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The natural history of human atherosclerosis : a histopathological approach

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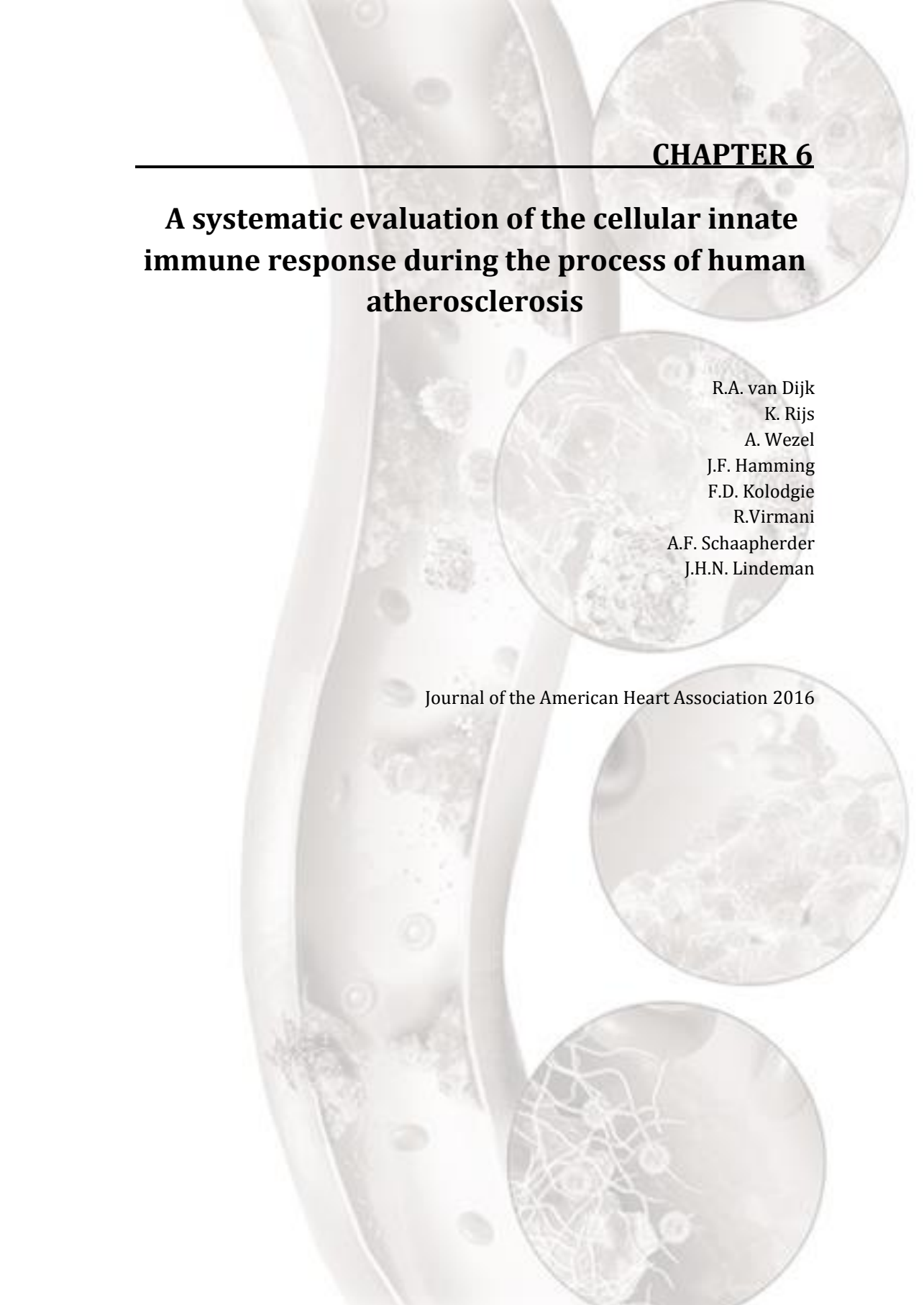


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CHAPTER 6

A systematic evaluation of the cellular innate immune response during the process of human atherosclerosis

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ABSTRACT

Background: The concept of innate immunity is well recognized within the spectrum of atherosclerosis, which is primarily dictated by macrophages. Although current insights to this process are largely based on murine models, there are fundamental differences in the atherosclerotic microenvironment and associated inflammatory response relative to the humans. In this light, we characterized the cellular aspects of the innate immune response in normal, non-progressive, and progressive human atherosclerotic plaques.

Material and methods: A systematic analysis of the innate immune response was performed on 110 well-characterized human peri-renal aortic plaques with immunostaining for specific macrophage subtypes (M1 and M2-lineage) and their activation markers neopterin and HLA-DR together with dendritic cells, NK cells, mast cells, neutrophils, and eosinophils.

Results: Normal aortae were devoid of LDL, macrophages, dendritic cells, NK cells, mast cells, eosinophils and neutrophils. Early, atherosclerotic lesions exhibited heterogeneous populations of (CD68+) macrophages whereby 25% were double positive "M1" (CD68+/ iNOS+/CD163-), 13% "M2" double positive (CD68+/iNOS-/CD163+) and 17% triple-positive for (M1) iNOS (M2)/ CD163 and CD68 with the remaining (~40%) only stained for CD68. Progressive fibroatheromatous lesions, including vulnerable plaques showed increasing numbers of NK cells and fascin positive cells mainly localized to the media and adventitia while the M1/M2 ratio and level of macrophage activation (HLA-DR and neopterin) remain unchanged. On the contrary, stabilized (fibrotic) plaques showed a marked reduction in macrophages and cell activation with a concomitant decrease in NK cells, dendritic cells, and neutrophils.

Conclusion: Macrophage "M1" and "M2" subsets together with fascin-positive dendritic cells are strongly associated with progressive and vulnerable atherosclerotic disease of human aorta. The observations here support a more complex theory of macrophage heterogeneity than the existing paradigm predicated on murine data and further indicate the involvement of (poorly-defined) macrophage subtypes or greater dynamic range of macrophage plasticity than previously considered.

INTRODUCTION

Macrophages and possibly other cellular components of the innate immune system are paramount in the initiation, progression and complications of atherosclerosis^{1,2,3}. At the same token, data from animal studies shows that these cells orchestrate atherosclerosis regression and plaque stabilization, underlining the central role of these cell types in the atherosclerotic process^{4,5,6}.

It is now recognized that macrophages constitute a highly heterogeneous and dynamic cell population that were initially labeled as pro-inflammatory M1 macrophages and tissue regenerative M2 macrophages, although more elaborative classifications have been brought forward and some even pointed out that macrophage differentiation is a continuum⁷. Experimental studies mainly involving mice implies a major shift in macrophage identity during the atherosclerotic process with pro-inflammatory processes (classically activated M1 macrophages) involved in the initiation and progression of the disease, and alternatively activated M2 macrophages) linked to resolution and repair⁸. Much of the theory in this area has been driven by *in vitro* studies exploring gene/protein expression patterns and functional attributes of monocytes or macrophages subjected to various treatments^{9,10}.

Knowledge on the innate immune system in the atherosclerotic process essentially relies on observations from rodent models¹¹. Yet, there are fundamental immunological and inflammatory differences between rodents and humans^{12,13,14}. Moreover, lesions in established murine atherosclerosis models fail to progress to advanced vulnerable plaques complicated by rupture hence inflammatory responses in advanced stages of the disease cannot be characterized in these models¹⁵. Consequently, it's becoming recognized that observations from animal models may not necessarily translate to the human atherosclerotic process, particularly when considering the inflammatory milieu^{16,17,18}.

In this regard we considered a systematic evaluation of the cellular components of the innate immune system (macrophages and their subtypes, dendritic cells, mast cells, natural killer cells, neutrophils and eosinophils) throughout the process of human atherosclerosis, particularly in relation to complicated plaques, relevant to symptomatic disease. To that end, we performed a systematic histological evaluation of these components using a unique collection of biobanked human arterial tissues that covers the full spectrum of lesion progression.

Results from this study confirm an extensive and dynamic inflammatory process involving specific cellular components of the innate immune system occurring throughout disease progression. Furthermore, it was concluded that a simple dichotomous classification system for macrophage differentiation falls short in the biological context.

MATERIAL AND METHODS

Patients and tissue sampling

Tissue sections were selected from a large tissue bank containing over 400 individual abdominal aortic wall patches (AAWPs) that were obtained during liver, kidney or pancreas transplantation (*viz.* all material was from cadaveric donors). Details of this bank have been described previously by van Dijk *et al.*¹⁹. All patches were harvested from grafts that were eligible for transplantation (*i.e.* all donors met the criteria set by The Eurotransplant Foundation) and due to national regulations, only transplantation relevant data for donation is available. Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical Committee in Leiden, Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies (<http://www.federa.org/?s=1&m=82&p=0&v=4#827>).

Histologic sections of AAWPs were stained by haematoxylin and eosin (H&E) and Movat pentachrome for classification of the lesions (in accordance with the modified AHA classification as proposed by Virmani *et al.*^{20, 21}). Classification was performed by two independent observers without knowledge of the character of the aortic tissue^{19, 20}. In all cases, the aortic tissue block showing the most advanced plaque from each patient was used for further analysis. The lesion types included in this classification are described below. In order to obtain a balanced and representative study group, 10 to 12 samples were randomly selected from representative lesion morphologies. A total of 110 samples were used for further immunohistochemistry staining and analysis.

Characterization of the lesions and histological definitions

A detailed description of plaque characterization, morphological analysis is provided in reference 19. Plaque morphologies included adaptive intimal thickening (AIT), intimal xanthoma (IX), pathological intimal thickening (PIT), early (EFA) and late fibroatheroma (LFA), thin cap fibroatheroma (TCFA), acute plaque rupture (PR), healed plaque rupture (HR) and fibrotic calcific plaque (FCP).

Immunohistochemistry

Single immunostaining for visualizing macrophages, dendritic cells (DC), mast cells, and neutrophils.

Histologic sections of formalin-fixed paraffin embedded aortic tissues decalcified in Kristensen fluid were immunostained using antibodies directed against the macrophage activation marker neopterin, HLA-DR (cell activation marker), fascin (dendritic cells), tryptase (mast cells), myeloperoxidase (MPO) and matrix metalloproteinase 8 (MMP8; neutrophils) and EG2 (eosinophils). The details and antibodies used for immunohistochemical staining are listed in Table I. Antigen

retrieval was heat-induced using either citrate (pH 6) or TRIS-EDTA (pH 9.2) buffers or proteolytic digestion with trypsin, when necessary. Antibody binding was visualized by a polymer-based HRP substrate (EnVision[®], Dako, A/S, Glostrup, Denmark) with diaminobenzidine (DAB) as the chromogen and Gill's hematoxylin as counterstain. Tissue sections from human tonsils and abdominal aortic aneurysms were used for positive controls while negative controls were performed by omitting the primary antibody.

