Inflammation and Immune System Contribution to the Etiology of Atherosclerosis

Mechanisms and Methods of Assessment

Dereck Salisbury ▼ Ulf Bronas

**Background:** Immune system activation and inflammation are intricately involved in the development and progression of atherosclerosis.

**Purpose:** The purposes of this review are to (a) discuss effects of inflammation and the immune system across the lifespan of atherosclerotic plaque, (b) review current recommended testing techniques for assessing inflammation using blood and within the atherosclerotic plaque, and (c) link basic research in inflammation and immunology with ongoing clinical research with potential to impact prevention and treatment interventions in atherosclerotic disease.

**Results:** The atherosclerotic process is typically initiated in the presence of endothelial dysfunction by increased uptake, entrapment, and deposition of lipids, especially low-density lipoprotein (LDL). Once inside the intima, LDL can become oxidized (LDLox), which promotes further endothelial cell activation/injury, stimulates adhesion molecule expression, and releases chemotactic factors that promote leukocyte–endothelial interactions. The process of atherogenesis is highly regulated by the innate and adaptive immune systems and systemic inflammatory response. In addition, proinflammatory mediators play a key role in the lifespan of the atherosclerotic plaque and its vulnerability, favoring eventual plaque fissure when exposed to increasing hemodynamic stress.

**Discussion:** The complex atherosclerotic process involves the innate and adaptive immune systems and systemic inflammatory activation. Incorporation of advances in understanding inflammation and immune system contributions to the etiology of atherosclerosis into intervention research allows the development of novel approaches to prevention and treatment.

**Key Words:** atherosclerosis • cardiovascular disease • immune system • inflammation • nursing research

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A framework describing inflammation as deeply intertwined with atherosclerosis and its manifestation in cardiovascular diseases (the inflammatory hypothesis) has developed over the past 2 decades (Ridker, 2009). The inflammatory hypothesis has evolved to encompass innate immune system response via various leukocytes (monocytes, macrophages, and neutrophils) and the adaptive immune response via lymphocytes (T helper [Th] cells) and its associated subtypes. Sophisticated technological advances have made it possible to advance understanding of immune cell activation and their protein mediators (cytokines/chemokines). These advances have enabled exploration of the role of inflammation and activation of the immune response in the etiology of atherosclerosis. The purposes of this review are to (a) discuss the etiology of inflammation and the immune system in the lifespan of the atherosclerotic plaque, (b) review the current recommended testing techniques for assessing inflammation in blood as well as within atherosclerotic plaque, and (c) link basic research on inflammation and immunology to ongoing clinical research that may impact therapeutic practice and symptom management of atherosclerotic disease.

**PATHOPHYSIOLOGY OF ATHEROSCLEROSIS**

**Involvement of the Immune System and Inflammatory Pathways**

Figure 1 outlines the pathophysiology of atherosclerosis from exposure to risk factors to development of a fibrous cap of plaque, highlighting the involvement of the immune system and inflammatory pathways. Endothelial cells are positioned as the innermost cell layer within all blood vessels. The endothelial cell lining was once thought of as an inert barrier but is now known to be a key regulator of angiogenesis, vasomotor tone, and vascular homeostasis via various mechanisms, including...
Autocrine, paracrine, and hormonal-like influences. The endothelial cell lining (endothelium) is integral to the protection of the arteries against atherosclerosis. Endothelial dysfunction has, therefore, been proposed to be the first step in the initiation of atherosclerosis.

**Endothelial Dysfunction** Endothelial dysfunction is induced by all known cardiovascular risk factors, including smoking, oxidized low-density lipoprotein (LDLox), hyperglycemia, hypertension, dyslipidemias, inflammation, and elevated free radicals or oxidative stress (Sauer & Wartenberg, 2008). Endothelial dysfunction results in a cascade of events highlighted by increased endothelial cell permeability; leukocyte, neutrophil, and platelet adhesion; and loss of anticoagulant and vasodilating capacity. Most of these processes are regulated by endothelial, immune, and foam cell (i.e., lipiHaden macrophage)-derived cytokines, which are elevated in response to a proinflammatory state. Major proinflammatory cytokines and acute phase reactants inherent to endothelial dysfunction and the atherosclerotic process include tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), IL-6, and C-reactive protein (CRP). TNFα, IL-6, and CRP have been reported as strong, independent risk factors for atherosclerotic cardiovascular disease (Cesari et al., 2003). These proinflammatory cytokines and acute phase reactants and other chemokines and cellular adhesion molecules (CAMs) involved in the atherosclerotic that can progress or slow the atherosclerotic process are listed in Tables 1 and 2.

