

Staging Lymphocyte Presence in Human Atherosclerosis: A Tale Told by Numbers

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The involvement of the adaptive immune system in the development of atherosclerosis has been underpinned by a wealth of experimental animal studies (reviewed in a previous work¹). Whereas T and B cells have been detected in human atherosclerosis, in this issue of *JAHA*, van Dijk et al. provide a next level of insight into the relevance of adaptive immunity to human disease.² This comprehensive histopathology study addresses temporal and spatial patterns for a broad range of T- and B-cell subsets during human plaque progression in perirenal aortic atherosclerosis specimen. Specifically, they delineated total T cells (CD3⁺), T helper (Th; CD4⁺), cytotoxic T cells (CD8⁺), naïve (45RA⁺) and memory T cells (45RO⁺), regulatory cells (Treg; FoxP3⁺), and Th-cell polarization (Th1 [CD4⁺Tbet⁺], non Th1 [CD4⁺Tbet⁻], and Th17 [CD4⁺/IL-17⁺]). In addition, CD20⁺ B cells and CD138⁺ plasma cells were assessed, as well as expression of B-cell maturation (AID/CD21) and lymphoid tissue (C-X-C motif chemokine 13; CXCL13) markers. The researchers even interrogated atherosclerotic plaque stages beyond rupture, providing a unique perspective on postevent processes after clinically silent cases of plaque rupture. Their major findings with regard to progression of disease include:

- 1 Progressive accumulation of CD4 and CD8 cells up to the stage of plaque rupture, with a striking decline in the aftermath of rupture, especially in the intima.
- 2 Over-representation of CD8 T cells in the earlier stages of disease, with more-balanced CD4-CD8 ratios later on.

- 3 Surprising abundance of non-Th1 cells (Th0 and Th2), virtual absence of Th17, and scarce presence of Treg throughout disease, with a predominant adventitial T- and B-cell residence in tertiary nonlymphoid follicles.

The CD4 and CD8 T-cell accumulation with disease progression, with up to 25-fold higher contents in ruptured plaque versus healthy arteries, is a first highlight of this study. It once more stresses the importance of including adventitia in the histological assessment, given that this compartment harbored the majority (70%!) of T cells. T cells were observed to reside in adventitial tertiary nonlymphoid organs, as reported for mice.³ Intriguingly, major changes in plaque T-cell patterns occurred during the transition from early to late fibroatheroma in whole plaque, intima, and plaque shoulder, but also in the media, typically smooth muscle territory. In fact, T cells could only be detected in the media, from the advanced atheroma stage onwards. The augmented plaque T-cell accumulation at this point in disease progression appears to echo the proinflammatory necrotic core expansion and the increased formation of intraplaque microvessels (angiogenesis) that occurs in advanced fibroatheroma, as previously shown in coronary lesions.⁴ Conceivably, adventitia-derived microvessels projecting into the intima could represent an additional entry point for circulating lymphocytes, as shown in *ApoE*^{-/-} mice,^{5,6} thus facilitating T-cell invasion into the plaque. In support of this notion, van Dijk et al. show T and B cells closely associated to microvessels.²

Another finding pertaining to T-cell dynamics is the profound T-cell decline subsequent to thrombus reorganization. Specifically, intimal non-Th1 and memory T cells appear to disappear, which is paralleled by a reduction in CXCL13 expression in tertiary nonlymphoid organs. Although speculation at this point, the reduced T-cell abundance could stem from halted migration and proliferation, enhanced T-cell migration, or apoptosis. Inflammation resolution in plaques with reorganized thrombi will likely limit chemokine secretion or availability, preventing influx and proliferation of T cells. During resolution, T cells may egress from plaques with reorganized thrombi resulting from increased lymphatic drainage. Finally, the rupture-associated

The opinions expressed in this article are not necessarily those of the editors or of the American Heart Association.

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J Am Heart Assoc. 2015;4:e001909 doi: 10.1161/JAHA.115.001909.

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influx of cholesterol-rich erythrocytes, together with oxidative stress generated by attracted phagocytes, will produce oxidized cholesterol, stimulating T-cell apoptosis.

A second important highlight is the dominance of CD8⁺ T cells in early stages of disease. This aligns well with human and murine findings,⁷ showing increased expression of interferon-gamma in CD8⁺CD28⁺ T cells in early plaques as well as in CD4⁺ T cells in advanced atherosclerosis. CD8⁺ T cells have been shown to exert both protective (regulatory CD8⁺ T-cells)⁸ and proatherogenic functions in *ApoE*^{-/-} mice (CD8⁺ effector cells).⁹ Establishment of the exact nature of plaque-associated CD8⁺ T cells will help to shed light on the functions of this subset in early atherosclerosis. The differential dynamics of CD4⁺ and CD8⁺ T-cell presence in human atherosclerosis hints to divergent chemotactic cues and/or plaque entry pathways for CD4⁺ and CD8⁺ T cells. In support, the location of CD8 and CD4 T cells appears to differ, with an even distribution of the former subset over plaque adventitia, media, and intima, whereas the latter predominantly invade the adventitia, compatible with earlier findings in experimental atherosclerosis.⁵

Similar to T cells, B cells also predominate the adventitia and tend to cluster into CXCL13⁺ follicles; unlike T cells, however, B cells are essentially lacking in the plaque intima. B cells were absent in early stages of disease, in line with previous studies reporting B-cell scarcity in carotid atherosclerosis.¹⁰ Plaque B cells mainly involve the CD21⁻AID⁻ immature subset. This contrasts with the confirmed AID⁺ mRNA expression in carotid plaque and adventitia, whereas CD138 was absent.¹⁰ However, it should be kept in mind that with the scanty presence of B and plasma cells in plaques, the sample size may have been insufficient to draw firm conclusions.