Double-labeling immunohistochemistry for visualizing Natural Killer (NK) cells and Low Density Lipoprotein (LDL) with elastin.

NK cells (T-bet⁺/CD4⁻) were identified by double labelling against T-bet (NK cells and T-helper1 cells) and CD4 (T-helper cells) with DAB and Vina Green chromogens, respectively, as described previously²². Dual staining for LDL and elastin was performed with an antibody directed against apolipoprotein B100 with DAB and counterstained by fuchsin.

Triple-labeling immunohistochemistry for M1 and M2 macrophage subpopulations

Details of all antibodies are listed in Table 1. Primary markers included CD68 (pan-MΦ macrophage marker with iNOS (inducible nitric oxide synthase, M1) and CD163 (macrophage scavenger receptor, M2). Macrophage phenotype was further validated with IL6 (M1) and dectin-1 (M2, major β-glucan receptor on macrophages). Antibody reactions were detected using an EnVision[®] + System – horseradish peroxidase (HRP) labeled polymer anti-mouse/anti-rabbit functioned as the secondary antibody (DAKO) and positive reactions were visualized with DAB and Vina Green chromogen or 4plus Biotinylated Universal Goat link kit (Biocare Medical, Concord, CA) in combination with 4plus streptavidin AP label for Ferangi Blue and Warp Red visualizations. For triple stainings a second heat induced citrate antigen retrieval was used following incubation with denature solution (Biocare Medical) to inactivate the preceding labels.

Quantification of the immunostained cells

Aortic lesion area was defined as the region between the lumen and first elastic lamina over a circumferential width of 1mm. For fibroathermatous plaques, the lateral distance was expanded to include flanking regions of the necrotic core incorporating shoulder regions. Regions of interest (ROI) were selected from the central region of the plaque and lateral shoulder regions with corresponding areas of underlying media and the adventitia (Figure 1). Three representative areas within separate ROIs involving the intima, media and adventitia were imaged at 200x magnification (Axiovision 4.6.3). A total of 9 images per atherosclerotic lesion, which were each analyzed.

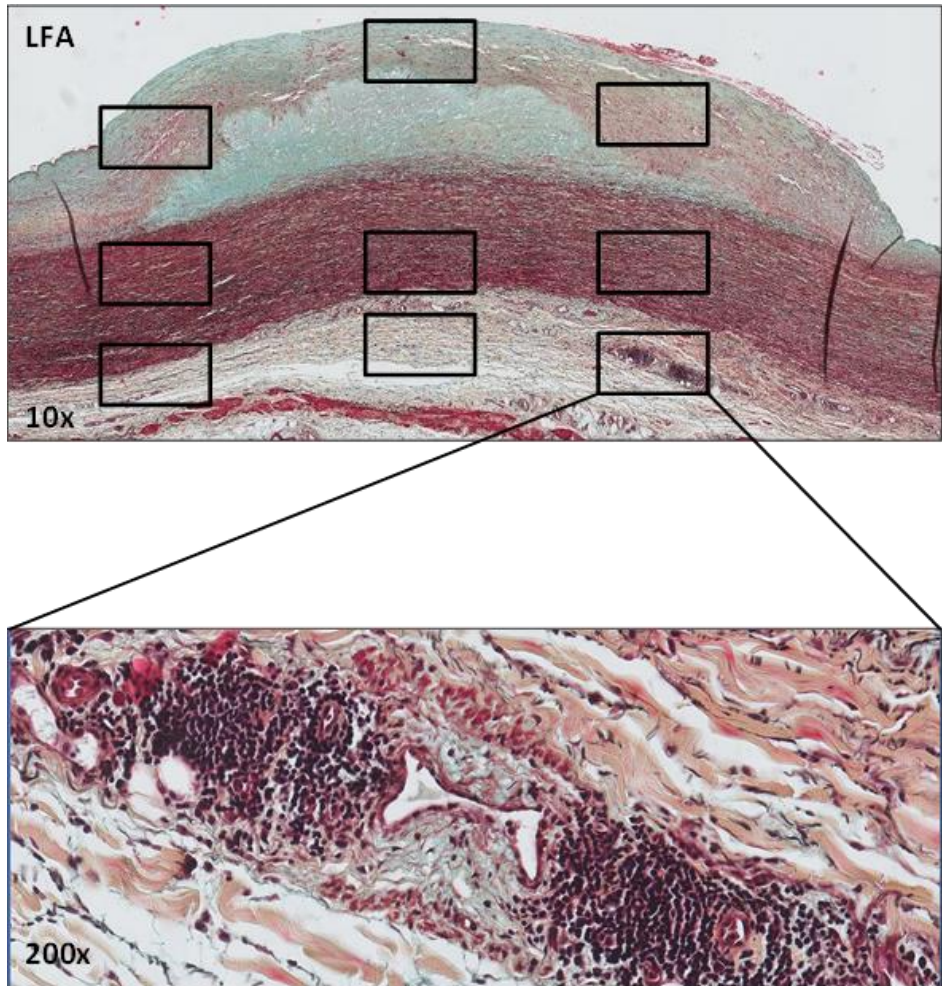


Figure 1. Defining the 9 regions of interest (ROI) within atherosclerotic lesions. Early aortic fibroatheroma stained by Movat pentachrome with regions of interest (ROIs, boxed areas) selected within the fibrous cap, flanking shoulders and underlying media and the adventitia. Immunostaining for select inflammatory markers within ROIs was quantitatively assessed with an image processing program (Image J; plug-in Cellcounter). An additional high resolution image of an adventitial inflammatory infiltrate at 200x magnification is shown to illustrate the cellular detail.

Positive staining for mast cells (tryptase), NK cells (T-bet⁺/CD4⁻), dendritic cells (fascin) and neutrophils (myeloperoxidase and MMP8) was quantified using Image J Colour Deconvolution. Tissue sections stained for macrophages (CD68), neopterin and HLA-DR were quantified and presented as a percentage of plaque area. Triple immunostains for macrophage subtypes were analyzed using the Nuance multispectral imaging system FX²³ based on unmixed colour spectra from DAB, Warp Red and Vina Green or Ferangi Blue.

Table 1. Antibodies used in the present study

Antibody, clone	Host isotype; subclass	Specificity	Pretreatment		Dilution	Reference/source
Apolipoprotein B	Goat (polyclonal), IgG	apoLipoprotein B	Tris/EDTA	pH 9.2	1:4000	Abcam
CD4	Rabbit (polyclonal)	T-helper cells	Citrate	pH 6.0	1:200	Abcam
CD68, KP1	Mouse (monoclonal), IgG1	Pan Macrophage	Tris/EDTA	pH 9.2	1:7000	DAKO Cytomation
CD163,10d6	Mouse (monoclonal) IgG1	"M2" Macrophages (triple stains)	Tris/EDTA	pH 9.2	1:200	Thermo scientific/neomarkers
Dectin-1, NBP2-13845	Rabbit (polyclonal)	"M2" Macrophages (triple stains)	Tris/EDTA	pH 9.2	1:50	Novus Biologicals
EG2	Mouse	Activated eosinophils	Trypsin	-	1:600	Pharmacia Diagnostics
Fascin, 55k-2	Mouse (monoclonal), IgG1	Dendritic Cells	Citrate	pH 6.0	1:800	DAKO Cytomation
HLA-DR, TAL.1B5	Mouse (monoclonal), IgG1	antigen presenting cells (APC)	Citrate	pH 6.0	1:200	DAKO Cytomation
IL-6, NYRhIL6	Mouse (monoclonal), IgG2	"M1" Macrophages (triple stains)	Citrate	pH 6.0	1:400	Santa Cruz
iNOS	Rabbit (polyclonal), IgG	"M1" Macrophages (triple stains)	Tris/EDTA	pH 6.0	1:400	Abcam
Myeloperoxidase	Rabbit (polyclonal)	Neutrophils	-	-	1:5000	Dako Cytomation
MMP8, MAB9081	Mouse (monoclonal) IgG2a	Neutrophils	Trypsin	-	1:1000	R&D systems
Neopterin	Rabbit (polyclonal)	Activated macrophages	Citrate	pH 6.0	1:500	Biogenesis
T-bet	Rabbit (polyclonal)	NK cells / T helper1-cells	Tris/EDTA	pH 9.2	1:200	Santa Cruz
Tryptase, AA1	Mouse (monoclonal), IgG1	Mast cells	Citrate	pH 6.0	1:3000	Dako Cytomation

Total numbers of M1 macrophages (CD68⁺/iNOS⁺ or CD68⁺/IL6⁺) and M2 macrophages (CD68⁺/CD163⁺ or CD68⁺/Dectin-1⁺) were expressed as a percentage of co-localized CD68 as well as the percentage of the triple positive immunostaining (IL6/Dectin-1/CD68 or iNOS/CD163/CD68) in addition to the percentage of CD68 without any co-localization.

Statistical analysis

Data are presented as means $\pm$ SEM. The means of cellular distribution were compared using One-way ANOVA. The relationship between the amount of positive cells in aortic layers (intima, media and adventitia) within a particular lesion types was analyzed by Spearman's correlation and compared with other lesion morphologies (SPSS 20.0; Chicago, IL). The Wilcoxon-Mann-Whitney test was used to analyze the non-normally distributed data of the mean total number of cells within each atherosclerotic phase and the Kruskal Wallis test was used to control for a type 1 error. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Study population

The male/female ratio among donors was 54.5% male and the mean age was ~47 years. There was a strong relationship between donor age and the stage of atherosclerosis¹⁹ morphology whereby normal aortic wall samples had a mean age of 17 years, while those with end-stage fibrocalcific plaques were 60 years of age. Cardiovascular risk factors of smoking were known in 34%, hypertension in 15% while two donors received statin therapy (Table 2).

Cellular aspects of the innate immune response

Normal and non-progressive lesions

The intima and media of the normal aortic wall are devoid of LDL, macrophages, dendritic cells, NK cells, mast cells, and neutrophils. Early, non-progressive atherosclerotic lesions inclusive of adaptive intimal thickening (AIT) and intimal xanthoma (IX)) showed traces of LDL (apolipoprotein B100) within the intima (Figure 2). By definition IX lesions showed extensive infiltration of (CD68⁺) macrophage foam cells (Figure 3), which was also heterogeneous, as shown by additional markers for M1 and M2 macrophages. Spectral imaging analysis of macrophage subtypes showed a predominance of "M1" (CD68⁺/ iNOS⁺/CD163⁻) over "M2" (CD68⁺/iNOS⁻/CD163⁺) macrophages (25% and 13%, respectively) (Figure 4) with approximately 17% of the macrophages co-expressing both (M1) iNOS and CD163 (M2) markers, with the remaining only positive for CD68.