**Innate Immune System** Endothelial dysfunction and the resulting increased membrane permeability may result in LDL

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*FIGURE 1.* Inflammation and immune system activation and contribution to the etiology of atherosclerosis and formation of a fibrous plaque.
infiltration of the arterial intima and its subsequent oxidation by various reactive oxygen and nitrogen species producing enzymes, including myeloperoxidase and inducible nitric oxide synthase (Glass & Witztum, 2001). LDLox is postulated to be a primary stimulus for monocyte–endothelial interactions mediated by increased expression of monocyte chemoattractant protein-1 (MCP-1) (Glass & Witztum, 2001).

### TABLE 1. Plasma and Serum Proinflammatory Biomarkers Linked to Atherosclerosis

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Classification</th>
<th>Primary sources</th>
<th>Function in atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein ^a</td>
<td>Acute phase reactant</td>
<td>Hepatocyte</td>
<td>Expression of CAMs and MCP-1</td>
</tr>
<tr>
<td>Tumor necrosis factor α ^b</td>
<td>Cytokine</td>
<td>Macrophage</td>
<td>Production of MMP-1 (↑ plaque fissure)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dendritic cell</td>
<td>Activation of complement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibroblast</td>
<td>↑ Endothelial dysfunction (inhibits dimethylarginine dimethylaminohydrolase and uncouples endothelial nitric oxide synthase)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipocyte</td>
<td>↑ Synthesis and release of acute phase proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B cell</td>
<td>↑ Leukocyte recruitment (via MCP-1, IL-8, CAMs)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Thrombogenesis (stimulates tissue factor production)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ fibrinolysis (inhibits tissue plasminogen activator production)</td>
</tr>
<tr>
<td>Interferon-γ ^c</td>
<td>Cytokine</td>
<td>Th1 cell</td>
<td>↑ Stimulation of macrophages and dendritic cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NKC</td>
<td>↓ Anti-inflammatory Th2 cell activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Proinflammatory Th1 cell development</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Expression of CAMs</td>
</tr>
<tr>
<td>Interleukin-1 ^d</td>
<td>Cytokine</td>
<td>Macrophage</td>
<td>↑ Synthesis and release of acute phase proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dendritic cell</td>
<td>↑ Protease activation (↑ plaque fissure)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Platelet activation</td>
</tr>
<tr>
<td>Interleukin-6 ^e</td>
<td>Cytokine</td>
<td>Macrophage</td>
<td>↑ Production of MMP-1 (↑ plaque fissure)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endothelial cell</td>
<td>↑ Leukocyte recruitment (via MCP-1, IL-8, CAMs)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipocyte</td>
<td>↑ Polarization of naïve CD+4 cells into Th1</td>
</tr>
<tr>
<td>Interleukin-12 ^f</td>
<td>Cytokine</td>
<td>Macrophage</td>
<td>↑ Proinflammatory cytokine production from macrophages</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dendritic cell</td>
<td>↑ Leukocyte chemotaxis</td>
</tr>
<tr>
<td>Interleukin-8 ^g</td>
<td>Chemokine</td>
<td>Macrophage</td>
<td>↑ Neutrophil chemotaxis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endothelial cell</td>
<td>↑ Stimulation of phagocytosis</td>
</tr>
<tr>
<td>Monocyte chemoattractant protein-1 ^i</td>
<td>Chemokine</td>
<td>Macrophage</td>
<td>↑ Migratory activity of monocytes and macrophages</td>
</tr>
<tr>
<td>Macrophage colony stimulating factor ^l</td>
<td>Chemokine/ cytokine</td>
<td>Endothelial cell</td>
<td>Regulator: macrophage survival, proliferation, differentiation</td>
</tr>
<tr>
<td>E-selectin ^h</td>
<td>Cell adhesion molecule</td>
<td>Endothelial cell</td>
<td>↑ Accumulation of blood leukocytes at the sites of inflammation</td>
</tr>
<tr>
<td>Intracellular adhesion molecule-1 ^m</td>
<td>Cell adhesion molecule</td>
<td>Endothelial cell</td>
<td>↑ Leukocyte–endothelial adhesion</td>
</tr>
<tr>
<td>Vascular cellular adhesion molecule-1 ^n</td>
<td>Cell adhesion molecule</td>
<td>Endothelial cell</td>
<td>Binds to integrins of type CD11a/CD18 or CD11b/CD18 (↑leukocyte–endothelial adhesion)</td>
</tr>
</tbody>
</table>

^Note. ↑ = increase in serum or plasma levels; ↓ = decrease in serum or plasma levels; CAMs = cellular adhesion molecules; CD = cluster of differentiation; IL-8 = interleukin-8; MCP-1 = monocyte chemoattractant protein-1; MMP-1 = matrix metalloproteinase-1; NKC = natural killer cell; Th1 = type 1 T helper cell; Th2 = type 2 T helper cell.

