Macrophage Polarization in Atherosclerosis

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Atherosclerosis and cardiovascular disease

Atherosclerosis complications

- Ischemia and cerebral infarction
  - Internal carotid artery

- Myocardial infarction
  - Anterior descending coronary artery

- Renal ischemia
  - Renal artery

- Intermittent claudication
  - Femoral artery
Atherosclerosis is an inflammatory disease

- Cells of the immune system: monocytes derived macrophages, T lymphocytes.
- Adhesion molecules: P Selectin, VCAM, ICAM
- Cytokine/Chemokines: IL-6, IL-1β, TNFα, CxCl4
- Chemoattractant & differentiation factors: MCP-1, G-CSF
- Acute phase proteins: CRP
- Inflammatory Transcription factor: NFκb
- Oxidative stress & ROS
Vulnerable plaque:

- Highly inflammatory and necrotic.
- High content of macrophages.
- High content of lipids.
- Prone to plaque rupture.
Macrophages – major mediators of inflammation in the atherosclerotic lesion.

- Monocytes recruited to the arterial intima differentiate into macrophages and the last undergo massive proliferation.
- Lesional macrophages play a key role in development of atheroma and plaque evolution.
- Macrophages comprise a very heterogeneous population in the site of lesion and have a dual and even opposite roles in the inflammatory processes that affect disease progression and plaque stability.

- The diversity of macrophage phenotypes specified by their plasticity of differentiation, according to environmental stimuli they meet.
**Macrophage Activation**

**a** Classical activation

IFN-γ

+ Microbial trigger (LPS)

MHC Class II

PD-L1

TNFα, IL-6, IL-12

NO, ROS

**b** Alternative activation

IL-4

IL-13

MHC Class II

PD-L2

MR, Dectin 1, Mgl1

Arginase 1

Ym-1, Fizz1

IL-10
Macrophage differentiation and features of distinct subtypes

**Pro-inflammatory M1** macrophages are induced by classical activation, including IFN-γ and microbial trigger such as LPS.
- Enriched in progressive plaques.
- Secrete pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and others.
- Express pro-inflammatory transcription factors NF-κB, activator protein 1.
- Produce high levels of inducible NO synthase and NO.

**Anti-inflammatory M2** macrophages are induced by alternative activation including IL-4 or IL-13.
- Enriched in early and regressive plaques.
- High endocytic activity, effective efferocytosis.
- Secrete anti-inflammatory cytokines, such as IL-10, TGF-β, IL-1R antagonist.
- Express high levels of arginase 1 and have increased secretion of collagen, which promotes tissue repair.
- Express mannose receptor 1 (CD206) and release IL-10.
Pro-atherogenic activity of macrophages

- Foam cell transformation and defective lipid metabolism.
- Production of inflammatory cytokines (IL-1, IL-6, TNF-α).
- Failure to migrate, prolong inflammation response.
- Undergo oxidative stress and apoptosis, by that promotion of necrotic core formation.
- Production of proteases and ROSs, promoting extracellular matrix degradation.
- Cause VSMCs apoptosis, that results in fibrous cap thinning.

• Inflammation amplification.
• Plaque destabilization.
• Formation and growth of vulnerable plaque.
Potential role of M2 macrophages in efferocytosis within atherosclerotic plaques

M2 macrophages localized in areas of neovascularization or outside the lipid core can phagocytose apoptotic M1 macrophages, contributing to the resolution of inflammation.

If efferocytosis is insufficient, dead M1 macrophages accumulate and undergo postapoptotic necrosis, leading to the formation of a necrotic core, which contributes to plaque instability and rupture.

Chinetti-Gbaguidi, G. et al. (2014) Macrophage subsets in atherosclerosis

*Nat. Rev. Cardiol.* doi:10.1038/nrcardio.2014.173
Macrophage balance affect the atheroma formation and evolution.

Environmental signals influence plaque-associated macrophages which can perform pro-atherogenic and anti-atherogenic functions through different effector molecules.

**M1/M2 macrophage balance affect the atheroma formation and evolution.**

In the early lesions that are small and stable it seems that pro-inflammatory response of M1 macrophages is well regulated and balanced by the M2 anti-inflammatory macrophages.

As the disease progress, the balance is shifted in favor of pro-inflammatory M1 macrophages, inflammation is getting out of control, causes extensive damage to the tissue end finally leads to dangerous complications.
## CRP and Plaque Stability


