Atherosclerosis newsletter

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Monocyte-derived macrophages play a pivotal role in the pathogenesis of atherosclerosis. Issues 351 and 352 contain several articles investigating related pathomechanisms or strategies for diagnostic or therapeutic exploitation.

Debris collected *in-situ* from spontaneously ruptured atherosclerotic plaque invariably contains large cholesterol crystals and evidence of activation of innate inflammation: Insights from non-obstructive general angioscopy

Spontaneous rupture of atherosclerotic plaques (SRAPs) has been demonstrated to be associated with the development and enlargement of cholesterol crystals (CCs) within the core of lipid rich plaques. While rupture of the plaque cap leads to atherothrombosis, rupture of the base of the plaque may lead to intra-plaque haemorrhage and release of CCs into the interstitial space where they can initiate an innate immune response. Komatsu et al. previously used non-obstructive general angioscopy to described a range of appearances of spontaneously ruptured atherosclerotic plaques (SRAPs) in the aorta *in-situ*, and confirmed that debris extruding from some SRAPs (puff-chandelier lesions) were rich in cholesterol crystals and leukocytes. In this study, the authors aimed to characterize the nature of the inflammatory infiltrate of this debris.

Debris was collected from puff-chandelier lesions at the time of angioscopy in patients with known coronary disease. Prepared specimens were examined with light microscopy, and immunostaining was used to detect markers of activation of the innate inflammatory pathway including CD68, NLRP3, caspase-1, IL-1β, IL-18, and IL-6.

Debris sampled from 20 puff-chandelier lesions were assessed. Microscopy confirmed the presence of large CCs, macrophages, fibrin, calcified gruel, lymphocytes, and neutrophils in 100%, 100%, 95%, 25%, 20%, and 15% of the specimens, respectively. Immunostaining confirmed the presence of CD68, NLRP3, IL-1β, and IL-6 within the debris in 100%, 90%, 80%, and 80%, of the specimens, respectively. CCs, NLRP3, caspase-1, IL-1β, IL-18, were also identified in the cytoplasm of macrophages.

Debris from SRAPs with a puff-chandelier appearance invariably contained large CCs associated with a range of activated leukocytes involved in innate inflammation. This observation

supports the thesis that the development and enlargement of CCs in the core of lipid rich plaques may precipitate traumatic and inflammatory injury that may lead to plaque rupture.

Cholesterol crystals drive metabolic reprogramming and M1 macrophage polarisation in primary human macrophages

It is now widely accepted that metabolism plays a major role in immune cell function, and that cells can preferentially utilise specific metabolic pathways to fine-tune their fate and function. Metabolic reprogramming of innate immune cells is emerging as a key player in the progression of a number of chronic diseases, including atherosclerosis, where high rates of glycolysis correlate with plaque instability and disease progression. O'Rourke et al. aimed to investigate if cholesterol crystals, which are key atherosclerosis-associated DAMPs (damage/danger-associated molecular patterns), alter immune cell metabolism and whether this impacts macrophage phenotype and function.

Primary human macrophages were treated with cholesterol crystals and expression of M1 (CXCL9, CXCL10) and M2-associated (MRC1, CCL13) macrophage markers, alarmins, and inflammatory cytokines was assessed by real-time PCR or ELISA. Cholesterol crystal-induced changes in glycolytic markers were determined with real-time PCR and Western blotting, while changes in cellular respiration and mitochondrial dynamics were examined via Seahorse analysis, fluorescence lifetime imaging microscopy (FLIM) and confocal microscopy. Treatment of macrophages with cholesterol crystals upregulated mRNA levels of *CXCL9* and *CXCL10*, while concomitantly downregulating expression of *MRC1* and *CCL13*. Cholesterol crystal-treated macrophages exhibited a significant shift in metabolism to favour glycolysis, accompanied by the expression of key glycolytic markers GLUT1, Hexokinase 2, HIF1α, GAPDH and PFKFB3. Furthermore, these effects were mediated upstream by the glycolytic enzyme PKM2, and direct inhibition of glycolysis or PKM2 nuclear localisation led to a significant reduction in cholesterol crystal-induced inflammatory readouts.