Table 2. Demographic data of the 110 studied aortic samples

	Male		Female	
N	60		50	
Mean age (years) [SD]	46.9	[19.8]	47.0	[15.7]
Mean length (cm) [SD]	178.9	[11.7]	165.6	[11.9]
Mean weight (kg) [SD]	80.0	[16.2]	63.9	[14.5]
Mean BMI (kg/m ²) [SD]	24.9	[3.0]	22.5	[4.3]
Patients with known history of nicotine abuse [percentage]	21	[35%]	17	[34%]
Patients with known history of hypertension* [percentage]	13	[21.7%]	10	[20%]
Patients with known diabetes [percentage]	0	[0.0%]	1	[2%]
<u>Cause of Death [n, percentage]</u>				
Severe head trauma	11	[18.3%]	7	[14.0%]
Cerebral vascular accident (CVA)	8	[13.3%]	10	[20.0%]
Subarachnoid bleeding (SAB)	15	[25%]	14	[28.0%]
Cardiac arrest	7	[11.6%]	0	[0.0%]
Trauma	1	[1.6%]	1	[2.0%]
Other	6	[10%]	3	[6.0%]
Unknown	16	[26.6%]	15	[30%]
<u>Medication [n, percentage]</u>				
Anti-hypertensives	11	[18.3%]	6	[12.0%]
Statins	1	[1.6%]	1	[2.0%]
Anti-coagulants	1	[1.6%]	2	[4.0%]
Other	5	[8.3%]	8	[16.0%]
None	31	[51.7%]	22	[44.0%]
Unknown	11	[18.3%]	9	[18.0%]

*Known antihypertensive medication / systolic blood pressure >140mmHg and diastolic >90mm Hg in the period preceding death. Abbreviations: SD = Standard Deviation.

Comparable results were obtained with a second set of validation markers IL6 (M1) and dectin-1 (M2) (Figure 4B).

Intimal xanthomas were further assessed for the general cellular activation marker HLA-DR and macrophage-specific marker neopterin (Figure 5 and 6). The profuse macrophage infiltration seen in IX is paralleled by increased expression of HLA-DR and neopterin in this early stage of the atherosclerotic process.

Mast cells (Figure 7) are also reported to express CD68 and may thus (partly) account for the CD68 double negative population. Tryptase/CD68 double staining indeed showed strong CD68 reactivity on mast cells although given the sparse number of mast cells in the intimal layer the overall effects are likely minimal unlike the interpretation of the macrophage data for the media and adventitia where mast cells populations are more prominent (Figure 7E). NK cells (Figure 8), dendritic cells (Figure 9) and neutrophils (Figure 10) are not identified in the intimal layer of normal aorta and non-progressive lesions. Moreover, the NK cells and dendritic cells that are seen in the media are located at the medial-adventitial border. No eosinophils (EG2⁺) were identified in the normal and non-progressive lesions.

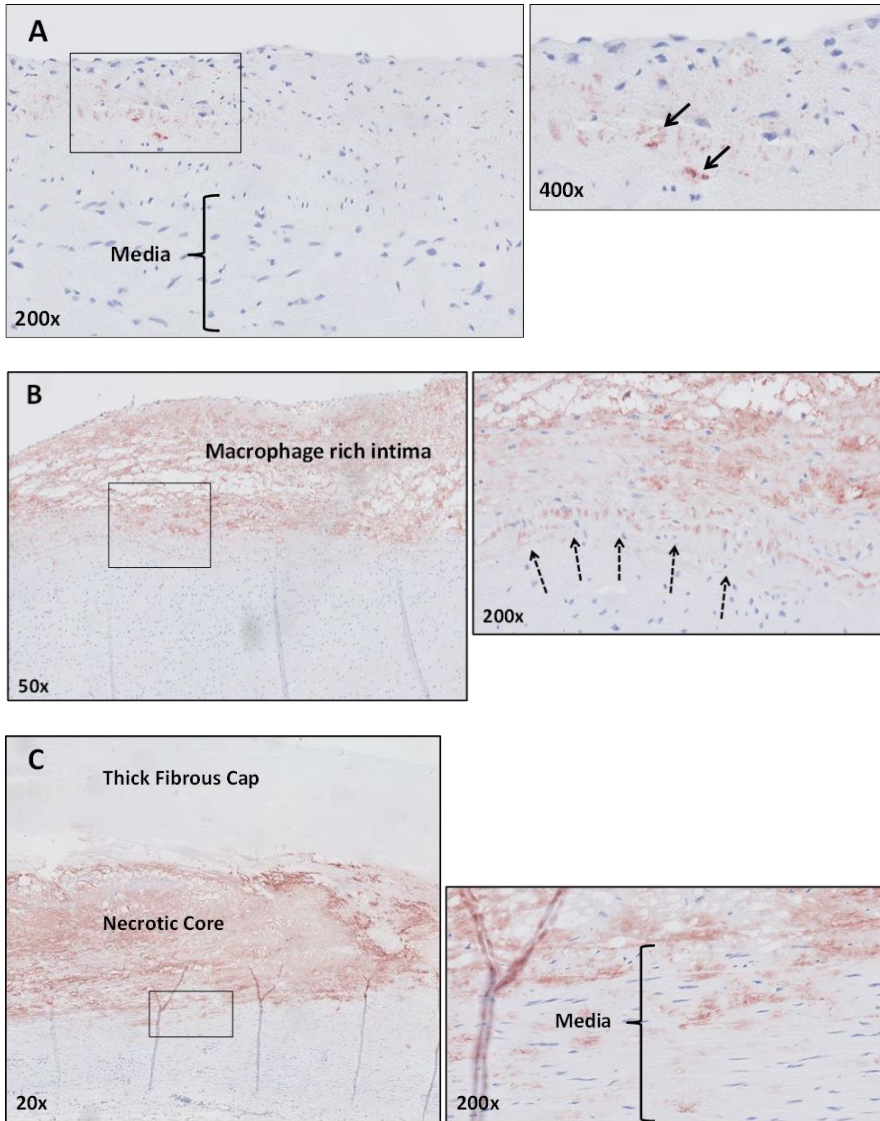


Figure 2. Apolipoprotein B100 (LDL) deposition. A. Representative image (200x) of an aorta with adaptive intimal thickening stained for Apolipoprotein B100 with a high resolution image at a 400x magnification. Apolipoprotein B100 accumulation is confined to the intima (black arrow). B. Representative image (50x) of an intimal xanthoma (IX) stained for Apolipoprotein B100 with a high resolution image at a 200x magnification. Increase in intimal ApoB100 deposition within the intima. Accumulation of ApoB100 is restricted to the intima (dotted arrows represent the internal elastic lamina). C. Representative image (20x) of a late fibroatheroma (LFA) stained for Apolipoprotein B100 with a high resolution image at a 200x magnification at the intimal-medial border. This stage of atherosclerosis is characterized by accumulation of ApoB100 within the media, coinciding loss of integrity of the internal elastic lamina. All ApoB100 samples were visualized with DAB and counterstained with haematoxylin.

Progressive atherosclerotic lesions

Progressive lesions of pathological intima thickening (PIT), early fibroatheroma (EFA) and late fibroatheroma (LFA) are characterized by loss of integrity of the intimal medial border zone as hallmarked by increasing apolipoprotein B100 infiltration in the inner media layer (Figure 2C) along with a major increase in the macrophage content both in the intima and adjacent media (Figure 3 and 4). In parallel, we observe increased levels of macrophage activation, as indicated by HLA-DR and neopterin expression. The percentage of M1 macrophages increases in late fibroatheroma (~27%) and slightly decreases, as the lesions progress towards a vulnerable thin cap fibroatheroma or plaque rupture. On the other hand, M2 macrophages slightly increase as early and late fibroatheromas (~13%) progress towards a thin cap fibroatheroma (~17%) while the number of monocytes and macrophages double positive for the M1 and M2 marker remains stable (Figure 4A and B). CD68⁺ cells negative for M1 and M2 markers slightly tend to increase in number as lesions progress towards plaque rupture, an observation that may (partly) reflect a major increase in the number of mast cells within the adventitia (Figure 7).

Progressive numbers of NK cells are found within the media and adventitia of these more advanced lesions (Figure 8). Fascin positive cells significantly increase in number in the intima and media (* $P < 0.0004$ and ‡ $p < 0.0001$) and remain stable within the adventitia.

Only 1 or 2 neutrophils are present in the intima (~8% of total) and the neutrophils identified in the media are located intravascular in the infiltrating *vasa vasorum* (Figure 10B and C).

Vulnerable atherosclerotic lesions

Thin cap fibroatheromas and plaque ruptures are characterized by a further increase in macrophage content in the intima and media, but the M1/M2 ratio and the level of macrophage activation (HLA-DR and neopterin) remain unchanged when compared to stable lesions (figure 4). Increased numbers of mast cells and NK cells, and fascin positive cells are found in all the vascular layers. Increasing numbers of intravascular neutrophils are observed in the media, and absent within the neointima and areas outside vessels. Overall, eosinophils are absent in the progressive and vulnerable stages.

Healed and stabilized lesions

Healed ruptures (HR) and fibrocalcific plaques (FCP) associated with vessel stabilization showed a marked reduction in the number of macrophages and decrease in cell activation, as indicated by reduced HLA-DR positivity and intimal neopterin expression. M1 macrophages increase in number whereas the M2 lineage remains stable. NK cells, dendritic cells, and neutrophils significantly decrease in number as lesions stabilize. Eosinophils remain absent.

