SHORT COMMUNICATION

Leukocyte Dynamics during the Evolution of Human Coronary Atherosclerosis

Conclusions from a Sevenfold, Chromogen-Based, Immunohistochemical Evaluation

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Atherosclerosis is a complex process with strong inflammatory component. We developed a straight-forward sevenfold staining protocol for simultaneous assessment of dominant leukocyte classes, vascularization, and expression of the putative foam cell maker CD36. The method was applied on human coronaries covering the full spectrum of atherosclerotic disease. Results confirm the progressive association of macrophages and T cells with the process and a global presence of mast cells. B cells are exclusively present in adventitial follicles that accompany the process plaque destabilization (thin cap and ruptured lesions) and are otherwise absent. Neutrophils are only present as part of the hemorrhage that accompanies plaque rupture. This study does not classify CD36 as a key factor in foam cell formation. Observed macrophage accumulation in the neointima of stabilized fibrous calcified plaques is consistent with a process of neoatherosclerosis. This study on human coronaries shows a progressive association of macrophage and T-cell abundance with plaque progression. Follicle-like structures are transiently present during the process of plaque destabilization. Plaque healing is accompanied by cessation of the inflammatory response but followed by a new cycle of atherosclerosis. (*Am J Pathol* 2018, 188: 1524–1529; <https://doi.org/10.1016/j.ajpath.2018.03.011>)

The atherosclerosis process is a complex pathology that is driven by an intricate interaction of metabolic and inflammatory factors.^{1,2} The process proceeds through a sequence of distinct stages (ie, disease initiation, plaque formation, plaque progression, and plaque rupture, followed by plaque healing and consolidation).³ Aspects of the inflammatory response are critically involved in the disease initiation and progression and its complications, but they also contribute to the resolution of the disease.

Insight in the interactions of the cellular and molecular inflammatory aspects in the atherosclerotic process critically relies on imaging tools, because only this approach allows for appreciation of the crucial spatial and contextual relationships between the different factors. At this point, most studies

depend on immunofluorescence for visualizing the spatial distribution of multiple targets of interest. Although this technique is well established, its visualization window is limited, and specific high-end scanning techniques are required to provide adequate contextual overviews. An additional challenge, particular in the context of atherosclerosis, is interference caused by the autofluorescence of the collagen and elastin-rich matrix.^{4,5} This interference can be avoided by using (near infra) red fluorochromes or two-photon microscopy⁶; then again, this limits the versatility

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Table 1 Details of the Antibodies Used

Antibody	Target	Manufacturer	Product no.	Clone	Source	Dilution	Chromogen
CD4/CD8	T-helper/cytotoxic T cells	Dako (Glostrup, Denmark)/Dako	M7310/M7103	4B12/C8-144B	Monoclonal mouse	1:200/1:300	DAB
CD20	B cells	Dako	M0755	L26	Monoclonal mouse	1:10,000	Bajoran
CD36	Scavenger receptor	Cell Signaling (Leiden, the Netherlands)	14347	D8L9T	Rabbit monoclonal	1:500	Ferengi blue
CD68	Macrophages	Dako	M 0814	KP1	Monoclonal mouse	1:2000	Vulcan red
Myeloperoxidase	Neutrophils	Dako	A0398	-	Polyclonal rabbit	1:7500	Deep space black
Tryptase	Mast cells	Dako	M 7052	AA1	Monoclonal mouse	1:3000	Stay green
Ulex/anti-ulex	Endothelium	Vector Labs (Amsterdam, the Netherlands)	L-1060/BA-0064	-	Goat polyclonal	1:2000	Vina green

DAB, diaminobenzidine.

of the approach and requires nonstandard microscopy techniques.

Traditional chromogen-based immunohistochemistry provides superior contextual information and thereby allows for a direct spatial interpretation of expression pattern. On the downside, multiple staining chromogen techniques are relatively poorly developed and considered technically challenging; as a consequence, they are not commonly used.⁷

In this context, we developed a straightforward, versatile, seven-color chromogen-immunohistochemistry platform for light microscopy, and we applied the platform to validate and extend earlier observations on the leukocyte dynamics obtained in the aorta^{8,9} for human coronary atherosclerosis.

Materials and Methods

Patients and Tissue Sampling

Material used in this study is of a large biobank of left coronary artery segments (>750 specimens). Use of the material is approved by the Medical and Ethical Committee of the Leiden University Medical Center (Leiden, the Netherlands). Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical Committee of the Leiden University Medical Center and the code of conduct of the Dutch Federation of Biomedical Scientific Societies. In all cases, permission for transplantation-orientated research was given, or permission was inherent to donation. Because of national regulations, only transplantation relevant data from donors were available for research (<http://www.federa.org/codes-conduct>, last accessed May 7, 2018).

