adjacent necrotic core and connective tissue matrix right is strongly positive. The area of CD206/CD163 positive macrophages does not stain for CD36(h) or TNFα (i) while adjacent peri-core foam cells stain heavily for these markers, respectively. Scale bars a.: 2mm. Scale bars b.-i: 200μm.
Figure 3. Rabbit Model of Simulated Hemorrhage Results in Increased Expression of IL-10 and MR in Macrophages, Which Can Be Reproduced In Vitro by Exposure of Monocytes to Hb:Hp Complexes

(a) Representative photomicrographs from sections from 28-day erythrocyte soaked rabbit sponge demonstrating dense iron staining (Fe) within macrophages (RAM11 stain) (left hand panels). After centrifugation and plating, cells harvested from sponges demonstrated staining for the macrophage marker RAM11 (green, nuclei blue) and heterogeneous mixture of ORO positive and negative (arrow) foam cells (right hand panels). (b) IL-10 levels in supernatant harvested from control and red blood cell (RBC) exposed macrophages for 24 hours. (c) Representative flow cytometry histograms (left) of MR surface expression with quantitation statistics (right) with data expressed as mean fluorescence intensity (MFI) ratio (MFI of FITC conjugated anti-MR Ab for macrophages harvested from RBC exposed sponges/MFI of macrophages from Ctrl sponges). Background MFI (Isotype control Ab) was subtracted from both. (d) IL-10 levels and representative histogram/quantitation of MR surface expression (e) as measured by flow cytometry in control and RBC ghost exposed macrophages. (f) Representative histogram/quantitation of MR surface expression by flow cytometry in foam cells (harvested from control sponges) exposed in vitro to Hb:Hp (1mg/ml) for 24 hours. (g) IL-10 levels in media and histogram/quantitation of MR expression (h) by flow cytometry from rabbit peripheral blood monocytes exposed in vitro to Hb (1mg/ml) for 7 days. (i) Representative histogram/quantitation of MR expression by flow cytometry and IL-10 levels in supernatant (j) from rabbit peripheral blood monocytes exposed in vitro to Hb:Hp (1 mg/ml) for 7 days. For all experiments, four separate experiments were conducted.
Figure 4. Human Monocytes Exposed to Hb:Hp Complexes Express Markers Consistent with M2 Macrophages but Are Distinct From IL-4 Induced M2 Macrophages

Representative histograms of mannose receptor (MR) (a) and CD163 (b) surface expression and quantitation (MR/CD163- MFI ratio) as measured by flow cytometry in human monocytes exposed to Hb:Hp (1mg/ml), IL-4 (15ng/ml) or LPS (100ng/ml) or media (Ctrl). (*p<0.05 versus Ctrl, **p<0.05 versus IL-4, ***p<0.05 versus LPS, ****p<0.05 versus Hb:Hp. (c) Representative flow cytometry dot plots with quadrant statistics of dual immunostaining for MR and CD163 in human monocytes exposed to Ctrl, LPS, IL-4, or Hb:Hp. (d) Confocal analysis to define CD163/CD206 positive macrophages in areas of plaque hemorrhage. Left, lower power light (2x) representative micrograph (Movat pentachrome staining) of human coronary fibroatheroma with evidence of hemorrhage (boxed area). Higher power confocal images (40x) of boxed area showing positive staining for CD163 (green-FITC) and CD206 (red-Alexa Fluor 647) with merge image (yellow) on far right. e) QPCR from RNA extracted from 13 human atherosclerotic coronary arteries for CD163 and MR transcripts with heavy (n=7, i.e. high mannose receptor) versus light (i.e. control) (n=6) MR staining by immunohistochemistry. *p<0.05 versus Ctrl. (f) Pearson correlation coefficients (r) and multiple regression analysis were calculated from QPCR ΔCt data for MR (x axis) and CD163 (y axis). Confidence intervals of 90% are indicated by the dashed arcs. p=0.02.
Figure 5. Cytokine Expression and Foam Cell Formation in Differentiated Macrophages in vitro
(a) TNF-α, MCP-1, IL-1 Ra, and IL-10 were quantitated in supernatants from human monocytes differentiated in the presence of control, Hb:Hp, IL-4, and LPS for 7 days. (N=4 separate experiments for each set of data). *p<0.05 vs. ctrl, **p<0.05 vs. IL-4, ***p<0.05 vs. LPS, ****p<0.05 vs. Hb:Hp. (b) Representative (40x) ORO staining of monocytes differentiated in the presence of normal media (control), IL-4 (15ng/ml), or Hb:Hp. After 7 days, one set of cells was exposed to oxLDL (30ug/ml) for 48 hours and ORO staining conducted. (c) Quantitation of ORO staining in (b) expressed as the number of ORO positive cells per 10x field (average of 4 different areas, n=4 experiments per group). *p<0.05 versus all. (d) 7 day old control and Hb:Hp differentiated macrophages were loaded with oxLDL (30ug/ml) for 48 hours and total, free, and esterified cholesterol quantitated. (Results of 3 wells/group shown. Similar results were obtained from 2 separate experiments). *p<0.05 vs. all, #p<0.05 versus Control oxLDL and Hp:Hp oxLDL, !p<0.05 versus Control oxLDL and Hb:Hp.
Figure 6. Intracellular Iron Affects ABC Transporter Expression and Cholesterol Efflux in M(Hb)
(a) ApoAI-mediated cholesterol efflux in M(Hb) versus control macrophages. Cholesterol loaded control or Hb:Hp differentiated macrophages were incubated with or without apoAI (100 µg/ml) for 24 hours and intracellular lipids determined. Results are expressed as the percent change of intracellular cholesterol amounts in the presence of apoAI relative to apoAI-free medium. *p<0.05 vs. corresponding control. (b) calcein fluorescence histograms and fluorescence quantitation of macrophages differentiated in control media or Hb:Hp. Black line represents fluorescence in the absence of calcein. Control macrophages were treated one hour prior to calcein staining with desferroxamine (DFO, 50µM) as a positive control. *p<0.05 vs. control, **p<0.05 versus DFO/Hb:Hp. (c) Representative example of western blotting for ferroportin in control versus Hb:Hp differentiated macrophages with quantitative densitometry (n=3 experiments) shown on right. *p<0.05 versus control. (d) QPCR for ABCA1 in 7 day control or Hb:Hp differentiated human monocytes. Additional sets of cells were treated overnight (day 6) with hepcidin (700nM) or vehicle (n=4 per group), *p<0.05 vs. all, **p<0.05 versus Hp:Hp and Hp:Hb hepcidin. (e) ApoAI-mediated cholesterol efflux in M(Hb) versus M(Hb) after overnight hepcidin treatment. Results are the mean of 4 experiments. *p<0.05 vs. control.
Figure 7. LXRα Transcriptional Activation Is Required for ABC Transporter Expression in Hb:Hp and SOD Differentiated Macrophages and Triggered by a Reduction in ROS