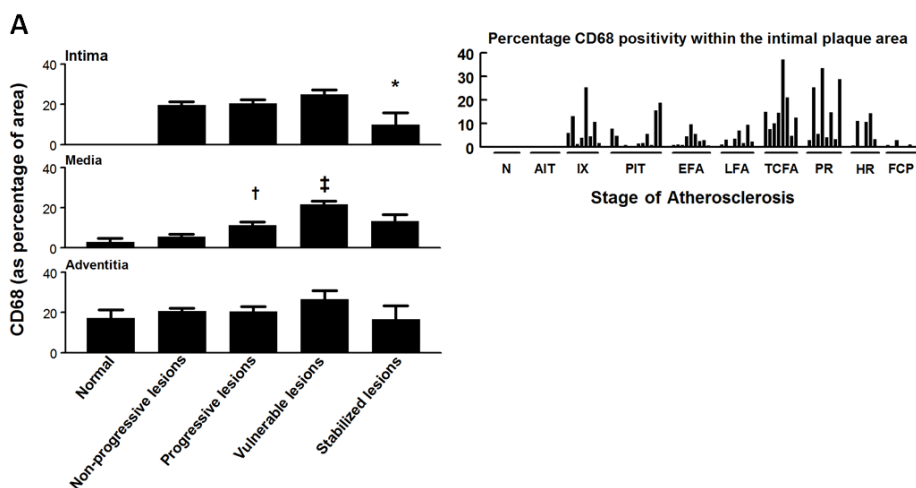


Figure 3. Macrophage (CD68+) distribution in normal, non-progressive, progressive, vulnerable, and stable atherosclerotic plaques. **A.** The mean percentage of CD68 positive area within various regions of interest (intima, media and adventitia) plotted by lesion morphology ($\pm$ SEM). An additional plot of the intima illustrates the percentage of CD68 positivity for individual plaque stratified by the stage of atherosclerosis. CD68 is seen from the stage of intimal xanthoma. CD68 positivity increases during disease progression and is maximal for vulnerable plaques, followed by a significant decrease when the lesions stabilize (* $p < 0.005$). CD68 positivity in the media increases with progressive atherosclerotic lesions and vulnerable lesions ($\dagger p < 0.004$, $\ddagger p < 0.001$) in contrary to the adventitia, which remained relatively constant except for a slight decrease for stabilizing lesions. **B.** Representative images of intimal xanthoma (IX), late fibroatheroma (LFA) and a thin cap fibroatheroma (TCFA) stained by Movat pentachrome with corresponding immunostain for macrophages (CD68; KP-1). Macrophages and macrophage foam cells essentially form the IX. The necrotic core in LFA stains highly positive for CD68, representing accumulation of macrophage remnants and is therefore was excluded during morphometric analysis. While the thick fibrous cap of the LFA is mainly negative for CD68, the shoulder regions show infiltration by macrophages and macrophage foam cells (see the 50x magnification) in addition to a notable presence within the media (see the 100x magnification). More advanced TCFA demonstrate increased macrophage accumulation in the cap. Total number of cases in figure A: 85 (normal 9, non-progressive lesions 18 (viz. AIT (10) and IX (8)), progressive lesions 29 (viz. PIT (12), EFA (9) and LFA (8)), vulnerable lesions 16 (viz. TCFA (8) and PR (8)) and stabilized lesions 13 (viz. HR (7) and FCP (6)). Abbreviations: N: normal, AIT: adaptive intimal thickening, IX: Intimal xanthoma, PIT: Pathological intimal thickening, EFA: early fibroatheroma, LFA: late fibroatheroma, TCFA: thin cap fibroatheroma, PR: plaque rupture, HR: healed rupture and FCP: fibrotic calcified plaque. For a detailed description concerning the classification see Material & Methods section.

B

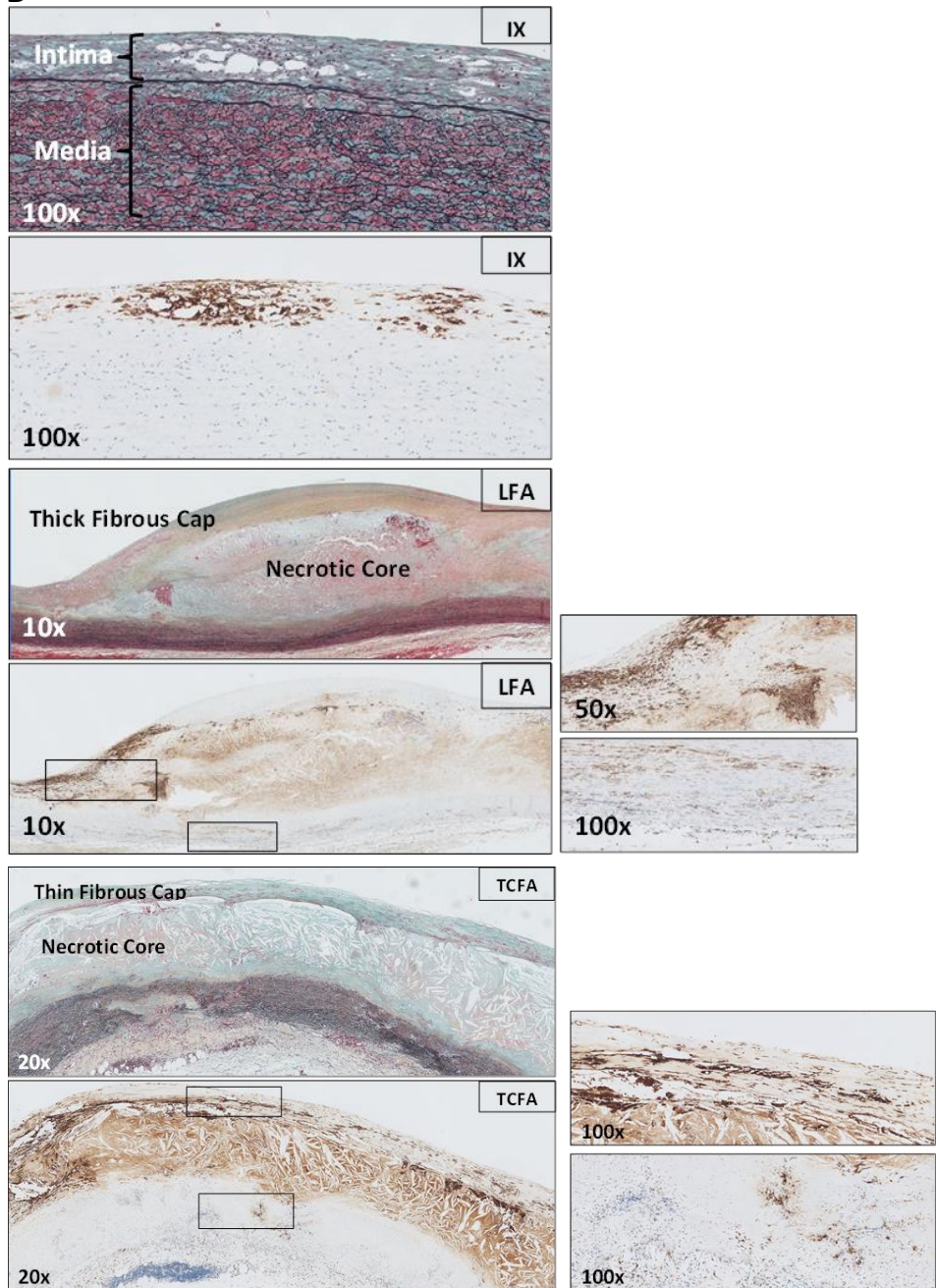


Figure 3. Continued.

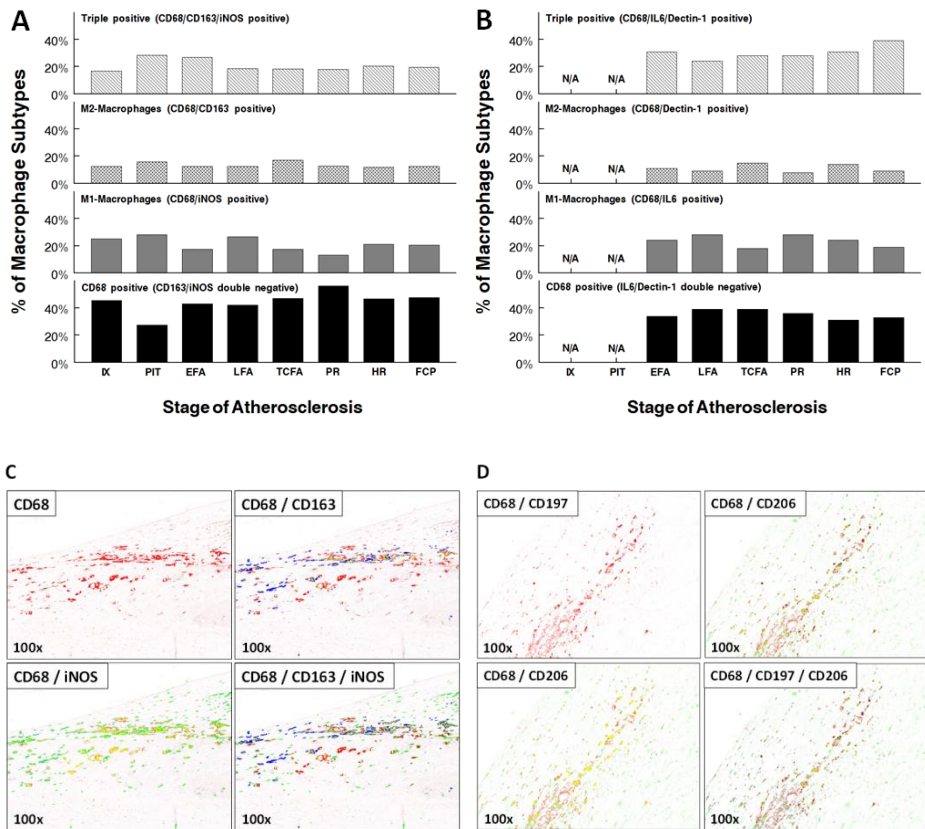


Figure 4. Macrophage subclass distribution (intima) during aortic atherosclerosis. **A.** Lesional macrophages were identified by the pan macrophage marker CD68, and respective subclasses identified based on the expression of iNOS (M1) and CD163 (M2). Various macrophage subtypes are plotted as a relative percentage of the total amount of CD68 cells for each atherosclerotic stage. As lesions progress towards a vulnerable phase (*i.e.* TCFA and PR) there is a slight decreases in the extent of M1 macrophages whereas the M2 lineage increases, resulting in a 1:1 ratio. Within healed and stabilized FCP the M1:M2 ratio is ~2:1. About 20-30% of the macrophages stain positive for both iNOS and CD163 and over 40% of the macrophages are double negative for the selective markers. **B.** Confirmation of M1 (iNOS) and M2 (CD163) expressing macrophages within various progressive, vulnerable lesions, and stable aortic lesions substituting IL6 and Dectin-1 as respective “M1” and “M2” markers. Nearly the same percentages and distribution of M1 and M2 macrophages was observed when compared with lesions triple-stained for iNOS and CD163 where ~ 20-30 percent of the macrophages express both IL6 and Dectin-1 and nearly 40% of the macrophages are double negative for both markers. **C.** Representative images of a shoulder region at a 10x magnification triple stained with CD68 (pan macrophage marker, Warp Red chromogen; Spectral image colour red), iNOS (inducible nitric oxide synthase; M1-macrophages, DAB; Spectral image colour green) and CD163 (macrophage scavenger receptor; M2-macrophages, Ferangi Blue; Spectral image colour blue). Yellow indicates the amount of co-localization between the various macrophage markers. **D.** Representative images of a shoulder region at a 9100 magnification triple stained with CD68 (pan macrophage marker, Vina Green chromogen; spectral image color red), CD197 (M1 macrophages, Vulcan Red; spectral image color green) and CD206 (M2 macrophages, Ferangi Blue; spectral image color blue). Yellow indicates the amount of colocalization between the various macrophage markers. EFA indicates early fibroatheroma; FCP, fibrotic calcified plaque; HR, healed rupture; IX, intimal xanthoma; LFA, late fibroatheroma; N/A, not applicable; PIT, pathological intimal thickening; PR, plaque rupture; TCFA, thin-cap fibroatheroma.

