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Monocyte recruitment and macrophage proliferation in atherosclerosis

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INTRODUCTION

In the famous 'Atlas of atherosclerosis: progression and regression', Herbert Stary [1] outlines in remarkable detail the development of human atherosclerosis. The photographs depicting lesions at all stages of development, from newborns to the elderly, capture in stunning detail the complexity of an evolving disease. Type I lesions develop, Stary explains, at sites of non-laminar flow and low shear stress, which disturbs endothelial function. Type II lesions are defined by macrophage foam cells, and type III preatheromas feature small pools of extracellular lipids. As the disease worsens, an easily discernible core of extracellular lipid marks a type IV atheroma, fibrous thickening corresponds to a type V atheroma, the appearance of fissures, haematoma, and thrombi denote type VI atheroma, and finally, calcification is a type VII complicated atheroma [1]. Each of these histological observations reflect a biological process; each has its corresponding cluster of scientists and clinicians devoted to understanding how it can be harnessed to prevent or treat disease. As the underlying pathology causing most myocardial infarctions and strokes, atherosclerosis is, after all, the deadliest disease in the world [2].

Among the many mechanisms that contribute to the development and complications of atherosclerosis, macrophage accumulation occurs early and persists throughout most of a lesion's evolution. The best evidence that macrophages are functionally important — rather than simply markers — can be found in animal studies. The most widely-used murine models of atherosclerosis, the *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, though far from perfect reflections of human disease, are nevertheless fair approximations to show how lesions develop. In both models, macrophages are prominent in early and advanced lesions, where they ingest oxidised lipoproteins via scavenger receptors and, as lipid-rich foam cells, become part of the disease's physical bulk [3]. Although many of the functions by which macrophages influence atherosclerosis have been deciphered, their ontogeny has continued to perplex. On the one extreme, lesional macrophages may be developing from resident precursors or stem cells through local differentiation and proliferation, requiring no input from the circulation. At the other extreme, macrophage accumulation may be the exclusive consequence of replenishment from blood monocytes, requiring no input from resident cells. Identifying the mechanisms can be therapeutically relevant. If macrophages are harmful to disease and originate only from local precursors, then approaches targeting the vessel wall and its environment should be explored. If, however, macrophages originate exclusively from blood monocytes, then targeting environments where monocytes arise, such as the bone marrow or spleen, may be the more effective strategy.

MONOCYTE RECRUITMENT IN ATHEROSCLEROSIS

In animal models of atherosclerosis, blood monocyte number associates with the severity of the disease (reviewed in [4]), and in humans leukocytosis predicts for cardiovascular events, although neutrophils appear to be more predictive than monocytes (reviewed in [5, 6]). Considerable evidence implicates monocytes as critical to the development of atherosclerosis [4, 7–18]. Monocytes arising in the bone marrow from haematopoietic precursors, and from the spleen via extramedullary haematopoiesis, enter the blood via the chemokine receptor CCR2, and circulate. Upon encountering the activated endothelium, monocytes infiltrate the vessel wall and differentiate to lesional macrophages. In the absence of the chemokine receptor CCR2 (and indeed while blocking CCR2, CX3CR1, and CCR5), atherosclerosis does not develop [8, 14, 16, 17, 19, 20], presumably because mice lacking CCR2 have severely diminished levels of circulating monocytes. It has been widely believed that the influx of monocytes is the key event that determines macrophage accumulation, but a few inconsistencies in the literature cast a shadow of doubt over this theory. Some studies have shown, for example, that interference of monocyte development or recruitment has little, if any, effect on established atherosclerosis [21–24]. More recent work on macrophage

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ontogeny shows that tissue macrophages do not depend on monocytes in the steady state [25] or during inflammation [26]. In models of atherosclerosis, a population of haematopoietic progenitors has been identified in the adventitia [27] revealing a potential alternative explanation for how disease evolves. The question then is whether the various findings are reconcilable, or whether they reflect inherent methodological limitations.

