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

Aloke V. Finn MD, Masataka Nakano MD, Rohini Polavarapu, BA, Vinit Karmali, MA, Omar Saeed, MD, XiaoQing Zhao, PhD, Saami Yazdani, PhD, Fumiyuki Otsuka, MD, Talina Davis, Anwer Habib, MD, Jagat Narula, MD, PhD, Frank D. Kolodgie PhD, Renu Virmani MD.
Macrophage Diversity

• Macrophages are the major inflammatory cells involved in the progression of atherosclerosis
• Macrophage infiltration into the arterial wall followed by uptake of oxidized LDL is marked by the formation of foam cells, a primary hallmark of atherosclerosis
• A newer concept is one of macrophage diversity-
  - Microenvironment drives these cells into morphologically and functionally distinct subtypes
Macrophage Diversity

Classically activated macrophage
+ Pro-inflammatory cytokines production
+ Antigen presentation & microbicidal activity
+ Expression of MHC class II molecules

M1 Macrophage
- Activated PPARγ

Monocyte
- IFN-γ, LPS

MHC class II
- Pro-inflammatory cytokines
- IL-6
- TNF
- IL-1

M2 Macrophage
- Mannose Receptor (CD206) Upregulation
- Anti-inflammatory cytokines
- IL-10
- IL-1Ra

Activated PPARγ
- IL-4, IL-13

Alternatively activated macrophage
- Anti-inflammatory cytokine production
- Cell growth and tissue repair
- Endocytic activity

Macrophage Diversity

- Some data support the presence of IL-4 induced M2 macrophages in human atherosclerosis (Bouhlel MA Cell Metabolism 2007, Chinetti-Gbaguidi Circ. Res. 2011)
- The M1/M2 concept is perhaps too black and white—there is more likely a spectrum of different macrophage subtypes
- Overall, little is known about the existence and function of macrophage subtypes in human atherosclerosis
- We have previously shown that intraplaque hemorrhage is associated with necrotic core enlargement through the release of cholesterol from red cell membranes (Kolodgie FD NEJM 2003)
- Here we examined the effects of hemorrhage on macrophage diversity and function in human atherosclerosis
Methods

• We used human atherosclerotic plaques to analyze macrophage differentiation in response to hemorrhage using traditional markers of M2 macrophages

• Areas of prior hemorrhage were identified by the presence of angiogenesis (CD31), red cells (glycophorin A), and iron (Perl stain) and these areas were compared to foam cell rich areas

• We confirmed the effects of hemorrhage on macrophage differentiation in vitro using human monocytes and explored the mechanisms underlying this phenotype
Macrophages are Important Cells For Hemoglobin Scavenging

Within areas of hemorrhage, RBC lysis and release of free Hb which contains iron occurs. The toxic effects of ferrous iron have been linked to oxidative stress through the fenton reaction where Fe$^{2+}$ oxidizes H$_2$O$_2$ leading to general of hydroxyl radicals and lipid peroxidation.

Among the important mechanisms to detoxify free hemoglobin (Hb) is haptoglobin, a plasma glycoprotein that binds free Hb and clears it from the plasma via uptake by CD163 (hemoglobin:haptoglobin receptor)—which initiates anti-oxidant effects such as induction of HO-1 (breaks down heme) and ferroportin (FPN), an iron exporter.

Human Plaque in Areas of Hemorrhage

Macrophages Express both CD163 and CD206 (Mannose Receptor)

Hp:Hb Ingestion by Monocytes Reproduces the Phenotype Seen in Human Plaques and Leads to a Distinct Mac Phenotype We Have Termed M(Hb)
M(Hb) are protected from foam cell formation

Control  IL-4  Hb:Hp-

Control  oxLDL  IL-4  oxLDL  Hb:Hp- oxLDL

# ORO+ cells/HPF

Control  oxLDL  IL-4  oxLDL  Hb:Hp- oxLDL
Macrophage Differentiation and Cholesterol Uptake: Live Cell Imaging

Control Macs  IL-4 M2  M(Hb)
What is the Mechanism of Lipid Handing in M(Hb)?