(a) 24 hour H$_2$O$_2$ production as measured by Amplex Red/peroxidase assay in control, M(Hb), and after overnight treatment of control and M(Hb) with hepcidin (700nM) (n= 4 per group). *p<0.05 versus all others, **p<0.05 versus Hb:Hp. (b) ABC transporter expression by QPCR in human monocytes differentiated for 7 days in control media versus media supplemented with SOD (75U/ml). (c) ABCA1 and ABCG1 expression by QPCR in 7 day differentiated control and M(Hb) macrophages 24 hours after transfection with scramble (Scr) or LXRα siRNA. *p<0.05 versus Control (Scr), **p<0.05 Hb:Hp (Scr), ***p<0.05 versus Hb:Hp (LXRα)/Control (LXRα). (n=4 experiments per group). (d) ABCA1 and ABCG1 expression by QPCR in 7 day differentiated control and SOD (75u/ml) macrophages 24 hours after transfection with scramble (Scr) or LXRα siRNA. (*p<0.05 versus all other groups, **p<0.05 versus SOD Scr and Ctrl LXRα siRNAs, respectively. N=4 per group.) (e) Monocytes differentiated in Ctrl, Hb:Hp, or SOD were transfected with a lentivirus encoding an LXRα responsive firefly luciferase reporter. After 96 hours cell were harvested and luciferase activity measured. Hb:Hp differentiated macrophages were treated with hepcidin (700nM) 18 hours before harvest. N=4 per group. *p<0.05 versus Ctrl, Hepcidin, and Hb:Hp Hepcidin.
Figure 8. Macrophage Diversity in Human Atherosclerosis

During intraplaque hemorrhage, exposure of monocytes to Hb:Hp complexes directs human monocytes into a M(Hb) differentiation program characterized by upregulation of CD163 and mannose receptors, increased expression of anti-inflammatory cytokines and the iron exporter ferroportin, which decreases intracellular iron. The latter reduces ROS, which activates LXRα-mediated transcription of ABC transporters. This program is distinct from macrophage foam cells, which demonstrate greater expression of the scavenger receptor CD36, uptake of oxidized LDL, reactive oxygen species, and production of pro-inflammatory cytokines. (ROS-reactive oxygen species, SR-scavenger receptor, oxidized LDL-oxLDL, ferroportin-FPN).
Table 1

Quantitative PCR Results for Nuclear Receptors, Scavenger Receptors, and ABC Transporters in M(Hb) versus control macrophages (n=4 experiments per group).

<table>
<thead>
<tr>
<th>Nuclear Receptor</th>
<th>Fold Versus Control (normalized to 1)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>LXRα</td>
<td>1.12±1.13</td>
<td>ns</td>
</tr>
<tr>
<td>Scavenger Receptor</td>
<td>Fold Versus Control (normalized to 1)</td>
<td>P value</td>
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<tr>
<td>SR-A1</td>
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<tr>
<td>SR-A2</td>
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<tr>
<td>CD36</td>
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</tr>
<tr>
<td>SR-B1</td>
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<td>0.03</td>
</tr>
<tr>
<td>ABC Transporter</td>
<td>Fold Versus Control (normalized to 1)</td>
<td>P value</td>
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<tr>
<td>ABCA1</td>
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</tr>
<tr>
<td>ABCG1</td>
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