Inflammation, Immune System, and Atherosclerosis

Inflammation, Immune System, and Atherosclerosis

Activated macrophages, Th1 lymphocytes, and foam cells within the evolving atherosclerotic lesion are all potent generators of proinflammatory cytokines, such as TNFα, IL-1β, and IL-6 (Libby, Aikawa, & Jain, 2006). Production of these cytokines results in vascular maladaptations, including endothelial dysfunction, increased LDL uptake, and oxidation. Moreover, the increased levels of proinflammatory cytokines increase the expression of CAMs (E-selectin, ICAM-1, and VCAM-1) on the arterial endothelium, which leads to attraction, adhesion, activation, and a subsequent increase in infiltration of monocytes, T lymphocytes, and neutrophils (Brevetti, Schiano, & Chiarieillo, 2006).

As with monocytes/macrophages and lymphocytes, activated neutrophils are highly involved in the complex biochemical etiology of atherogenesis. Proinflammatory cytokines, in particular, TNFα, bind to the vascular endothelium and activate nuclear factor kappa B (NF-κB). NF-κB is a transcription factor known to upregulate endothelial gene expression. NF-κB induces secretion of proinflammatory cytokines and chemokines and adhesion molecules (Ciszzer, Wang, Lakatta, & Ungvari, 2008). Concurrently, the proinflammatory cytokines, NF-κB, and CRP increase expression of P- and L-selectin (which reside on the surface of the neutrophil) (Bevilacqua et al., 1995; Brevetti et al., 2006) and prime the vasculature for leukocyte-endothelial interactions. Upon neutrophil “capturing” (loose” binding to the endothelium), the selectins, primarily P-selectin, mediate the neutrophil rolling necessary for the activation of reduced nicotinamide adenine dinucleotide phosphate oxidase (Zarbock & Ley, 2009). Firm adhesion is then mediated by E-selectin, ICAM-1, and VCAM-1 on the endothelium as well as integrins and the CD11/CD18 complex on the neutrophils (Kakkar & Lefer, 2004)—the latter is upregulated by P-selectin and platelet-activating factor. Once fully adhered to the endothelium, the now activated neutrophil can transmigrate through the activated endothelium and into the source of activation (i.e., the developing atherosclerotic plaque; Kakkar & Lefer, 2004).

Adaptive Immune System Cells of the adaptive immune system, such as CD4+ (helper) T cells and B cells (to a lesser extent), are also found in the initial sites of atherosclerotic lesions (Ait-Oufella, Sage, Mallat, & Tedgui, 2014; Hansson, 2005). T cells are reactive to LDLox and heat shock protein 60 and function as antigens bound to major histocompatibility complex class II molecules present on phagocytic, antigen-presenting cells (macrophages, dendritic cells, and B cells; Hansson, 2005). Antigen-presenting cells, particularly macrophages, release IL-1 and IL-12 that polarize naïve CD4+ cells into type 1 T helper cells (Th1; de Jager & Chiariello, 2006). The resulting polarization to Th1 lymphocytes is followed by the production of interferon γ (IFNγ), a cytokine that stimulates macrophage activation, augmentation of the synthesis of TNFα and IL-18, and increased efficiency of antigen presentation by macrophages (Hansson, 2005). Therefore, the presence of Th1 lymphocytes within the developing atherosclerotic plaque further increases the phagocytic capabilities of the innate immune cells, such as monocytes and neutrophils.