A third highlight involves the plaque T-cell polarization patterns, with a surprising enrichment of non-Th1 cells (Th0, Th2, and $\gamma\delta$ T-cells) over Th1 cells in plaque, a complete absence of Th17, and a surprising scarcity of Treg. Although activity and location, rather than mere numbers, may be decisive, these data seem to disqualify the impact of Th1, and, to a lesser extent, Th17 and Treg, on human atherosclerosis and suggest more-prominent roles for Th2 and possibly $\gamma\delta$ T cells. While hardly any data are available on a role of $\gamma\delta$ T cells in atherosclerosis, the observed Th2 enrichment contrasts with findings in experimental models and with the reported inverse association of circulating Th2, but not Th1, cell counts with human cardiovascular disease.¹¹ The actual contribution of Th17 in atherosclerosis is still subject to controversy, its effects appear to be rather subtle,¹² and this study seems to underscore this view.

Conceivably, however, B-cell-, Th1-, Treg-, and/or Th17-mediated immunity may not be driven from within the plaque, but from plaque-draining lymph nodes or even the periphery,

considering also their predilection for adventitial and microvessel clustering. This is supported by a similar B-cell IgG repertoire in human adventitia and draining lymph nodes, whereas different clonotypic markers appear to be present in the plaque.¹⁰ For Th1 cells, peripheral Th1 could act by controlling B-cell antibody class switching or cytotoxic T-cell instruction, rather than by exerting effector functions. For Treg and Th17, this is a less likely option given that, in other chronic inflammatory disorders, these subsets were observed to accumulate at the inflammatory hotspot, in close proximity to effector T cells to provide effective suppression.

In this regard, the present study touches upon fundamental differences between human and mouse atherosclerosis, prompting more anthropocentric, rather than mouse-model-based, strategies to dissect atherosclerosis related adaptive immune responses. Several factors could explain this cross-species discrepancy. First, unlike in murine extreme hypercholesterolemia models, human atherosclerosis is a multifactorial disease, involving a.o. hypertension, hyperglycemia, and obesity, accompanied by mild hyperlipidemia. Second, the rapid development of atherosclerosis in murine models, compared to decades of progression, in human arteries represent a wholly different process. In human disease, immune cell dynamics and turnover are, presumably, more critical than in mice. Finally, murine models on a C57Bl/6 background are inherently Th1 skewed.¹³ Human T-cell-driven immune responses are more balanced. As van Dijk et al. show, Th1 are not the dominant subset in human plaque and represent only 5% to 10% of all CD4 T cells. This contrasts with previous studies reporting a Th1 dominance in blood¹¹ and carotid plaque.¹⁴ In these studies, Th1 skewing was associated with atherosclerosis, but also with cytomegalovirus. While serology status was not given in van Dijk et al.'s cohort, this may explain differences with published data.

Although this study raises provoking questions regarding the presence and relevance of specific adaptive immune cell subsets in human atherosclerosis, some caution should be taken in generalizing these findings to the patient presenting with acute myocardial infarction or stroke. Analysis, however elaborate, of a single vascular bed by a single technique, as well as the age and health status of the donors, pose some limitations. First, data are lacking on circulating or lymphoid T- and B-cell presence in this patient cohort, rendering it impossible to link plaque-resident to peripheral immunity and to exclude patients with aberrant lymphocyte status. Although several studies show a close association between circulating and coronary plaque-resident Tregs and memory T cells,^{15,16} others observed increased activation of plaque-resident versus circulating T (HLADR⁺CD3⁺) and B cells (CD20⁺CD69⁺).¹⁷ Second, though immunohistochemistry is particularly apt to pinpoint the arterial location of immune cells, its discriminative capacity is limited to 2 or 3 subset markers, in

contrast to flow cytometry. In general, the use of single markers has some disadvantages. For instance, CD3 also recognizes natural killer T cells, whereas expression of foxp3 is not confined to CD4 Treg, but may extend to CD8 Treg, Breg, and macrophage subsets. Third, plaque morphology and content depends on vascular bed, age, and gender. In fact, the frequency of advanced plaques in the carotid artery harboring Tregs is 82% (31 of 39),¹⁸ as compared to only 4% of advanced atheroma in the aorta shown here² (2 intima/45 progressing and vulnerable atheroma). Plaque Treg,¹⁸ Th17,¹⁹ and mature B-cell¹⁰ density seems slightly higher in carotid arteries, possibly owing to more-effective infestation of carotid over aorta plaques. The fact that, in this study, plaque (including adventitia) were interrogated from younger, and generally healthier, donors than usual may have impacted the findings as well. Indeed, diabetes is known to enhance to CD45RO⁺ memory T-cell presence in coronary atherosclerosis,²⁰ whereas also gender and age are likely to affect plaque morphology and adaptive immunity. In future, this valuable cohort may be expanded to be able to dissect gender/age/disease effects of adaptive immunity in atherosclerosis.

In conclusion, the present study raises important questions about the presence and relevance of adaptive immunity in human atherosclerosis, which will prompt us to revisit some of the prevailing murine-derived conceptions of adaptive immune responses in atherosclerosis. Moreover, it may foster new investigations into the immune-cell responses subsequent to plaque rupture and thrombus reorganization.

Disclosures

None.

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Key Words: Editorial • atherosclerosis • immune system • lymphocyte