This study provides further insights into how atherosclerosis-associated DAMPs impact immune cell function and highlights metabolic reprogramming as a potential therapeutic target for cholesterol crystal-related inflammation.

Monocyte subsets, T cell activation profiles, and stroke in men and women: The Multi-Ethnic Study of Atherosclerosis and Cardiovascular Health Study

Observational studies and experimental models suggest an important role of immune response and regulation in ischemic stroke pathogenesis. Despite mechanistic data implicating unresolving inflammation in stroke pathogenesis, data regarding circulating immune cell phenotypes – key determinants of inflammation propagation *versus* resolution – and incident stroke is lacking.

Feinstein et al. aimed to comprehensively define associations of circulating immune phenotypes and activation profiles with incident stroke.

The authors investigated circulating leukocyte phenotypes and activation profiles with incident adjudicated stroke in 2104 diverse adults from the Multi-Ethnic Study of Atherosclerosis (MESA) followed over a median of 16.6 years. Cryopreserved cells from the MESA baseline examination were thawed and myeloid and lymphoid lineage cell subsets were measured using polychromatic flow cytometry and intracellular cytokine activation staining. Multivariable-adjusted associations of cell phenotypes with incident stroke and ischemic stroke were assessed using Cox regression models.

Associations of intermediate monocytes, early-activated CD4+ T cells, and both CD4+ and CD8+ T cells, producing interleukin-4 after cytokine stimulation, with higher risk for incident stroke were observed; effect sizes ranged from 35% to 62% relative increases in risk for stroke. In addition, differentiated and memory T cell phenotypes were associated with lower risk for incident stroke. In sex-stratified analyses, positive and negative associations were strong among men but null among women.

Circulating IL-4 producing T cells and intermediate monocytes were significantly associated with incident stroke over nearly two decades of follow-up. These associations were stronger among men and not among women. These findings have implications for future investigation of cellular triggers of inflammation and stroke.

In vivo detection of urokinase-type plasminogen activator receptor (uPAR) expression in arterial atherogenesis using [^{64}Cu]Cu-DOTA-AE105 positron emission tomography (PET)

Urokinase-type plasminogen activator receptor (uPAR) is known to be involved in pathophysiological processes such as wound healing and cancer metastasis. Its role in arterial atherogenesis as a molecular imaging target is not well-established. Khare et al. aimed to non-invasively visualize uPAR expression in atherosclerosis using a novel uPAR-targeting positron emission tomography (PET) tracer [^{64}Cu]Cu-DOTA-AE105.

The authors investigates uPAR expression in human atherosclerotic plaques and cultured cells. A retrospective analysis was performed on patients, who underwent combined PET/CT (n = 10) to measure [^{64}Cu]Cu-DOTA-AE105 uptake in five large arteries, divided into a high and low-risk group based on coronary artery calcium score (CAC score).

Single-cell flow cytometric analysis showed a significantly upregulated uPAR expression upon stimulation in THP-1 monocytes. Flow cytometric and microarray analyses of freshly excised human atherosclerotic plaques showed 73.9 ± 2.9% of mononuclear phagocyte system (MPS) cells expressing uPAR and a greater than 7-fold higher gene expression of plasminogen activator urokinase receptor (PLAUR), integrin subunit alpha X (ITGAX), and cluster of differentiation 163 (CD163). The tissue-to-

background ratios (TBRmax) in five large arteries showed a higher [^{64}Cu]Cu-DOTA-AE105 uptake in the group with high CAC score compared to the group with low CAC score, significantly higher in the ascending aorta and the abdominal aorta.

uPAR is abundantly expressed by MPS cells in atherosclerotic plaques and can be visualized by the novel PET tracer [^{64}Cu]Cu-DOTA-AE105 that may non-invasively detect extracellular matrix remodeling during atherogenesis.