Inflammation Activated macrophages, Th1 lymphocytes, and foam cells within the evolving atherosclerotic lesion are all potent generators of proinflammatory cytokines, such as TNFα,
The major regulating chemokine that controls chemotaxis of the neutrophil to the source or site of inflammation (i.e., the forming atherosclerotic lesion) is IL-8, which is regulated by TNFα (Gerszten et al., 1999; Stillie et al., 2009). At the site of inflammation, the activated neutrophils undergo a respiratory burst, which is an autoimmune response characterized by the rapid release of reactive oxygen species (primarily O₂⁻ and other oxidants such as the heme enzyme myeloperoxidase) designed to rid the area of cellular damage and debris. In addition, TNFα upregulates nicotinamide adenine dinucleotide phosphate oxidase, inducible nitric oxide synthase, and xanthine oxidoreductase, all of which are well-known producers of the potent radical superoxide (O₂⁻) — this further promotes LDL oxidation and subsequent foam cell generation (Griendling & Alexander, 1997; Picchi et al., 2006). These processes show the intricate relationship of inflammation and oxidative stress to the generation and accumulation of foam cells in the arterial intima as well as the resulting fatty streaks.

Development of Atheromas
Accumulation of foam cells and formation of fatty streaks result in the proliferation of smooth muscle cells. The proliferating smooth muscle cells migrate from the arterial media and then wall off the fatty streaks with connective tissue, creating the fibrous cap of the plaque. This atheroma is an advanced, complicated lesion. When the artery can no longer compensate for the space-occupying lesion by dilation, the lesion protrudes into the lumen and reduces blood flow (Ross, 1999). The relative degree of luminal narrowing associated with cardiovascular signs and symptoms differs depending on the diameter of the artery. However, it is commonly thought that a large partial arterial occlusion is needed to reduce blood flow in coronary or iliac/femoral arteries to the point that symptoms of angina pectoris or claudication, respectively, are experienced. This large degree of arterial occlusion likely reflects a longstanding, stable, calcified atheroma that may be relatively resistant to rupture or fissure. Lesser degrees of arterial plaque development, which may not even protrude into the lumen because of compensatory remodeling, have the most unstable (vulnerable) plaques. Commonly, rupture of vulnerable plaques results in ischemic events.

Plaque Rupture
An advanced lesion is also subject to remodeling, primarily regulated by proinflammatory cytokines. Cytokines such as TNFα and CRP both impair the smooth muscle cell production of collagen (required for fibrous cap repair) and directly promote the disruption of the thin, vulnerable fibrous cap covering the lipid-laden lesion (Libby, 2002). CRP exacerbates plaque disruption by upregulating matrix metalloproteinase-1, an enzyme known to degrade the basement layer of the endothelium and the fibrous cap of the atheroma (Bisoendial, Kastelein, & Stroes, 2007). Disruption of vulnerable plaque can lead to thrombotic events, such as acute coronary syndrome, limb-threatening ischemia, major and minor strokes, and death. The proinflammatory cytokines TNFα and CRP both play an active role in the thrombogenic process after plaque rupture through the upregulation of tissue factor (Khan et al., 2010) and plasminogen activator inhibitor-1 (Biemond et al., 1995).

Methods for the Assessment of Immune Response and Inflammation
Reliable and valid techniques enabling measurement of serum- and plasma-based inflammatory mediators and other molecular- and immune-based measurement technologies are available. Immunological assessments most commonly reported in the literature involve analyses of blood-borne circulating immune proteins, such as cytokines and chemokines, CAMs, and acute phase reactants (Leng et al., 2008; Walsh et al., 2011). These proteins include, but are not limited to, IL-1, IL-6, IL-8, TNFα, CRP, ICAM-1, VCAM-1, and E-selectin. In addition, circulating blood leukocytes (CD4+ T cells, CD8+ T cells, Th1, Th2, Th17, Treg, B cells, neutrophils, monocytes) and plasma immunoglobulin concentrations are often measured from the same sample concurrently (Leng et al., 2008; Walsh et al., 2011).

Methods used to assess these biomarkers are commonly based on enzyme-linked immunosorbent assay (ELISA), multiplex arrays, or chemiluminescent assays. Close attention must be paid to sample collection details (e.g., plasma vs. serum, physical activity before sample collection, and diurnal variations), sample preparation, and storage to properly perform the analysis of the biomarkers.

Cytokine Analysis
Sample and Anticoagulant Choice Before blood sampling, the decision must be made whether biomarker analysis will be
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performed using plasma, serum, or less commonly, whole blood. Plasma contains fibrinogen, and other clotting factors collected using evacuated tubes containing an anticoagulant (such as ethylenediaminetetraacetic acid, sodium, or lithium heparin that chelate calcium ion) that are used to prevent coagulation. When compared with ethylenediaminetetraacetic acid plasma, sodium heparin plasma showed the most constant recovery of all cytokines tested (TNFα, IFN, IL-1, IL-2, IL-4, IL-6, IL-10, IL-13, IL-15, IL-17, and IL-18) and, therefore, is the preferred anticoagulant (de Jager, Bourcier, Rijkers, Prakken, & Seyfert-Margolis, 2009). Serum is the liquid fraction of whole blood collected after blood is allowed to clot. Serum collection tubes contain clot activators; however, this method does not allow collection of peripheral blood mononuclear cells from the same vial, which means that, oftentimes, plasma will be the product of choice to maximize the value of blood drawn in a minimal number of tubes from study participants.