Receptors involved in Lipid Uptake

<table>
<thead>
<tr>
<th>Scavenger Receptor</th>
<th>Fold Versus Control (normalized to 1)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-A1</td>
<td>0.17±0.20</td>
<td>0.006</td>
</tr>
<tr>
<td>SR-A2</td>
<td>0.15±0.16</td>
<td>0.009</td>
</tr>
<tr>
<td>CD36</td>
<td>0.09±0.04</td>
<td>0.004</td>
</tr>
<tr>
<td>SR-B1</td>
<td>0.54±0.32</td>
<td>0.03</td>
</tr>
</tbody>
</table>

ATP Binding Cassette (ABC) Transporters involved in apo-AI mediated cholesterol efflux to HDL (i.e. reverse cholesterol transport)

<table>
<thead>
<tr>
<th>ABC Transporter</th>
<th>Fold Versus Control (normalized to 1)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC A1</td>
<td>4.18±2.99</td>
<td>0.03</td>
</tr>
<tr>
<td>ABCG1</td>
<td>29.92±31.98</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Understanding how M(Hb) Handle Lipid Receptors May Allow For the Development of Strategies to Prevent Foam Cell Formation.
Circulating iron is regulated by hepcidin, a secreted hepatocyte peptide that plays a key role in iron homeostasis. Hepcidin binds to the iron exporter ferroportin (FPN), expressed on the surface of macrophages, and promotes degradation of this protein. Hepcidin-induced downregulation of FPN thus inhibits iron export from macrophages.
What is the mechanism of ABC transporter expression in M(Hb)? Could it be ROS mediated and related to iron handling?

Iron efflux plays an important role in ABCA1 expression in M(Hb). Higher intracellular iron quenches calcein.
Intracellular Iron Affects ABC Transporter Expression and Cholesterol Efflux in M(Hb)

*\(p<0.05\) versus Control

*\(p<0.05\) versus Hb:Hp Hepcidin
Reduction of ROS Mediates LXRα Activation of ABC Transporters in M(Hb)

![Graph showing reduction of ROS Mediates LXRα Activation of ABC Transporters in M(Hb)](image)

* *p<0.05 versus all
**p<0.05 versus Hb:Hp

![Graph showing fold increase in transcript](image)

* *p<0.05 versus Control (Scr), **p<0.05 Hb:Hp (Scr), ***p<0.05 versus Hb:Hp (LXRα)/Control (LXRα)

![Graph showing relative LXRα reporter luciferase activity](image)

* *p<0.05 versus Ctrl and Hepcidin
**p<0.05 versus Hb:Hp hepcidin
***p<0.05 versus Hb:Hp
M(Hb) in Human Atherosclerosis

**Foamy Macrophage**
Stimulus: oxLDL
+ Cytokine production – pro-inflammatory
+ MHC class II expression
↑Lipid uptake
↑Reactive Oxygen Species (ROS)

**M(Hb) Macrophage**
Stimulus: Hp:Hp
+ Cytokine production – anti-inflammatory
↓lipid uptake
↑cholesterol efflux
↓intracellular iron and ROS

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**Anti-inflammatory cytokines**
- IL-10
- IL-1Ra

**Pro-inflammatory cytokines**
- IL-6
- TNF
- IL-1

**Mannose Receptor Upregulation**

**CD163 Upregulation**

**Free cholesterol/sterols**

**HDL**
Therapeutics: Can Manipulation of Macrophage Intracellular Iron Reduce Foam Cell Formation and Lesion Progression?

- Iron Hypothesis—theorized that iron depletion protects against atherosclerosis
- Initially presented as an explanation for sex differences in CV disease and increase in disease after menopause in women
- Our data demonstrate that lowering intracellular iron decreases ROS which increases ABC transporter expression
- This may in part explain how modulations in macrophage iron controls cholesterol efflux proteins

Conclusion

• We have demonstrated a novel non-lipid driven macrophage phenotype driven by monocyte ingestion of Hb:Hp

• Hb, not IL-4, is an important stimulus for macrophage differentiation in human atherosclerosis

• Iron metabolism through ROS plays an important role in macrophage lipid handling
  – The exact mechanism by which lowering ROS triggers LXRα activation requires further investigation

• Perhaps the iron hypothesis may in part be explained by its effects on macrophage lipid handing

• Manipulation of macrophage intracellular iron may be of therapeutic value for prevention of CAD by increasing macrophage cholesterol efflux but awaits confirmatory data
Acknowledgements

• Finn Lab
  • Vinit Karmali
  • Rohini Polavarapu
  • Talina Davis
  • Omar Saeed

• Collaborators
  • Renu Virmani (CVPath)
  • Frank Kolodgie (CVPath)
  • Masataka Nakano (CVPath)

• Advisors
  • Bob Taylor (Emory)
  • David Harrison (Vanderbilt)

• Funding: Carlyle Fraser Foundation, National Institutes of Health