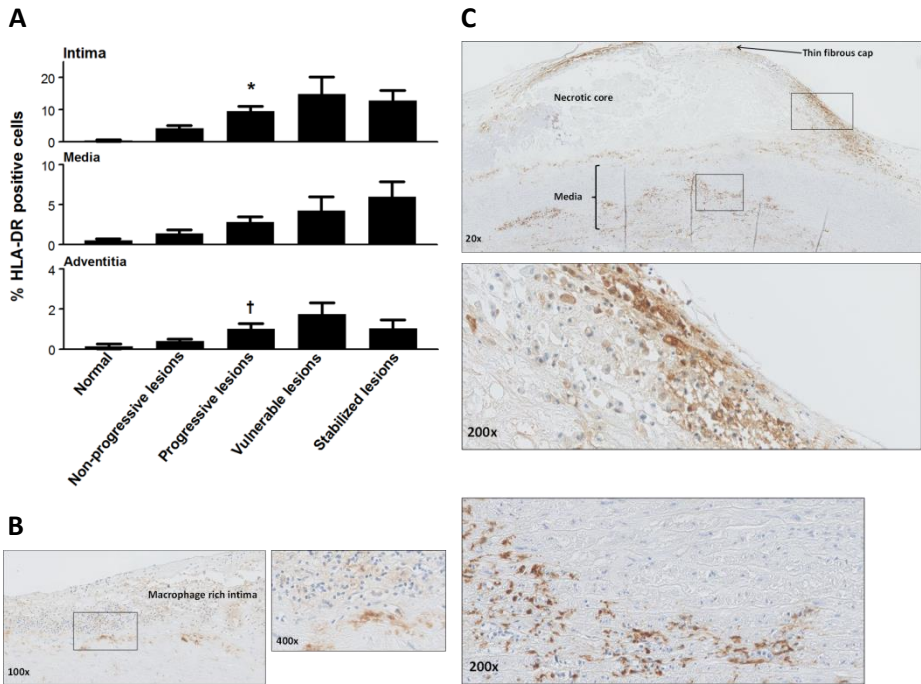


Figure 5. HLA-DR expression during aortic atherosclerosis. **A.** The mean percentage of HLA-DR expression (intima, media and adventitia) based on lesion morphology. HLA-DR expression increases within all the vascular layers (significant within the intima and the adventitia with progressive atherosclerosis (* $p < 0.002$; † $p < 0.01$)) and tend to decrease in the stabilized phase. **B.** Representative image of an intimal xanthoma (IX) stained for HLA-DR at 100x magnification with a high-resolution image at a 400x magnification. HLA-DR staining is clearly seen in macrophage rich areas located in the intima. **C.** Representative image of a thin cap fibroatheroma (TCFA) stained for HLA-DR at 20x magnification with a more detailed (200x magnification) of the macrophage foam cell rich shoulder region and media. Expression of HLA-DR is more extensive in advanced and vulnerable stages of atherosclerosis. Total number of cases in figure A: 89 (normal 8, non-progressive lesions 24, progressive lesions 34, vulnerable lesions 14 and stabilized lesions 9). The large solid bars in figure A represent the mean percentage of HLA-DR within the aortic wall section per atherosclerotic phase $\pm$ SEM. All sections were developed with DAB and counterstained with Mayer's haematoxylin.

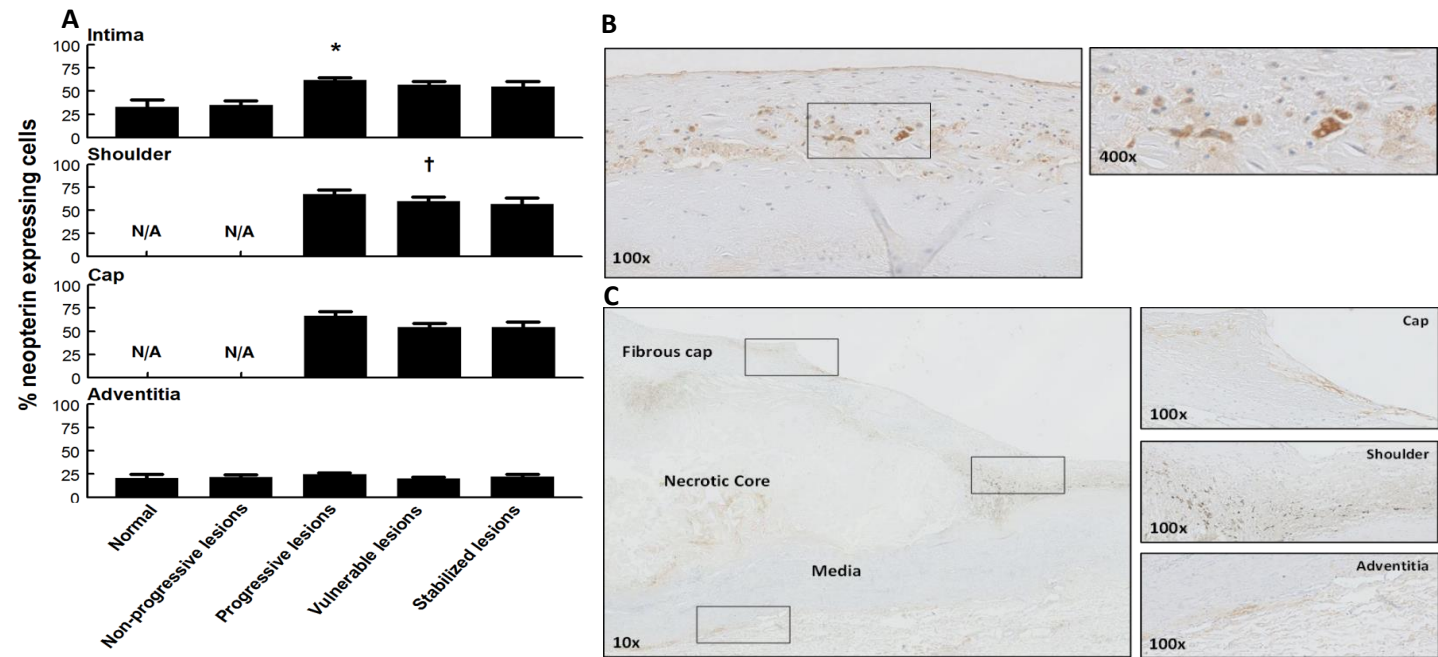


Figure 6. Neopterin expression during aortic atherosclerosis. **A.** The mean percentage of neopterin (intima, media and adventitia) based on lesion morphology. Neopterin expression is minimal in early atherosclerosis, but increases during progressive disease (* $p < 0.0007$) afterwards, remains relatively unchanged for stable healed plaque ruptures and fibrocalcific plaques. There is a small, but significant decrease in neopterin expression within the shoulder regions of vulnerable plaques ($\dagger p < 0.043$). Adventitial neopterin staining is relatively minimal and stable throughout all lesion morphologies. **B.** Representative image of an intimal xanthoma (IX) stained for neopterin with a high resolution image at a 400x magnification. The neopterin expression is seen in areas containing macrophage foam cells. **C.** Representative image of a healed rupture (HR) stained for neopterin with high resolution details of the cap, shoulder and adventitia at a 100x magnification. Neopterin expression is present within the various areas of the atherosclerotic lesion. Total number of cases in figure A: 102 (normal 7, non-progressive lesions 23, progressive lesions 35, vulnerable lesions 22 and stabilized lesions 15). The large solid bars in figure A represent the mean percentage of neopterin expression within the aortic wall section per atherosclerotic phase $\pm$ SEM. All sections were developed with DAB and counterstained with Mayer's haematoxylin.

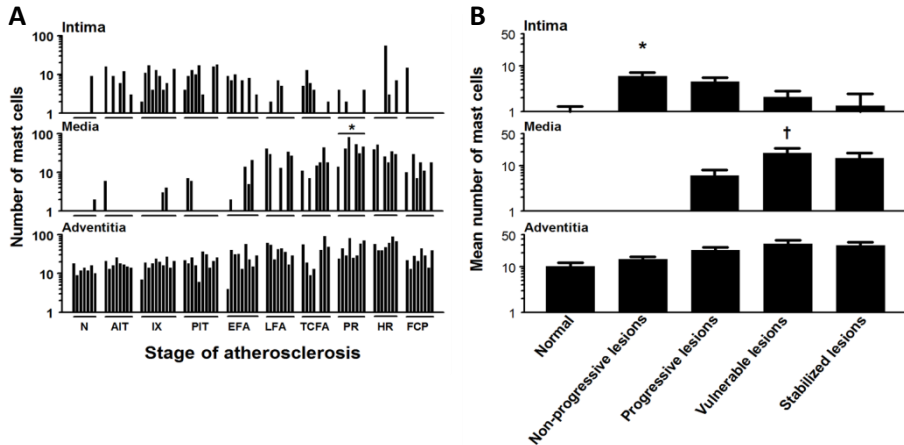


Figure 7. Mast cell (tryptase+) distribution during aortic atherosclerosis. **A.** Mast cells are constitutively present in the intima and adventitia in the aortic wall. The media practically remains devoid of mast cells until the progressive lesions. A significant increase in mast cells is seen in the media of ruptured plaques (* $p < 0.01$). **B.** The highest number of mast cells within the intima is present in the non-progressive phase ($p < 0.01$) while showing a gradual decrease with lesion progression achieving a minimum for stable fibrocalcific plaques. The media remains almost devoid of mast cells in the normal aorta's and in the non-progressive lesions but significantly increases in vulnerable plaques ($\dagger P < 0.0001$) in contrast to adventitial mast cells, which remain relatively stable throughout the atherosclerotic process. **C.** Representative image of a normal aorta stained for tryptase with high-resolution images of the intima (400x magnification) and adventitia (200x magnification), as indicated by the black arrows. Note the adventitial mast cells are scattered and mainly located near *vasa vasorum*. **D.** Representative image of a late fibroatheroma with a large necrotic core stained for Tryptase at 20x magnification with high-resolution images of media and adventitia at a 400x magnification. Mast cells are relatively more and remain in close proximity to the *vasa vasorum*. **E.** Representative image of the adventitia of an intimal xanthoma double stained for CD68 (Vina Green) and tryptase (Warp Red). CD68 positive mast cells are identified (arrows) and using the Nuance multispectral imaging system FX the co-localization can be separately analyzed. Total number of cases in figure A and B: 83 (normal 7, non-progressive lesions 18 (viz. AIT (8) and IX (10)), progressive lesions 27 (viz. PIT (10), EFA (9) and LFA (8)), vulnerable lesions 16 (viz. TCFA (8) and PR (8)) and stabilized lesions 15 (viz. HR (7) and FCP (8)). In figure A, the vertical axis of the intima, media and adventitia is presented as a log-scale. Each solid bar in figure A represents the number of positively stained mast cells within the intima, media and adventitia of one aortic plaque. The large solid bars in figure B represent the mean total number of mast cells within the entire aortic wall per atherosclerotic phase $\pm$ SEM. For abbreviations and a detailed description concerning the classification see Material & Methods section. All sections were developed with DAB and counterstained with Mayer's haematoxylin.