**Time of Sampling** Several cytokines are under neuroendocrine control (particularly by glucocorticoids) and exhibit diurnal rhythm. Specifically, proinflammatory cytokines such as IL-1, IL-6, TNFα, and IFNγ are linked to melatonin release and peak early in the morning (Petrovsky & Harrison, 1998). Therefore, it is currently recommended that blood samples be obtained in the morning, and the time of blood sampling stays consistent when samples are drawn on multiple days (e.g., during an intervention trial).

**Influence of Exercise** Intense and prolonged bouts of exercise result in large but transient increases in leukocytes and systemic levels of cytokines, including IL-6, IL-10, IL-1ra, TNFα, monocyte chemotactant protein-1, and CRP. IL-6 appears to be the most influenced by exercise and has been reported to rise 5 pg/ml and 10 pg/dl immediately after 1 hour and 2 hours of strenuous, glycogen-depleting exercise (Nieman, 2012). IL-6 also influences several anti-inflammatory cytokines, including IL-10 and IL-1ra, and as a result, physical activity and exercise influence each of these cytokines (Keller et al., 2005). Therefore, it is recommended that patients refrain from intense physical activity and exercise for a minimum of 1 day before cytokine analysis.

**Assay Options** ELISA and multiplex arrays are used for the measurement of biomarkers for inflammation. A direct comparison of the advantages and disadvantages of ELISA and multiplex arrays is provided in Table 3.

ELISA is the most common laboratory technique for the measurement of inflammation in human subjects. ELISA enables sensitive detection of the antigen (cytokine/chemokine of interest) and has been considered the “gold standard” technique. ELISA has been utilized for many years, and results are highly quantitative and reproducible (Leng et al., 2008). Only one cytokine at a time can be measured in a given aliquot sample necessitating a high volume of sample when more than one cytokine/chemokine is analyzed. In addition, commercially available kits differ in quality (Leng et al., 2008).

A more recent advancement in the quantification of cytokines/chemokines came with the production of multiplex arrays. Multiplex arrays allow the measurement of multiple cytokines/chemokines in the same sample at the same time (Leng et al., 2008; Zhou et al., 2010). Several multiplex arrays, which are becoming increasingly utilized in laboratories, include flow cytometry, chemiluminescence, and electrochemiluminescence (ECL). Flow cytometric, bead-based multiplex assays are the most common (Leng et al., 2008; Zhou et al., 2010). Luminescent immunoassays are variations of the standard ELISA, where an enzyme converts a substrate to a reaction product that emits photons of light instead of developing a visible color. Luminescence is often described as the emission of light from a substance as it returns from an electronically excited state to ground state. Chemiluminescence is the light produced by a chemical reaction. The chemiluminescent substance of interest is excited by theoxidation and catalysis-forming intermediates. When the excited intermediates return back to their stable ground state, a photon is released, which is detected by the luminescent signal instrument. ECL is a kind of luminescence produced during electrochemical reactions in solutions. In electrogenerated chemiluminescence, electrochemically generated intermediates undergo a highly exergonic reaction to produce an electronically excited state that then emits light upon relaxation to a lower-level state. The wavelength of the emitted photon of light corresponds to the energy gap between the two states (Forster, Bertoncello, & Keyes, 2009).

ECL combines analytical advantages of chemiluminescence analysis (absence of background optical signal) with ease of reaction control by applying electrode potential. As an analytical technique, it presents outstanding advantages over other common analytical methods because of its versatility and good temporal and spatial control compared with chemiluminescence. Enhanced selectivity of ECL analysis is reached by variation of electrode potential, thus controlling species that are oxidized/reduced at the electrode and take part in ECL reaction (Fähnrich, Pravda, & Guilbault, 2001).