Proximal left coronary artery segments were collected during aortic valve dissection for tissue banking. In short, the aortic valve is carefully removed from the intact heart at the Euro Heart Valve Bank (Beverwijk, the Netherlands). The surrounding tissue was removed, and the valve was banked for transplantation. The removed surrounding fat tissue containing a segment of the proximal left coronary

artery (approximately 2 to 3 cm) was formaldehyde fixed and used in this study.

The formaldehyde-fixed tissue was decalcified (Kristensen's solution) to allow proper sectioning. Coronary arteries were divided in consecutive 5-mm segments and paraffin embedded, and 4- μ m tissue sections were prepared from each segment. Movat pentachrome staining was performed for each 5-mm segment, and each tissue block was classified (revised classification of the American Heart Association, as proposed by Virmani and colleagues³). In sections with multiple lesions, grading was dictated by the most advanced lesion present.

Immunohistochemistry

Sections were immunostained using antibodies directed against CD4, CD8, CD20, CD36, CD68, myeloperoxidase, tryptase (mast cells), and ulex/anti-ulex. Details of antibodies used for immunohistochemical staining are listed in Table 1.

This multiple staining protocol is based on repetitive staining cycles, each separated by a neutralization step. The following neutralization strategies were evaluated: heat-induced antigen retrieval with Tris-EDTA, pH 9.0, or citrate, pH 6.0, buffer (10 minutes in preheated buffer maintained at 95°C in a household vegetable steamer); commercial denaturing solution (DNS001L; Biocare, Duiven, the Netherlands), SDS-glycine buffer, or commercial stripping buffer (21059; Thermo Fisher Scientific, Bleiswijk, the Netherlands).

For the actual staining experiments, citrate, pH 6.0, heat-induced antigen retrieval was used as the initial antigen retrieval step and commercial stripping buffer in the following staining steps. Antibody binding was visualized using a polymer-based horseradish peroxidase secondary system [EnVision (Dako A/S, Glostrup, Denmark) or Vectastain ABC-HRP Kit (Amsterdam, the Netherlands) for ulex] and the alkaline phosphatase-based mouse or rabbit MACH 2 polymer system (Biocare Medical, Concord, CA), along with matching chromogens (Table 2).

Table 2 Chromogens Used for Visualization and Their Stability for the Various Elution Methods

Chromogen	Product no.	EDTA (pH 8.0), 10 mmol/L	Citrate (pH 6.0), 10 mmol/L	Glycine–10% SDS (pH 2.0), 25 mmol/L	Glycine-NaOH (pH 10.0), 100 mmol/L	Stripping buffer
DAB	K400711–2*	++	++	++	++	++
Bajoran	BJP811†	++	+	–	++	–
Vina green	BRR807A†	–	–	–	–	–
Romulin AEC	RAEC810†	++	++	–	+/–	++
Deep space black	BRI4015†	++	++	+/–	+	+/–
Warp red	WR806†	++	++	+	++	+
Ferengi blue	FB813S†	++	++	++	++	++
Vulcan red	FR805†	++	++	++	++	+
Stay green	Ab156428‡	++	++	++	++	++
Stay yellow	Ab169561‡	++	++	++	++	++

+, slight loss of staining; –, full loss of staining; ++, stable staining; +/–, severe fading of staining.
*Dako.
†Biocare Medical.
‡Abcam (Cambridge, UK).

Slides were not counterstained. Tissue sections from human tonsils and abdominal aortic aneurysms were used as positive controls; negative controls were performed by omitting the primary antibody. Images were acquired on a Philips Ultra Fast Slide Scanner (Philips Digital Pathology Solutions, Best, the Netherlands), and color deconvolution analysis was performed on the Nuance multispectral imaging system (Perkin Elmer, Oss, the Netherlands).

Results

This multiple staining platform is based on repetitive, simultaneous, double-staining procedures that combine horseradish peroxidase and alkaline phosphatase–labelled secondary antibodies. The efficacy of various heat retrievals and stripping buffers to neutralize or strip (erase) the signal of the preceding primary and secondary antibodies was evaluated. Although conventional citrate or EDTA heat retrievals adequately neutralize the preceding staining sequence, the required heating step results in tissue damage and potentially damages epitopes of interest. To minimize heat-induced tissue damage, different antibody elution procedures were evaluated. In our hands, commercial denaturing solution was insufficient for signal clearance, whereas glycine-SDS or high pH buffers required incubation at 50°C or more for effective clearance. Superior results, both with respect to antibody elution and epitope stability, were obtained with commercial stripping buffer intended for Western blot, which could both be performed at 37°C.

The method further relies on the stability of the chromogens. Table 2 shows that the stability of the precipitates formed varies, with stable precipitates formed by chromogens, such as diaminobenzidine and Ferengi blue, but full elution of precipitated Vina green in an aqueous environment.