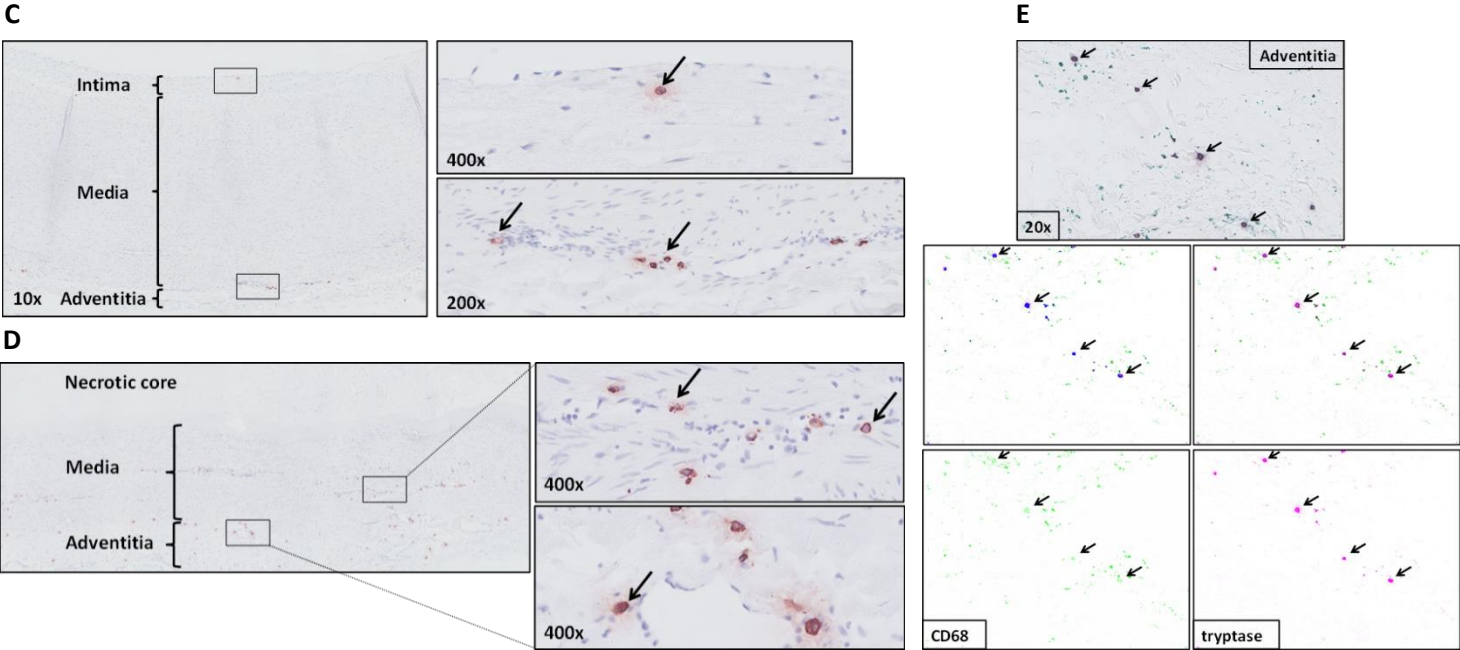


Figure 7. Continued.

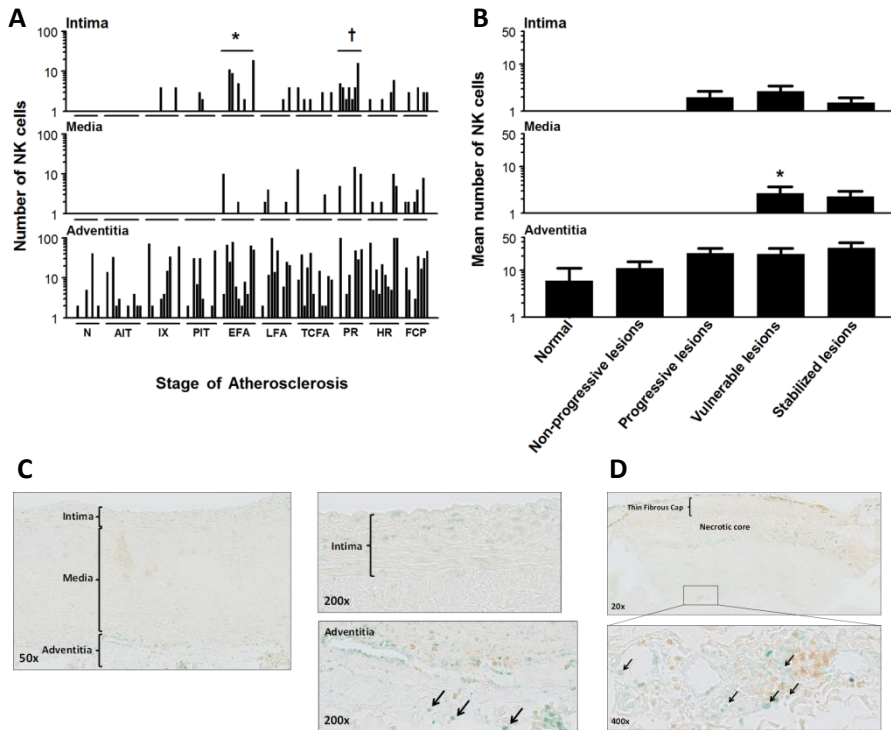


Figure 8. NK cell (T-bet⁺/CD4⁻) distribution during aortic atherosclerosis. **A.** A significant increase in NK cells is seen in the intima in EFA (* $p < 0.01$) and in ruptured plaques († $p < 0.011$). The media practically remains devoid of NK cells in progressive atherosclerosis. NK cells are constitutively and similarly present in the adventitia throughout the disease process. **B.** NK cells are minimally present in the aortic intima and media. NK cells are largely confined to the adventitia and the medial-adventitial border zone and the number of NK cells increases during the atherosclerotic process. A small but significant increase in NK cells in the media is seen in the vulnerable phase (*viz.* TCFA and PR). (* $p < 0.001$). **C.** Illustrative images of a non progressive lesion (AIT) showing T-Bet⁺/CD4⁻ cells (methylgreen; black arrows). Note T-helper cells (CD4⁺ single positive) cells in the adventitia (brown; diaminobenzidine chromogen) and the T-helper1 cells (CD4 and T-bet double positive cells). **D.** Representative low power image of a TCFA dual immunostained for CD4/T-bet with a high resolution image of the adventitia at 20x magnification. Note the predominant adventitial location of NK cells (Vinagreen; black arrows). The lesion is a consecutive slide from the Movat and CD68 shown in Figure 3B. Total number of cases in figure A and B: 100 (normal 8, non-progressive lesions 23 (*viz.* AIT (12) and IX (11)), progressive lesions 31 (*viz.* PIT (10), EFA (11) and LFA (10)), vulnerable lesions 20 (*viz.* TCFA (12) and PR (8)) and stabilized lesions 18 (*viz.* HR (10) and FCP (8)). Each solid bar in figure A represents the number of positively stained NK cells within the intima, media and adventitia of a single lesion while the large solid bars in figure B represent the mean total number of NK cells within the aortic wall section per atherosclerotic phase $\pm$ SEM. For abbreviations and a detailed description concerning the classification see Material & Methods section. All sections were developed with Vina green and DAB and counterstained with Mayer's haematoxylin.

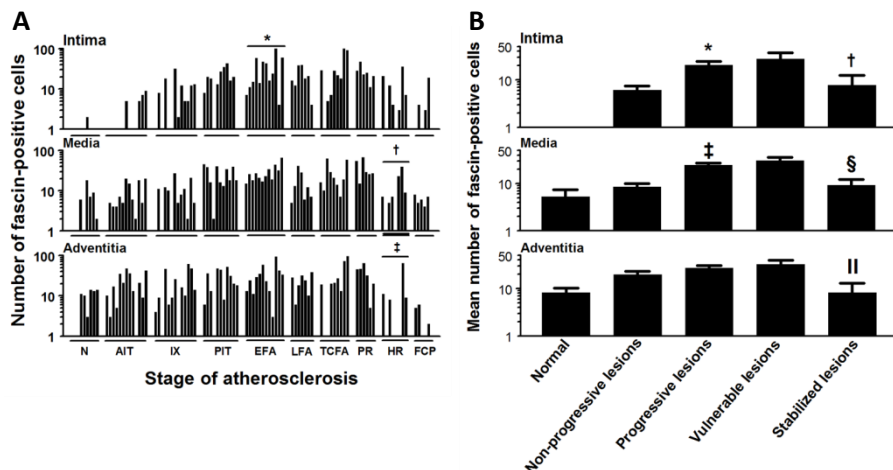


Figure 9. Dendritic cell (fascin+) distribution during aortic atherosclerosis. **A.** Dendritic cells (DCs) are mainly localized to the adventitia near the medial border and gradually increase in number with lesion progression such that DC's are minimally present in the normal intima and intima of non-progressive lesions while significantly increased in progressive atherosclerotic plaque (* $p < 0.046$). On the contrary, a significant decrease in dendritic cells is seen in stabilizing lesions ($\dagger p < 0.001$ and $\ddagger p < 0.029$). **B.** The amount of dendritic cells in the intima and media increases significantly in the progressive phase (* $P < 0.0004$, $\ddagger p < 0.0001$). In post ruptured stabilized atherosclerotic lesions the number of fascin-positive cells decrease in all the vascular layers ($\dagger p < 0.015$, $\S p < 0.0004$ and $\parallel p < 0.002$). **C.** Representative image (100x) of a non-progressive lesion (IX) stained for fascin. Only a few dendritic cells are identified in the deeper intima. **D.** Representative image (10x) of TCFA stained for fascin. This example of a TCFA is from a consecutive slide as the example seen in figure 6C. Note the abundance of fascin-positive cells in the intima, media and adventitia. **E.** Illustrative image (10x) of a FCP stained for fascin with a high-resolution image of the adventitia (200x) showing a relative decrease in fascin-positive cells in all vascular layers. Total number of cases in Figure A and B: 92 (normal 8, non-progressive lesions 26 (viz. AIT (13) and IX (13)), progressive lesions 30 (viz. PIT (11), EFA (12) and LFA (7)), vulnerable lesions 15 (viz. TCFA (9) and PR (6)) and stabilized lesions 13 (viz. HR (8) and FCP (5)). The vertical axis of the intima, media and adventitia in figure A and B is presented as a log-scale. Each solid bar in figure A represents the number of fascin-positive cells within the intima, media and adventitia of one aortic plaque. The large solid bars in figure B represent the mean total number of fascin-positive cells within the aortic wall section per atherosclerotic phase $\pm$ SEM. For abbreviations and a detailed description concerning the classification see Material & Methods section. All sections were developed with DAB and counterstained with Mayer's haematoxylin.