**Special Recommendations for CRP** Most of the inflammatory mediators that were discussed (cytokines and chemokines, CAMs, and acute phase reactants) are commonly measured in research settings but are not used in clinical assessment. The exception is high-sensitivity CRP. Analysis of data from 3,000 participants in the Framingham Heart Study found that high CRP levels improved clinical prediction of first-time heart attack or stroke by 5%–15% (Wilson et al., 2008). In multivariable analyses that included age, gender, systolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, diabetes mellitus, current smoking, hypertension treatment,
and homocysteine, the log CRP level remained significantly related to the development of hard coronary heart disease endpoints (coronary death, myocardial infarction, stroke) and general cardiovascular disease (CVD), and provided moderate improvement in discrimination among events. The authors concluded that, if the predicted 10-year likelihood of myocardial infarction risk using the Framingham Risk Calculator was “intermediate,” strong consideration should be given to measuring CRP (Wilson et al., 2008). A complete guide to the levels of evidence and recommendations for the clinical measurement of CRP is available in the 2010 ACCF/AHA Guidelines for Assessment of Cardiovascular Risk in Asymptomatic Adults (Greenland et al., 2010).

CRP levels appear to be stable and comparable across age and gender, despite some findings showing increasing levels of CRP with advancing age and differences between men and women (Ferri et al., 2007). CRP also carries considerable intraindividual variability, so two to three repeated measurements at 2-week intervals are recommended for CVD risk stratification and to reduce the potential confounding intra-individual variability (Pearson et al., 2003). Because CRP production is partially mediated by IL-6 (which exhibits diurnal variation), plasma or serum samples should be obtained in the morning (Ferri et al., 2007). Current CVD risk categories for CRP include low (<1 mg/dl), moderate (1–3 mg/dl), and high (>3 mg/dl) and CRP levels > 5 mg/dl being an indicator of an acute inflammatory response or chronic autoimmune disorders (Bisoendial et al., 2007).

**Freeze Duration and Sample Stability** Another important factor that may affect the validity of analysis is sample storage time. As with all assays, experimental variation should be minimized; all samples should be collected, and plasma or serum should be frozen at ~80°C so that the samples can be analyzed as a batch. However, the amount (level) or stability of the cytokines deteriorates over time even in frozen samples (de Jager et al., 2009). Some (IL-13, IL-15, IL-17) degraded within 1 year of storage. Levels of IL-1α, IL-1β, IL-5, IL-6, and IL-10 degraded by over 50% in 2–3 years. IL-2, IL-4, IL-12, and IL-18 were much more stable, maintaining their initial levels out to 3 years after the initial storage.

**Freeze/Rethaw and Sample Stability** It is common practice to avoid multiple freeze-thawing cycles before analysis (de Jager et al., 2009), with stability of cytokine levels from sodium heparin plasma aliquots stored at ~80°C and subjected to multiple freeze-thaw cycles. IL-6 and IL-10 were found to be stable throughout multiple freeze-thawing cycles, whereas IL-4, IL-13, IL-15, IL-17, TNFα, IFNγ, and CXCL8 (IL-8) levels either rose (IL-4 and TNFα) or dropped (IL-13, IL-15, IL-17, IFNγ, and CXCL8) after one or more freeze-thaw cycles. These data indicate that samples for cytokine measurements should not have been subjected to repeated freeze-thaw cycles.

**Imaging Techniques**

Noninvasive and molecular imaging techniques are less frequently utilized but are intriguing research techniques for quantifying inflammation within the atherosclerotic plaque. Although traditional noninvasive imaging techniques—such as magnetic resonance imaging and computed tomography (CT)—provide meaningful information about plaque structure and vulnerability, they fail to yield specific quantifications regarding inflammation and immune cell activation. However, novel techniques, such as single photon emission CT, microwave radiometry (MR), and positron emission tomography (PET), have been utilized in clinical trials to further

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**TABLE 3. Enzyme-Linked Immunosorbent Assay (ELISA) and Multiplex Array Comparisons: Measurement of Inflammation in Plasma and Serum**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td>ELISA</td>
<td>• Highly quantitative&lt;br&gt;• Generally reproducible&lt;br&gt;• “Gold standard”&lt;br&gt;• Widely used in clinical research&lt;br&gt;• Good for comparison purposes</td>
<td>• Only one cytokine measured at a time per sample aliquot, increasing costs&lt;br&gt;Performance varies with antibody quality&lt;br&gt;Performance varies with kit manufacturer</td>
</tr>
<tr>
<td>Multiplex array</td>
<td>• High throughput multiplex analysis&lt;br&gt;• Less sample volume needed&lt;br&gt;• Efficiency in time and cost&lt;br&gt;Can evaluate levels of one given inflammatory molecule in the context of multiple others&lt;br&gt;Can perform repeated measures of the same cytokine panels in the same subjects under the same experimental assay conditions&lt;br&gt;Can reliably detect different proteins across broad dynamic range of concentrations</td>
<td>• Experience with technique is limited (although good correlations to ELISA have been reported)&lt;br&gt;Multiple data interpretation requires careful knowledge of molecular pathways leading to cytokine regulation&lt;br&gt;Performance varies with antibody quality&lt;br&gt;Performance varies with kit manufacturer</td>
</tr>
</tbody>
</table>