<table>
<thead>
<tr>
<th>Variable</th>
<th>Unstable (n = 33)</th>
<th>Stable (n = 29)</th>
<th>P</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Median</td>
<td>66</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>48-83</td>
<td>42-79</td>
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<tr>
<td>Gender (male)</td>
<td>30 (90.9%)</td>
<td>27 (93.1%)</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m2)</td>
<td>25 (19-40)</td>
<td>23 (19-29)</td>
<td>NS</td>
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<tr>
<td>Hypertension</td>
<td>17 (51.5%)</td>
<td>12 (41.4%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>13 (39.4%)</td>
<td>7 (24.1%)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoker</td>
<td>13 (39.4%)</td>
<td>9 (31%)</td>
<td>NS</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>12 (36.4%)</td>
<td>10 (34.5%)</td>
<td>NS</td>
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<td>Lipid-lowering drugs</td>
<td>7 (21.2%)</td>
<td>5 (17.2%)</td>
<td>NS</td>
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<td>Antiaggregant therapy</td>
<td>14 (42.4%)</td>
<td>12 (41.4%)</td>
<td>NS</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>7 (21.2%)</td>
<td>6 (20.7%)</td>
<td>NS</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>5 (15.2%)</td>
<td>5 (17.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Cerebral infarction</td>
<td>14 (42.4%)</td>
<td>11 (33.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>Neurologic event</td>
<td>27 (81.8%)</td>
<td>9 (31%)</td>
<td>&lt;.001</td>
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<tr>
<td>Macrophage count (cells/10 fields)</td>
<td></td>
<td></td>
<td>&lt;.001</td>
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<tr>
<td>Median</td>
<td>19</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>4-45</td>
<td>1-6</td>
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<tr>
<td>High-sensitivity C-reactive protein (mg/L)</td>
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<td>&lt;.001</td>
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<tr>
<td>Median</td>
<td>27.1</td>
<td>4.1</td>
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<tr>
<td>Range</td>
<td>1.8-1.65</td>
<td>0.03-560</td>
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</table>

**Plaque morphology**

**hs CRP levels vs. Macrophages**
CRP is Expressed in Macrophages and is up-Regulated under Diabetic States

CRP mRNA expression in THP-1 macrophages

CRP Protein Content (Arbitrary Units)

Glucose Concentration (mM)

10
20
30
40

*
CRP in atherosclerotic plaques

- C-Reactive Protein Colocalizes With the Terminal Complement Complex in the Intima of Early Atherosclerotic Lesions of Human Coronary Arteries
  *Arterioscler Thromb Vasc Biol.* 1998;18:1386-1392

- Local Generation of CRP in diseased Coronary artery Venous Bypass Grafts and normal Vascular tissue
  *Jabs WJ. Circulation* 2003;108:1428-1431
1. To analyze CRP expression in different subtypes of macrophages (M1 versus M2) at the mRNA and protein levels, and to question whether CRP selective expression involves NFkB signaling pathway.

2. To determine whether oxidative stress can affect the macrophage phenotype balance between pro-inflammatory and anti-inflammatory macrophages, and further modulate macrophage CRP expression.
Validation of M1 induction system:

**M1 activation:**

*Figure 1: Influence of IFN-γ + LPS treatment on IL-6 secretion by M1 macrophages*

**Figure 1:** Determination of IL-6 levels in media from THP-1 macrophages incubated with increasing concentrations of IFN-γ (30ng/ml, 60ng/ml, 90ng/ml) in the presence of LPS at 100ng/ml for 24 hours. p=0.012 (60ng vs.30ng, n=3). * p<0.001 (30ng vs. 0ng, n=3).
Validation of M2 induction system:

**M2 Activation:**

Figure 2A: Influence of IL-4 treatment on IL-10 secretion by M2 macrophages

![Graph showing IL-10 concentration vs. IL-4 concentration](image)

- +290% vs 0 ng; p=0.003

Determination of IL-10 levels in media from THP-1 macrophages incubated with increasing concentrations of IL-4 (30ng/ml, 60ng/ml, 90ng/ml) for 24 hours. p=0.003 (60ng vs. 0ng, n=3).

Figure 2B: % CD206 positive macrophages vs. IL-4 treatment

![Graph showing CD206 expression vs. IL-4 concentration](image)

- US - unstained sample, p=0.02 (60ng vs. 0ng).

CD206 expression in macrophages incubated with increasing concentrations of IL-4 (30ng/ml, 60ng/ml) for 24 hours. US- unstained sample, p=0.02 (60ng vs. 0ng).
CRP expression in M1/M2 macrophages on mRNA and protein levels

**Figure 3A:** Determination of CRP mRNA level in M1/M2 macrophages by Real Time PCR. Control – untreated cells. M1: IFN-γ 60ng/ml + LPS 100ng/ml. M2: IL-4 60ng/ml. p=0.004 (M1 vs. control, n=3).

**Figure 3B:** Determination of CRP intracellular level in M1/M2 macrophages by immunohistochemistry. Control – untreated cells. M1: IFN-γ 60ng/ml + LPS 100ng/ml. M2: IL-4 60ng/ml.
Confocal microscope images of CRP immunohistochemistry in M1/M2 macrophages (X100, FITC and PI staining)

Control (untreated cells)

M1 macrophages

M2 macrophages
NF-κB activation level was assessed by measuring concentration of p50 in nuclear lysates from M1/M2 macrophages by ELISA technique.

p50 is one of the subunits of NF-κB complex. In inactivated state the subunits of NF-κB reside in cytosol, but upon activation they form homo- and hetero-dimers (p50/p50, p50/p65) and translocate to nucleus.