A third point of consideration is the stability of the epitopes of interest. We aimed at identifying different leukocyte class by combining CD20 (B cells), the putative

foam cell marker CD36 [fatty acid translocase (recommended name, platelet glycoprotein 4)], CD68 (macrophages), myeloperoxidase (neutrophils), tryptase (mast cells), and ulex/anti-ulex (endothelium). A CD4/CD8 antibody mixture proved superior to CD3 staining for the identification of T cells. With the exception of CD4/CD8, the stripping procedure did not influence epitope recognition. T-cell staining was therefore included in the initial staining cycle.

The feasibility of the method is illustrated on the abdominal aortic aneurysm section (Figure 1). This tissue was chosen for illustration purposes because the disease involves almost all cellular components of the inflammatory spectrum. On establishment of an adequate staining procedure, the method was used to map the cellular immune response during atherosclerosis progression in left common coronary artery samples.³ Thirty representative tissue sections covering the full spectrum of the disease (three different sections for each stage of the disease) were randomly selected from the biobank for the sevenfold staining. Means ± SD age of the donors included was 51 ± 11 years, and 64% of the samples were from a male donor.

With the exception of occasional adventitial mast cells, macrophages, and T cells, leukocytes were absent in normal coronary samples. Adaptive intimal thickening and intima xanthoma are characterized by the presence of scattered intimal macrophages in both intima and adventitia. Most macrophages were negative for the scavenger receptor CD36 (Figure 2).

Appearance of an extracellular lipid pool (pathologic intimal thickening) and a necrotic core (early and late fibroatheroma) hallmark transition to progressive disease.³ These stages are characterized by macrophage and T-cell abundance in the shoulder areas of the acellular core. The necrotic core was notably enriched for the scavenger receptor CD36, whereas most macrophages/foam cells were negative for this receptor. Transition to so-called vulnerable lesions (ie, thin cap fibroatheroma and ruptured plaque) was accompanied by appearance of T- and B-cell rich adventitial

Figure 1 **Top panel:** Positive control (aortic abdominal aneurysm) illustrating the sevenfold staining. T-cell [CD4/8⁺: diaminobenzidine (DAB; brown)], B-cell [CD20⁺: Bajoran (purple)], macrophage [CD68⁺: Vulcan red (red)], mast cell [tryptase⁺: Stay green (dark green)], neutrophil [myeloperoxidase⁺: deep space black (black)], scavenger receptor [CD36⁺: Ferengi blue (blue)], and endothelial cell [ulix: Vina green (bright green)] staining. **Bottom panels:** Color deconvolution using the Nuance spectral imaging system, illustrating separation of the individual chromogens. Mast cells are absent in the field used for color deconvolution. Original magnification: $\times 200$ (**top panel**); $\times 100$ (**bottom panels**). B, CD20 (Bajoran, B cells); E, ulix (Vina green, endothelium); MC, mast cell; M ϕ , CD68 (Vulcan red, macrophages); N, myeloperoxidase (space black, neutrophils); P, plain image; SR, CD36 (Ferengi blue); T, CD4/8 (DAB, T cells).

tertiary follicles and presence of macrophages in the cap. Neutrophils are now observed as part of the hemorrhage that accompanies plaque rupture.

Plaque healing and consolidation (scarring and fibrous calcified plaque³ formation) after plaque rupture were accompanied by cessation of inflammation, with minimal leukocytes present in the fibrous calcified plaque lesions. Yet, abundant CD36-negative subendothelial macrophages can be observed in neointimal areas that overlay the consolidated lesion.

Discussion

We herein present a systematic evaluation of the cellular aspects of the inflammatory process in coronary atherosclerosis. This evaluation is based on a straightforward and versatile platform for multiplexed chromogen-based immunostaining. Data presented incorporate seven target epitopes that were stained in four successive cycles.

Theoretically, the staining could be extended by additional cycles, yet further extension is limited by the availability of chromogens and overlap between the different colors. Overlap (and steric hindering by the diaminobenzidine precipitate)¹⁰ is also a limitation when performing colocalization studies; in our hands, the best results for costaining are achieved with red (Vulcan red) and blue (Ferengi), and these chromogens were chosen for CD68/CD36, for which colocalization was anticipated. Other combinations of chromogens do not result in secondary colors that are easily discriminated by the human eye, yet spectral imaging was successfully applied for the interpretation of colocalization of triple stainings for macrophage classification.⁹

Although coelution of the high-contrast chromogen Bajoran in stripping buffer limits its use to the final staining cycle, it opens up the opportunity for repetitive staining cycles on a single tissue section. This then allows generation of successive overlays of scans or images of the exact same area of interest.