Glutamine synthetase in human carotid plaque macrophages associates with features of plaque vulnerability: An immunohistological study

Glutamine is generated from glutamate and ammonia solely by the enzyme glutamine synthetase (GLUL) in a two-step reaction, the rate of which depends on cofactor availability (divalent metal ions), substrate supply, and the expression and activity of the *GLUL* gene. Sorto et al. previously demonstrated a 2.2-fold upregulation of *GLUL* mRNA in stroke-causing carotid plaques when compared with plaques from asymptomatic patients. In this study, the authors compared in the same cohort *GLUL* mRNA expression with plaque gross morphology, and the colocalization of immunodetectable GLUL protein with histopathological changes and molecular and mechanical mediators linked to plaque development.

Endarterectomy specimens from 19 asymptomatic and 24 stroke patients were sectioned longitudinally and immunostained for GLUL, CD68, α-smooth muscle actin, iron, heme oxygenase-1 and CD163, and graded semiquantitatively in every 1 mm^2. The amounts of cholesterol clefts and erythrocytes were graded. The fibrous cap thickness within each 1 mm^2 area was measured. The association between the local pathological findings was analyzed by a hierarchical mixed modelling approach.

The previously found correlation between *GLUL* mRNA and clinical symptomatology was supported by the increased *GLUL* mRNA in diseased tissue and increased local GLUL immunoreactivity in areas with multiple different atherosclerotic changes. A longer symptom-to-operation time correlated with lower *GLUL* mRNA but few outliers had a significantly higher *GLUL* mRNA levels, which persisted throughout the post-symptomatic period. Plaque ulceration associated with 1.8-fold higher *GLUL* mRNA. Macrophages were the main GLUL immunoreactive cells. GLUL immunostaining colocalized with erythrocytes, iron, CD163, and heme oxygenase-1. The correlations between local variables were consistent in both asymptomatic and stroke-causing plaques. An inverse correlation was found between the fibrous cap thickness and local GLUL immunoreactivity. Considerable variability in interplaque expression pattern of GLUL was present.

These results connect macrophage GLUL expression with carotid plaque features characterizing plaque vulnerability.

Semaglutide reduces vascular inflammation investigated by PET in a rabbit model of advanced atherosclerosis

Atherosclerosis is a chronic inflammatory vascular disorder and a hallmark of coronary artery disease and stroke. Atherosclerotic disease progression involves a multitude of complex pathological processes such as imbalance of cholesterol homeostasis and chronic low-grade arterial inflammation with macrophage accumulation, ultimately leading to endothelial dysfunction. Jensen et al. investigated the effects of semaglutide, a long acting glucagon-like peptide-1 receptor agonist, on atherosclerotic inflammation and calcification using a multimodality positron emission tomography and computed tomography (PET/CT) approach.

Atherosclerotic New Zealand White rabbits were randomized to an intervention (n = 12) or placebo group (n = 11) receiving either semaglutide or saline-placebo. PET/CT imaging was performed before and after 16 weeks of intervention. Three different radiotracers were used: [^{64}Cu]Cu-DOTATATE for imaging of activated macrophages, [^{18}F]FDG imaging cellular metabolism and [^{18}F]NaF PET visualizing micro-calcifications. Tracer uptake was quantified by maximum standardized uptake value (SUVmax) and target-to-background-ratio (TBRmax). Animals were euthanized for autoradiographic imaging and histological analyses.

A reduction in activated macrophage tracer-uptake was observed in the semaglutide group. When imaging cellular metabolism, an attenuation of SUVmax and TBRmax was observed in the semaglutide group. No difference in uptake of the micro-calcification tracer between the two groups was found. Values of macrophage density in the vessel wall were significantly correlated with SUVmax values of the activated macrophage and cellular metabolism tracers.

The results showed that semaglutide decreased vascular uptake of tracers imaging activated macrophages and cellular metabolism but not micro-calcifications compared to a saline placebo. This supports the hypothesis that semaglutide reduces atherosclerotic inflammation by means of decreased activated macrophage activity.