By measurement of p50 concentration in nuclear lysates the relative level of NF-κB complex activation was interpreted.

Is NFκB Involved in M1 and M2 phenotypes induction and further CRP expression?
NF-κB activation in M1/ M2 macrophages

Figure 4: NFκB activation in M1/M2 macrophages

Figure 4: Determination of NF-κB activation in M1/M2 macrophages by measurement of p50 protein in nuclear cellular extracts. Control – untreated cells, M1: IFN-γ 60ng/ml + LPS 100ng/ml; M2: IL-4 60ng/ml. M1: +79% increase, p=0.05 vs. control, n=2
Effect on NF-κB inhibition on M1/M2 differentiation markers: IL-6 and IL-10 secretion respectively

**Figure 5A**: NFκB inhibition effect on IL-6 secretion by M1 macrophages

- **Control**: 0.82 pg/ml
- **M1**: 315.31 pg/ml
- **Treatments**: None, + parthenolide

**Figure 5B**: NFκB inhibition effect on IL-10 secretion by M2 macrophages

- **Control**: 5.31 pg/ml
- **M2**: 4.08 pg/ml
- **Treatments**: None, + parthenolide

**Figure 5**: Effect of pretreatment of macrophages with parthenolide (NFkB inhibitor) A) on IL-6 secretion by M1 macrophages. B) On IL-10 secretion by M2 macrophages. Control – untreated cells. M1: IFN-γ 60ng/ml + LPS 100ng/ml. M2: IL-4 60ng/ml.
Effect of NF-κB inhibition on CRP mRNA and protein level in M1/M2 macrophages:

**Figure 6A:** NFκB inhibition effect on CRP mRNA level in M1/M2 macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CRP mRNA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.8</td>
</tr>
<tr>
<td>M1</td>
<td>1.3</td>
</tr>
<tr>
<td>M2</td>
<td>0.8</td>
</tr>
<tr>
<td>+ NFKB inhibitor</td>
<td>0.4</td>
</tr>
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</table>

-39.9%

**Figure 6B:** NFkB inhibition effect on CRP intracellular level in M1/M2 macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CRP intracellular level (IOD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>20</td>
</tr>
<tr>
<td>M1</td>
<td>50</td>
</tr>
<tr>
<td>M2</td>
<td>20</td>
</tr>
<tr>
<td>+ NFkB inhibitor</td>
<td>10</td>
</tr>
</tbody>
</table>

-43%

**Figure 6:** Effect of pretreatment of macrophages with parthenolide (NFκB inhibitor) A) on CRP mRNA level in M1/M2 macrophages by Real Time PCR. B) on CRP intracellular level (IOD by immunohistochemistry. Control – untreated cells. M1: IFN-γ 60ng/ml + LPS 100ng/ml. M2: IL-4 60ng/ml.
Effect of the Ox-LDL treatment on IL-6/IL-10 secretion by THP-1 macrophages

Figure 7A: Ox-LDL impact on IL-6 secretion by THP-1 macrophages

Figure 7B: Impact of ox-LDL treatment on IL-10 secretion by macrophages

Figure 7: Determination of A) IL-6 level, B) IL-10 level in media from THP-1 macrophages treated with increasing concentrations of ox-LDL (0ug/ml, 10ug/ml, 20ug/ml, 30ug/ml) for 24 hours. p= 0.012 (10ug/ml vs. control, n=3).
Our data support the conclusion that elevated expression of CRP is characteristic of M1 pro-inflammatory macrophages rather than M2 phenotype and it is mediated through NFkB activation. Oxidized LDL could be one of the endogenous trigger for a new phenotype of macrophages: Mild M1 & M2 combination.

⇒ Switching the induction of macrophages from M1 pathway to M2 pathway could reduce CRP deleterious effects as well as the inflammatory cascade in the atherosclerotic plaque and antioxidants could be potent triggers to this anti inflammatory pathway

⇒ Arterial M1 macrophages could be the source of CRP identified in atherosclerotic lesions.
Macrophage activation: Glancing into Diversity

Main macrophage subtypes found in atherosclerotic lesions

Stimuli present in atherosclerotic lesions drive the differentiation of monocytes towards different macrophage phenotypes. a | M1 macrophages release proinflammatory cytokines. b | M(Hb), Mhem, and M2 macrophages are resistant to lipid accumulation, possess iron-handling capacities, and have anti-inflammatory effects. c | Mox macrophages display an antioxidant gene expression profile. d | M4 macrophages, like M1 macrophages, are proinflammatory, but lack the capacity for phagocytosis. Abbreviations: COX-2, cyclooxygenase; CXCL4, C-X-C motif chemokine 4; HMOX-1, haem oxygenase (decycling) 1; LDL, low-density lipoprotein; LXR, liver X receptor; MMP-7, matrix metalloproteinase-7; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR, toll-like receptor; TNF, tumour necrosis factor.