MACROPHAGE PROLIFERATION IN ATHEROSCLEROSIS

The many observations that monocytes are required for the development of atherosclerosis have contributed to the perception that a single infiltrating monocyte yields one, terminally-differentiated, macrophage. However, increased cellularity could also result from proliferation of cells within the plaque. Evidence for macrophage proliferation in atherosclerotic lesions has been reported in humans, rabbits, and mice [28–34], but its importance relative to monocyte influx has not been assessed. *In vitro* experiments have shown that macrophages can proliferate in response to oxidised low density lipoprotein, possibly involving PI-3-kinase [35–37]. Collectively, these observations prompted us to re-examine how atherosclerosis develops.

To determine the relative importance of monocyte influx and macrophage proliferation in atherosclerosis, we used several methodologies that merged the history of proliferation (constant bromodeoxyuridine [BrdU] delivery) with the history of location (parabiosis). Our experiments led us to five major conclusions [38].

Firstly, macrophage turnover in lesions is rapid. Using BrdU, a nucleotide analogue that incorporates into the genome during cell division, we show that after four weeks the entire macrophage population in the intima is replaced.

Secondly, turnover is largely the result of local macrophage proliferation rather than monocyte recruitment. To arrive at this conclusion, we employed a combination of parabiosis and BrdU tracking experiments; parabiosis is a surgical method that establishes a shared circulation between two mice and which can discriminate between local and blood cell origins.

Thirdly, cell recruitment is important in early lesions but, as atherosclerosis develops, local macrophage proliferation dominates. To this end, we compared recruitment and proliferation in early and established atherosclerosis.

Fourthly, non-proliferating, circulating monocytes infiltrate lesions avidly and can differentiate to proliferating macrophages. We performed adoptive transfer and parabiosis experiments, confirming earlier work that monocytes migrate to lesions continuously [16, 19].

Fifthly, type 1 scavenger receptor class A (SR-A) contributes to local macrophage proliferation. The data to support this last observation utilised mixed chimeric mice with a BrdU-chase method, allowing for direct comparison of macrophage proliferation in wild type and SR-A-deficient cells.

On the basis of these observations, we proposed a unified scenario (Fig. 1). Lesions grow, we speculate, because of a multi-phasic numerical escalation of the monocyte-macrophage lineage. The escalation involves the production of

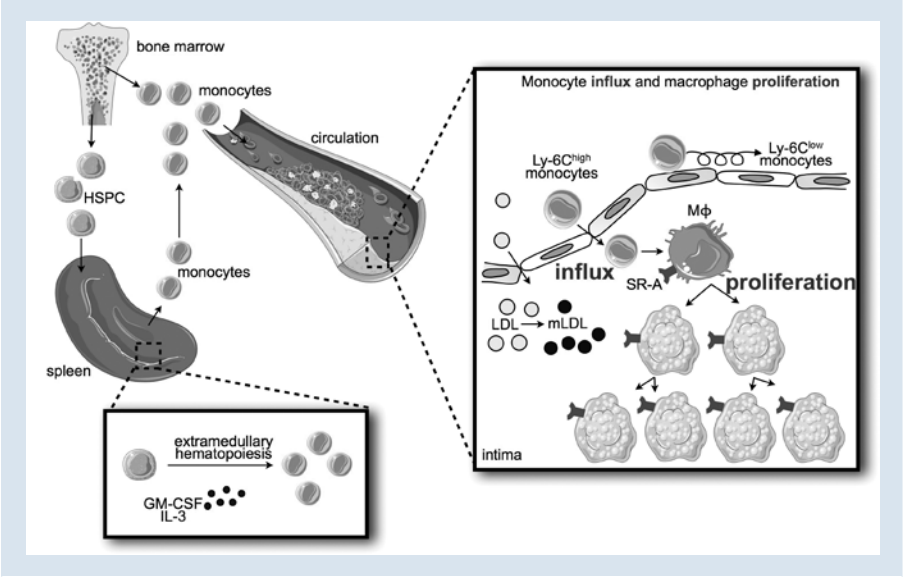

Figure 1. Model of monocyte influx and macrophage proliferation in atherosclerosis. Monocytes are produced in the bone marrow and spleen. Upon mobilisation, monocytes circulate and infiltrate the growing lesion. In lesions, monocytes differentiate to macrophages. Lesional macrophages then proliferate in response to environmental cues; HSPC — haematopoietic stem and progenitor cells; LDL — low density lipoprotein; mLDL — modified low density lipoprotein; IL-3 — interleukin-3; GM-CSF — granulocyte macrophage colony stimulating factor; SR-A — scavenger receptor class A; MΦ — macrophage. Cell and organ images courtesy of Servier Medical Art Image Bank (http://www.servier.com/Powerpoint-image-bank)

monocytes from haematopoietic stem and progenitor cells in the bone marrow and spleen, monocyte mobilisation and circulation, their infiltration to the lesion, differentiation, and eventual proliferation.