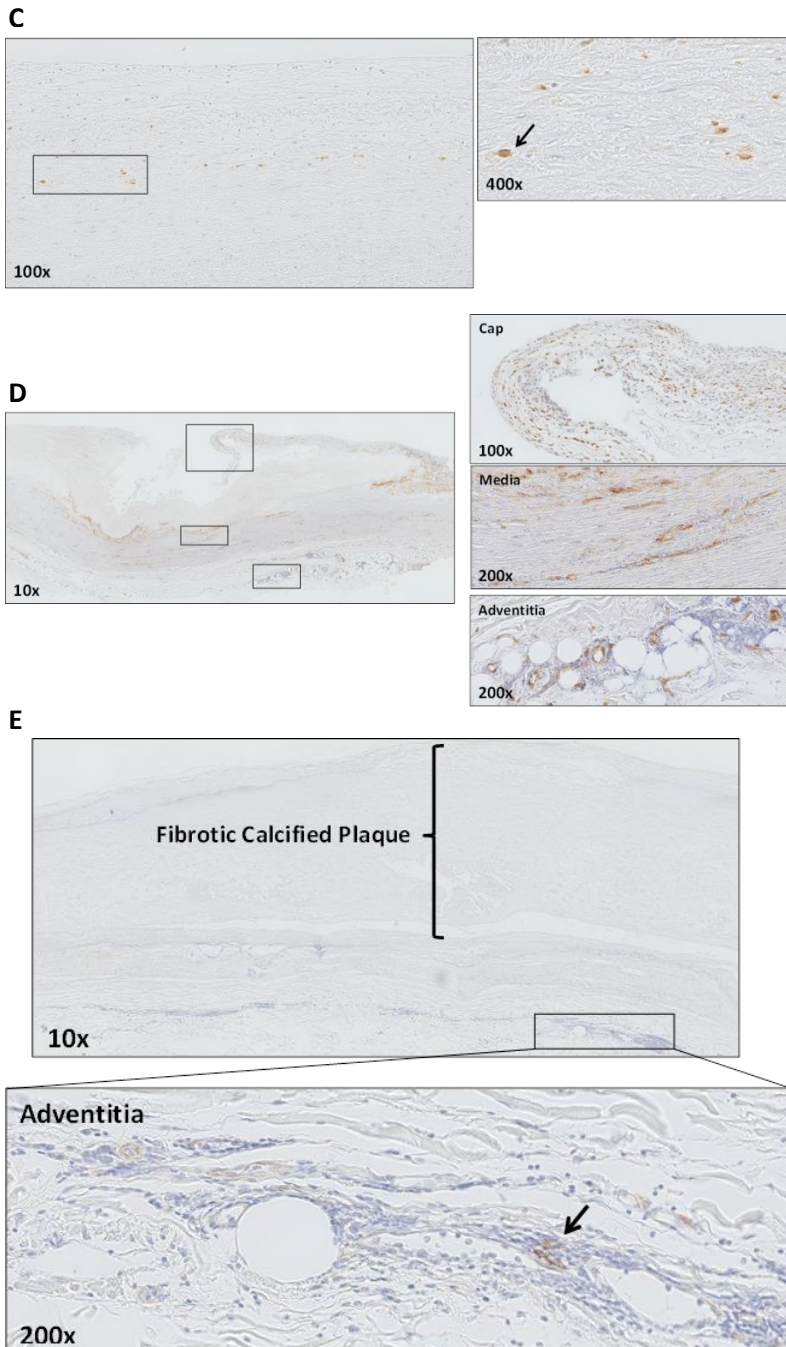


Figure 9. Continued.

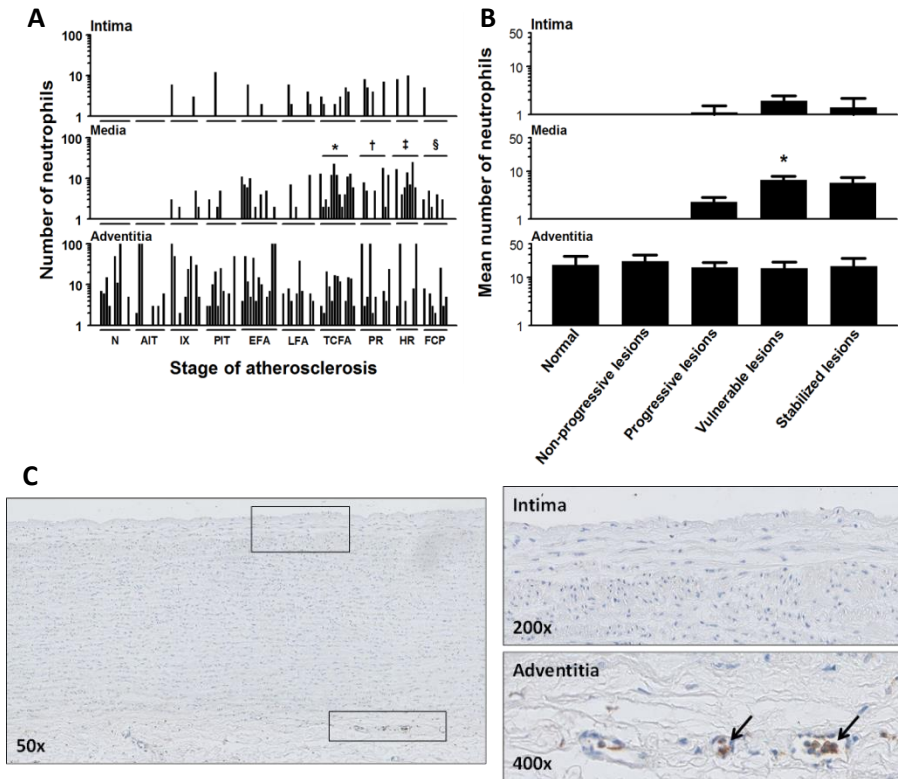
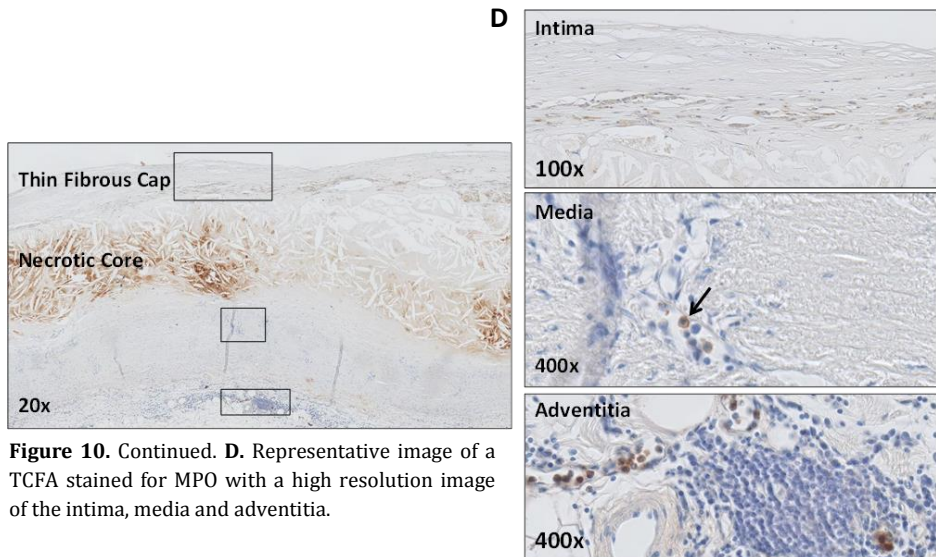


Figure 10. Neutrophil (myeloperoxidase+) distribution during aortic atherosclerosis. **A.** Minimal presence of intimal neutrophils during the atherosclerotic process. Medial neutrophils significantly increase in TCFA and PR (* $p < 0.0002$, † $p < 0.033$) and healing ruptures (‡ $p < 0.007$) and decrease in FCP (§ $p < 0.0002$). **B.** Overall the mean amount of neutrophils within the media significantly increase in vulnerable lesions (* $p < 0.0005$). **C.** Illustrative image (50x) of a normal aorta stained for MPO with high resolution images of the intima (200x) and the adventitia (400x). The identified neutrophils are all intravascular located in the vaso vasorum (black arrows). This example of a TCFA is again a consecutive section of the Movat and CD68 provided in Figure 3B and 8D. Neutrophils in the media and adventitia are located within the infiltrating vaso vasorum (black arrows). Total number of cases in figure A and B: 108 (normal 11, non-progressive lesions 22 (*viz.* AIT (11) and IX (11)), progressive lesions 35 (*viz.* PIT (11), EFA (13) and LFA (11)), vulnerable lesions 24 (*viz.* TCFA (13) and PR (11)) and stabilized lesions 16 (*viz.* HR (7) and FCP (9)). The vertical axis of the intima, media and adventitia in figure A and B is presented as a log-scale. Each solid bar in figure A represents the number of neutrophils within the intima, media and adventitia of one aortic plaque. The large solid bars in figure B represent the mean total number of neutrophils within the aortic wall section per atherosclerotic phase $\pm$ SEM. For abbreviations and a detailed description concerning the classification see Material & Methods section. All sections were developed with DAB and counterstained with Mayer's haematoxylin.



DISCUSSION

To a large extent, murine studies have emphasized the pivotal role of the innate immune system in the development and progression of atherosclerosis¹¹ although there are fundamental metabolic, inflammatory and immunologic differences that exist between various mouse strains, and more importantly between mouse and man. As such, definitive evidence on how observations of lipoprotein oxidation, inflammation and immunity translate to human atherosclerotic disease is still lacking²⁴. Consequently, it has been pointed out that studies in the human atherosclerotic process are essential to ensure the data obtained from experimental murine atherosclerosis models is relevant^{17,25}. This explorative study is based on histological observations from a unique biobank of human aorta, which allows evaluation of the full spectrum of atherosclerotic disease.

Our findings confirm a predominance of macrophages. Multiplex staining for M1/M2 macrophages failed to show a significant predominance in M1 macrophages in the foam cell rich areas, which is inconsistent with a dichotomous classification system for macrophage phenotype differentiation²⁶ as been described for murine models where M2 macrophages dominate the later stages of atherosclerotic disease. Moreover, quantitative findings for mast cells, NK cells, eosinophils and neutrophils do not imply these cell types as critical contributors to plaque destabilization.

LDL and innate immunity in lesion progression

Accumulation of LDL fragments is the primary trigger for the atherosclerotic process and is directly linked to activation and perpetuation of the innate immune response²⁷. In the present study, Apo B100 was used to visualize pro-atherogenic lipoprotein particles mainly consistent with low-density lipoprotein (LDL) accumulation although antibody staining is not specific for oxidation products. In an earlier study we extensively reported on oxidation specific epitopes, apolipoprotein B100 and apo(a) in a wide range of human coronary and carotid atherosclerotic lesions and demonstrated that plaques in early atherosclerotic lesions are enriched in apolipoprotein B100²⁸.