Figure 2 Leukocyte dynamics in the process of cardiac atherosclerosis (sevenfold staining). Representative stages (Virmani classification) shown in the image are as follows: normal (A), intima xanthoma (IX; B), pathologic intimal thickening (PIT; C), early fibroatheroma (EFA; D), thin cap fibroatheroma (TCFA; E), ruptured plaque (RP; F), and fibrous calcified plaque (FCP; G). **A:** Minimal macrophages, T cells, and mast cells are present in a normal coronary. **B:** Incident foam cells, but absent extracellular lipid pools, characterize the IX. The IX overview (B, left panel) shows a cluster of intimal foam cells. Higher-magnification images (B, middle and right panels) show diffuse (B, middle panel) and an intimal cluster (B, right panel) of CD68⁺ foamy macrophages. Arrowheads indicate the foam cells. In IX, all macrophages are negative for the scavenger receptor (CD36⁺). **C:** PIT is characterized by presence of a lipid pool (LP; C, right panel). These lipid pools are best appreciated in Movat staining and are poorly visible in this plain section. There is an abundance of macrophages (most of which are CD36⁺) in the area overlying the lipid pool. **D:** EFA is hallmarked by presence of bona fide necrotic core (nc), which stains positive for the scavenger receptor (CD36⁺). There are clusters of macrophages overlying the necrotic core [arrowheads in the lower-magnification image (D, left panel)] and macrophage heterogeneity with CD36⁺ (red) and CD36⁺ (purple) macrophages (D, right panel). **E and F:** Plaque destabilization [TCFA (E) and RP (F)] is accompanied by (B- and T-cell rich) tertiary follicle formation in the underlying adventitia (arrowheads) and a cap rich in elongated heterogenic (CD36⁺/CD68⁺) CD68⁺ cells. The high-magnification inset in the right panel TCFA image illustrates the close relationship between vaso vasorum (green) and the tertiary follicle. Diffuse neutrophils (indicated by arrows in right panel in F) accompany the hemorrhage in RP. **G:** Plaque healing (HR; not shown) and scarring (FCP) is accompanied by cessation of inflammation, vanishing of adventitial follicles (HR), and, in FCP lesions, appearance of neoatherosclerosis (clusters of neointimal CD68⁺ cells; arrowheads) in the neointima over the consolidated old lesion (G, middle panel). Scale bars: 2 mm (A–G, left panels); 200 μ m (A–G, right panels).

The staining platform was developed to validate the earlier mapping of the innate and adaptive immune responses in human aortic atherosclerosis for the human coronary.^{8,9} This validation was considered relevant considering the clear topographic variations in atherosclerosis susceptibility,¹¹ the location-specific risk profiles,^{12,13} and the distinctive immunologic makeup of the various vascular beds,¹⁴ but also as an independent validation of the earlier findings for the aorta.

In the process of developing platform, and in retrospect in earlier stainings, poor performance of the CD3 staining was noted, with all CD3 antibodies tested underestimating the anticipated CD3 count (on the basis of the sum of separate CD4 and CD8 stainings). This underestimation may reflect a high susceptibility of the CD3 epitope to organ/tissue

procurement and/or impaired CD3 recognition on paraffin-embedded tissue. To avoid this problem, we decided to use a CD4/CD8 cocktail for identifying T cells.

Findings for the leukocyte subsets for the coronary mimic those reported earlier for the aorta, with endogenous presence of mast cells and absence of infiltrating neutrophils.³ An apparent absence of intact neutrophils may reflect their short tissue half-life in the absence of survival signals, such as CXCL-8, although our observations do not exclude presence of submicroscopic neutrophil fragments, such as neutrophil extracellular traps.¹⁵

A notable confirmation in this study is the exclusive and apparently transient presence of adventitial tertiary follicles in the area underlying a vulnerable plaque. As such, the study extends our former observations that a change in the

inflammatory footprint accompanies plaque destabilization from the human aorta⁹ to the human coronary. These observations show that the observations for the aorta translate to coronary artery disease.

CD36 was included as a putative foam cell marker,¹⁶ and it was anticipated that this would allow differentiation between macrophages and foam cells. Remarkably, the data in this study characterize CD36/CD68 double-positive cells as a (minority) subclass of macrophages/foam cells. Although we cannot exclude that CD36 expression in these early phases is below the detection threshold of the immunohistochemistry, the observational data herein challenge the concept of critical involvement of CD36¹⁵ in early foam cell formation for the human context.

A further notable finding is the subendothelial macrophage accumulation in areas overlying the consolidated lesions in the end-stage fibrous calcified plaques. This phenomenon presumably reflects the repetitive character of atherosclerosis with novel lesions (referred to as neoatherosclerosis in the context of in-stent stenosis¹⁷) forming on top of old fibrotic lesions. The underlying barrier formed by fibrotic remains of the former lesion and absence of neovascularization suggest that these lesions principally rely on a luminal exchange, in contrast to primary lesions, which are supported by an extensive medial-adventitial vasa vasorum network.¹⁸

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