*Note: ELISA = enzyme linked immunosorbent assay.<br>Leng et al., 2008. bZhou, Fragala, McElhaney, & Kuchel, 2010."
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The most utilized PET technique—18-F fluorodeoxyglucose PET/CT (FDG-PET/CT)—has allowed the study of leukocytes within the atherosclerotic plaque. In a recent review, it was concluded that FDG-PET/CT has many advantages over other imaging modalities: (a) high contrast resolution; (b) quantification of plaque volume and metabolic activity; (c) dynamic, real-time in vivo imaging; and (d) minimal operator dependence (Mehta, Torigian, Gelfand, Saboury, & Alavi, 2012). Furthermore, FDG-PET-CT represents a stable and reproducible phenotype over time; has high sensitivity for the detection of inflammation; and has high interreader and intrareader reliability, particularly in femoral and carotid arteries (Joshi, Rosenbaum, Bordes, & Rudd, 2011; Mehta et al., 2012; Silvera et al., 2009). A complete review of a FDG-PET/CT testing protocol is available in Mehta et al.

Another utilized method in PET-CT scanning of the atherosclerotic plaque involves use of the 11C-labeled PET tracer PK11195 (Gaemperli & Kaufmann, 2012; Lamare et al., 2011; Pugliese et al., 2010). PK11195 is a specific ligand of the translocator protein (18 kDa), a protein that is highly expressed in activated cells of the mononuclear phagocyte lineage (Veenman & Gavish, 2006). The stimulated monocytes express more than 2,000,000 binding sites for PK11195, and this increase is paralleled by an enhanced expression in CD11a and CD11b surface antigens and augmented production of IL-1, IL-8, and TNFα, indicating that translocator protein overexpression is a marker of activated phagocytes (macrophages; Canat et al., 1993).

The most recent advancement in plaque imaging—MR—is a noninvasive method that converts electromagnetic radiation from internal tissue at microwave frequencies and provides accurate measurement of the temperature of the patient’s internal tissue, including the atherosclerotic plaque (Toutouzas et al., 2012). MR use in atherosclerotic populations is based on research showing increased temperature heterogeneity in ex vivo atherosclerotic specimen of human carotid arteries; this thermal heterogeneity is indicative of inflammatory atherosclerotic plaque (Canat et al., 1993). In addition, both experimental and clinical studies have demonstrated the effectiveness of MR in detecting an inflammatory, vulnerable plaque (Toutouzas et al., 2012). MR therefore appears to be a promising technique for the identification of vulnerable atherosclerotic plaque; however, additional study of diagnostic accuracy is needed.

APPLIcations in Nursing Science

Understanding of immunology, inflammation, and methods of assessment is essential for the full comprehension of the atherosclerotic process and the biobehavioral and biological treatments used to attenuate plaque progression and rupture. Plasma inflammatory biomarkers, such as CRP, now have importance in risk stratification and prognosis of cardiovascular disease, particularly when used in conjunction with other established risk factors (Wilson et al., 2008). Compared with invasive or noninvasive imaging modalities, biomarkers offer the advantage of being relatively risk free, less expensive, and applicable to a wide range of populations at risk. Furthermore, like traditional cholesterol evaluation, plasma inflammatory biomarkers can be performed and interpreted in outpatient primary care settings that are the most appropriate for patient follow-up and discussions of preventive interventions (Tsimikas, Willerson, & Ridker, 2006). Because biomarkers are usually measured in the blood, their tissue or organ etiology often cannot be determined, and when abnormal, it may be difficult to localize the site of abnormality (Tsimikas et al., 2006). In addition, systemic inflammatory biomarkers may offer a more global assessment of the response to various pharmaceutical (i.e., 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors [statin drugs], beta-1 adrenergic blockers, angiotensin-converting enzyme inhibitors, and cyclooxygenase inhibitors) and therapeutic lifestyle (i.e., dietary change and aerobic exercise) therapies.