Apolipoprotein B100 expression was limited to the intimal layer in aortic wall samples classified as normal, adaptive intimal thickening and intimal xanthoma. On the contrary, transition to pathological intimal thickening, the earliest form of progressive atherosclerosis, is characterized by the accumulation of apolipoprotein B100 within the medial wall, which in fact is much earlier than previously reported²⁹. Demonstration that the transition to irreversible atherosclerosis is associated with a loss of internal elastic lamina integrity hallmarks the beginning of medial and adventitial involvement in atherosclerosis, a phenomenon that warrants further investigation^{30, 31}.

Macrophage subtypes

Macrophages play a critical role in innate and acquired immunity, and are in varying degrees involved in all phases of atherosclerotic lesion development particularly in regards to lipid core formation, lesion remodeling, and degradation of the fibrous cap, as seen in advanced symptomatic disease³². Macrophage accumulation in the intimal layer of the vessel wall is observed in early, non-progressive atherosclerotic lesions and is thought to be essentially driven by the accumulation of oxidized LDL^{33, 34}. In the present study, the overall number of CD68-positive macrophages increased with advancing disease, in concordance with previous studies. Among the various lesion types, macrophage infiltration was most prominent in TCFAs particularly in regions of the fibrous cap, and least for healing ruptures and fibrocalcific plaques¹⁹. It is generally assumed that macrophages, through release of matrix metalloproteinases directly contribute to plaque destabilization^{7, 32}. The overall amount of lesional macrophages as recognized in the present study however is less pronounced compared with murine atherosclerosis⁷ and affords a different perspective on the complexity of human plaques¹⁹ particularly in reference to animal models.

Contrary to murine atherosclerosis, we found no evidence of a macrophage subclass shift during initiation, progression, rupture and ultimate stabilization of human atherosclerotic lesions. Overall, approximately 20-30% of macrophages in

early to advanced plaques are double positive for both M1 and M2 markers challenging the paradigm of a clear M1-M2 dichotomy. This finding was further confirmed using alternative M1 and M2 markers (IL-6/Dectin-1), respectively in combination with CD68 to exclude the possibility that the iNOS/CD163/CD68 combination was inadequate in identifying all M1/M2 macrophages. Moreover, ~40% of the intimal macrophages were double negative for iNOS/CD163 or alternatively, IL-6/Dectin-1. CD68 was equally expressed in tryptase positive mast cells, which may partially contribute to macrophages double negative for M1 or M2 markers.

Taken together, a simple dichotomous classification scheme as proposed for macrophage differentiation in mice falls short in the biological context and complexity of human disease. Clearly, more than 2 subtypes of macrophages with presumably functionally different roles might be involved in human atherosclerosis⁸. As mentioned, the current immunostaining techniques likely fall short in identifying known and unknown macrophage subsets in human disease for lack of specificity. Single-cell analyses could provide invaluable insights into studying macrophage heterogeneity, but is technically challenging and beyond the scope of this paper. Along these same lines, a functional equivalence to murine macrophages remains unclear where caution is warranted with directly extrapolation of findings from mouse to humans or *vice versa*.

Dendritic Cells

Dendritic cells are professional antigen-presenting cells and constitute a uniquely interface between innate and adaptive immune system³⁵. The identification of dendritic cells in human and mouse vascular wall has stimulated interest in the role of these cells in the pathogenesis of several acute and chronic vascular disorders, including atherosclerosis³⁶. How dendritic cells influence the initiation and progression of atherosclerosis remains unclear. On basis of their pivotal role in antigen presentation, activating T cells, and secreting cytokines, and perhaps also their ability to become foam cells, it has been suggested that dendritic cells are critically involved in the pathogenesis of atherosclerosis^{37,38}.

Unfortunately, attempts to visualize dendritic cell subtype markers (CD80, CD86, DC lamp, DC sign) failed on formalin-fixed paraffin embedded (FFPE) material, as demonstrated in a previous study³⁹. As such, dendritic cells could only be recognized by the general DC-marker fascin, which has been showed to correlate with DC maturation into antigen presenting cells⁴⁰

There was a strong positive relationship between progressive disease and fascin positive dendritic cells in all vascular layers. The accumulation of dendritic cells within atherosclerosis-prone areas of normal aorta further supports the involvement of immune mechanisms from very early stages of the disease⁴¹.

Dendritic cells residing in the media and adventitia were mainly located near the infiltrating *vaso vasorum* and adventitial infiltrates of T cells. Dendritic cells are known to patrol this region of the plaque sampling antigens and presenting them to T lymphocytes. Our previous study regarding the adaptive immune response in human atherosclerosis identified such organized tertiary lymphoid structures in the adventitia²².

The current observations indicate a prominent role of dendritic cells in human atherosclerosis. However, different roles for dendritic cells in atherogenesis or plaque progression have been identified in mouse models while a clear attribution to dendritic cells and the exact molecular mechanisms engaged remain unresolved. The identification of human homologs of mouse dendritic cell subsets and further work in this area will aid to promote translational approaches of single cell studies.

Mast cells

Abundance of mast cells in the shoulder region of human coronary atheromas spurred interest in a possible role of these cells in the atherosclerotic process⁴². Although a role for mast cells is supported by a rodent studies, a role in human atherosclerosis remains unclear with the current knowledge^{43,44}.

Mast cells were most prominent in the media and adventitial layers and were independent of the phase of atherosclerosis. Similarly, intimal mast cell infiltration constitutes an early event in the atherosclerotic process, which subsequently plateaus. Specifically, our data do not imply an association between mast cell content and plaque destabilization and on the contrary, suggest an apparent decrease in progressive and vulnerable atherosclerotic lesions. Moreover, we did not find extracellular mast cell granules associated with fibrous cap thinning in contrary to the proposed active participation in substantial degradation of the extracellular matrix and local weakening of inflamed atherosclerotic lesions, a situation well documented in diseases affecting the connective tissue^{45, 46, 47}. Altogether, these findings fail to point to a direct relationship between intimal mast cell content and cap destabilization.

Considering medial and adventitial mast cells were typically located within or in close proximity to the infiltrating *vaso vasorum* it's conceivable that these cells are involved in plaque neovascularization in advancing and vulnerable atherosclerotic lesions. Yet an alternative explanation would be that mast cells enter through and home in the proximity of the infiltrating *vaso vasorum*^{26,36}. The accumulation of mast cells in shoulder regions of human coronary atheromas may be similarly secondary to the neovascularization. Overall, these observational data neither support nor refute a role for mast cell in the progression and complications of atherosclerosis. Such a role can only be assessed in clinical studies. Although aggressive immunosuppression for abdominal aortic aneurysms appeared to have

no effect on resident mast cells, including macrophages and SMCs, a similar study on atherosclerosis related end-points is urgently missing⁴⁸.

Natural killer (NK) cells

Natural killer cells are cytotoxic lymphocytes that are part of the innate immune system. Apart from their cytotoxic action, they are an important early source of interferon- γ (IFN γ) and tumor-necrosis factor (TNF) in response to acute injury and infections. NK cells can prime macrophages to secrete pro-inflammatory cytokines and play a key role in viral defense, tumor surveillance and tolerance during pregnancy⁴⁹. In the context of atherosclerosis, it is demonstrated *in vitro* that NK cells infiltrate the vessel wall and promote atherosclerotic lesion development in murine models of the disease⁵⁰. Along these lines, deficiency of functional NK cells has been found to reduce lesion size⁵¹. At this point a role of NK cells in clinical atherosclerosis remains still unclear.

We tested several classical NK cell markers (CD56, NKp30 and the PEN5 epitope) in order to visualize NK cells, *yet all* these markers failed on FFPE material and therefore selected an alternative strategy of applied double staining for the Th1/NK cell specific transcription factor T-bet in combination with CD4 staining. CD4⁺/T-bet⁺ cells classified as “NK cells” were abundant in abdominal aortic aneurysm tissue, but minimal in atherosclerotic samples. The few NK cells present in the intima were most common to advanced and vulnerable stages of atherosclerosis. While CD56 immunostaining clearly identified aortic ganglia (and NK cells in tumor tissue), samples of aortic atherosclerosis were negative. In essence, NK cells failed to present as a major local factor contributing to human atherosclerotic disease despite previous indications suggesting marginally decreased systemic NK cell activity in advanced atherosclerosis in the elderly⁵².

Neutrophils

Neutrophils are emerging as a potential new player in the atherosclerotic process primarily based on murine studies suggesting their involvement in the initiation of the atherosclerotic process, as well as plaque angiogenesis in later stages of the disease^{53, 54}. Moreover, neutrophil infiltration at the level of a disrupted plaque could potentially contribute to plaque progression due to release high amounts of reactive oxygen species and the pro-oxidant enzyme myeloperoxidase⁵⁵. Yet, a role for neutrophils in human atherogenesis, atheroprogession, and atherosclerotic plaque destabilization is still unclear based on available data⁵⁶.

Selective immunostaining suggested that neutrophils were mainly present in the *vasa vasorum*, or in the post-hemorrhagic hematoma due to surgical manipulation where the specific neutrophil markers MPO or MMP8 failed to identified tissue-infiltrating neutrophils with no association of atherosclerotic progression or

plaque vulnerability. The failure to detect neutrophil infiltrates within atherosclerotic tissue however, may be related to the short half-life of these cells although our earlier protein-based studies showed negligible MMP8 and CXCL8 expression thus indicating an absence of neutrophil remnants within the atherosclerotic aorta wall^{57,58}. To summarize, these observational data do not support a primary role of neutrophils in human atherosclerosis, but rather indicates that neutrophil presence is related to surgical manipulation of the aorta during removal⁵⁹.

Eosinophils

Were fully absent during the entire process of human atherosclerosis.

Limitations

Numerous inflammatory cell phenotypes identified within well- defined morphological stages of atherosclerosis emphasize the complexity of the innate immune response and atherosclerotic disease. This study was performed on aortic sections of deceased individuals, where continuous data represents incidental findings from a large series of patients and therefore may not necessarily reflect longitudinal data. Another limitation of the study is the fact the all findings are based on IHC using paraffin-embedded tissue sections, which precludes use of other relevant and confirmatory markers, particularly those for dendritic cells. Although IHC has the advantage of showing the spatial relationships, multiple staining is challenging, and allows only limited marker sets. Consequently, findings in this study should be considered in this context. Moreover, differences in plaque inflammation may also exist among various vascular beds, although our previous work clearly shows that aortic atherosclerosis follow a similar pattern of disease progression as for coronary tissue^{19, 20, 21}.

In conclusion, the presence of various macrophage subsets and fascin-positive dendritic cells are strongly associated with atherosclerotic disease of human aorta, particularly for progressive and vulnerable fibroatheromatous plaques. A less prominent role however, is suggested for mast cells and NK cells, while a direct relation with neutrophils and eosinophils was not established. More interestingly, macrophage heterogeneity is far more complex than the current paradigm predicated on murine data and supports the involvement of additional (poorly-defined) macrophage subtypes or perhaps a greater dynamic range of macrophage plasticity. Clearly, there is a divergence in observational data between murine models of atherosclerosis from our human atherosclerosis studies, which point to a limited translational aspect of animal findings.

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