Lesional macrophage proliferation, therefore, locally augments macrophage numbers in plaques.

IMPLICATIONS AND FUTURE DIRECTIONS

The finding that atherosclerosis evolves by both monocyte influx and local macrophage proliferation raises many questions [39, 40]. If local macrophage proliferation is a pathological event that propagates lesional instability, then future therapeutics may need to deliver agents that specifically target macrophage proliferation in lesions. Alternatively, if 'surges' of monocyte recruitment in response to certain triggers destabilise lesions, then targeting monocyte recruitment at crucial moments may be the desired clinical goal. Recent studies have shown that myocardial infarction accelerates atherosclerosis by augmenting lesional monocytes and macrophages [41], which supports the idea that the relative contribution of monocyte migration and macrophage proliferation fluctuates in response to external stimuli. Critical issues that remain unanswered include the phenotype and function of proliferating macrophages, the molecular mechanisms that regulate proliferation, and the contribution of each process to lesional stability.

Macrophages can be classified into functionally distinct subsets *in vitro* and *in vivo* [42–44]. During differentiation, macrophages undergo either classical M1 or alternative M2 activation, although other subsets have been described. M1 macrophages express inflammatory cytokines and nitrogen and oxygen intermediates, whereas M2 macrophages participate in tissue remodelling, wound healing, and immune regulation. Highly phagocytic, M2 macrophages express scavenging molecules, mannose and galactose receptors, and produce ornithine and polyamines through the arginase pathway. It is not known whether proliferating macrophages are M1, M2, or whether they fall somewhere in between. Proliferation could either be a common event to any macrophage or a specialised process of a specific state.

Scavenger receptors promote lipoprotein ingestion, macrophage apoptosis, apoptotic cell and debris clearance, and signal transduction [45]. SR-A fosters the uptake of oxidised low density lipoproteins [46, 47], which can induce peritoneal macrophage proliferation *in vitro* [48]. The *in vivo* data reveals a complex SR-A biology, positioning the receptor as a mobiliser of the TLR4-JNK-IFNβ signalling pathway [49] that contributes to lesion complexity through its influence on apoptosis and inflammatory gene expression [50]. It remains to be determined whether SR-A mediates cell division directly, by its effects on downstream cell transduction pathways, or indirectly through the physical act of lipoprotein ingestion.

Another important issue that needs to be resolved is the relative importance of monocyte recruitment and macrophage proliferation to lesional instability. Macrophage proliferation may be a protective response to increase the number of cells capable of sequestering lipids. Alternatively, proliferating macrophages may be more inflammatory and more susceptible to apoptosis than non-proliferating macrophages. It is also possible that waves of monocyte recruitment destabilise lesions. Studies utilising human plaques can be particularly informative. Quantification and localisation of proliferating and non-proliferating monocytes and macrophages in the context of precise lesional definitions will allow investigators to generate a 'signature' of the relative contribution of these processes to human disease. Laser-capture microscopy on sections with high vs. low concentrations of proliferating macrophages, for example, can correlate proliferating regions with SR-A expression, oxidative stress, reverse cholesterol transport, and inflammatory gene expression, among others. Future treatment strategies to prevent the generation of vulnerable lesions might interfere with lesional macrophage proliferation (drug-eluting stents) or with monocyte influx after events such as myocardial infarction. Because our understanding of these processes is still rudimentary, much more basic science is required to determine whether the delivery of myeloid cell-specific anti-proliferative or anti-migratory agents to patients could be an option.

Without monocytes and macrophages, atherosclerosis will not develop, but the cells are also required for host defence. Currently, preclinical trials aim to test the benefits of blocking leukocyte recruitment [51, 52], and indeed, pharmaceutical companies may be thinking seriously about bringing these approaches to humans. If macrophage proliferation is a major mechanism by which atherosclerosis develops, as our studies suggest, then alternative, perhaps more precise, approaches to targeting leukocytes in atherosclerosis should be explored.

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