Pharmacological therapies that have been utilized in pathologic conditions such as rheumatoid arthritis, Crohn’s disease, and psoriasis that directly target the inflammatory process have a potential in the treatment of unstable atherosclerotic plaque. Such pharmacological agents include monoclonal antibodies that target proinflammatory cytokines upstream of CRP and inhibitors of TNFα (infliximab and adalimumab), IL-6 (tocilizumab), IL-1 (canakinumab), and CRP 1,6-bis(phosphocholine)-hexane and CRP42; Thompson, Nidorf, & Eikelboom, 2013).

Comprehensive understanding of inflammatory and immune system biomarkers and the collection and analysis of specimens to measure them is essential to research on the prevention and management of many chronic diseases, infections, and other conditions involving chronic inflammation and immune activation. These biomarkers are relevant across the spectrum of biopsychosocial research in nursing science involving pathophysiological pathways or intervention research. These biomarkers are also being used for research in depression, aging and frailty, sleep disorders, muscle wasting disease or sarcopenia, osteoporosis/osteopenia, neurologic diseases, anemias, obesity, endocrine disorders, pain, fatigue, pregnancy, and even disability and fall prevention (Hunt, Walsh, Voegeli & Roberts, 2010a, Hunt, Walsh, Voegeli and Roberts, 2010b), and the approach is in line with the current strategic plan for the U.S. National Institute of Nursing (National Institute of Nursing Research, 2011).
Although the inflammation-atherosclerosis link is well recognized, currently, there is no therapy with established effectiveness that uniquely targets unstable plaque (Thompson et al., 2013). Because evidence about biological mechanisms linking inflammation with progression of atherosclerosis and plaque instability is strong and plausible, therapy-focused research to slow and even prevent atherosclerosis is underway. Ongoing clinical trials are investigating various cytokine inhibitors and antagonists including canakinumab (Ridker, Thuren, Zalewska, & Libby, 2011), adalimumab (clinicaltrials.gov identifier: NCT01088165), etanercept (clinicaltrials.gov identifier: NCT01372930), the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statin drugs) atorvastatin (clinicaltrials.gov identifier: NCT00920101) and fluvastatin, (clinicaltrials.gov, identifier: NCT01045512), and aerobic exercise training (clinicaltrials.gov identifier: NCT01353737) to reduce low-grade systemic inflammation and progression of atherosclerosis. The Canakinumab Anti-inflammatory Thrombosis Outcomes Study trial is evaluating the effectiveness of canakinumab (a human monoclonal antibody targeted at IL-1B) treatment compared with placebo in the prevention of recurrent cardiovascular events in patients with previous myocardial infarction and elevated high-sensitivity CRP (Ridker et al., 2011); effectiveness of canakinumab in this situation would add to validation of the inflammatory hypothesis in atherosclerotic disease.

Another major focus in clinical trials is the attenuation of atherosclerosis through vaccination. Vaccination can be targeted at three sites: circulation (lipid antigens), the atherosclerotic lesion (lipid and inflammatory antigens), and lymphoid organs (inflammatory antigens; de Jager & Kuiper, 2011). IL-12 and IL-15 are the two proinflammatory cytokines currently targeted in vaccination clinical trials. Vaccination for IL-12 is an attempt to inhibit polarization of naive CD+4 cells into Th1 cells, whereas vaccination for IL-15 has the potential to reduce chemotaxis and attenuate proinflammatory cytotoxic production by macrophages (de Jager & Kuiper, 2011). Vaccination attempts are also focusing on LDLox via the innate, antiatherogenic B-1 B cells and their ability to offer protection through secretion of natural immunoglobulin M (IgM) antibodies (LDLox IgM antibodies). The LDLox IgM antibodies are believed to have two distinct and separate physiological functions: (a) inhibit foam cell formation and (b) accelerate the clearance of apoptotic cells—the latter being especially important in plaque stability (Ait-Oufella et al., 2014). Some of these interventions may enter clinical practice over the next decade, making it essential that practicing nurses have a firm understanding of the pathways involved in the treatments.

Conclusion

Immune system activation and inflammation are intricately involved in the development and progression of atherosclerosis. Advances in methods for the assessment of immune response and inflammation are critical to enhancing the current understanding of the role of inflammation in the etiology of atherosclerosis, designing and evaluating more effective biopsychosocial prevention and treatment interventions, and elucidating the mechanisms underlying interventions that work.

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