Atherosclerosis 225 (2012) 461–468

Contents lists available at SciVerse ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Distribution of macrophage polarization markers in human atherosclerosis

J. Lauran Stöger [a,c], Marion J.J. Gijbels [a,b,c], Saskia van der Velden [c], Marco Manca [b], Chris M. van der Loos [d], Erik A.L. Biessen [b], Mat J.A.P. Daemen [d], Esther Lutgens [c], Menno P.J. de Winther [c,*]

[a] Dept. of Molecular Genetics, Cardiovascular Research Institute Maastricht, Maastricht University, 6229ER Maastricht, The Netherlands
[b] Dept. of Pathology, Cardiovascular Research Institute Maastricht, Maastricht University, The Netherlands
[c] Dept. of Medical Biochemistry, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands
[d] Dept. of Pathology, Academic Medical Center, Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 16 April 2012
Received in revised form
3 September 2012
Accepted 7 September 2012
Available online 26 September 2012

Keywords:
Human pathology
Coronary artery disease
Innate immunity
Macrophages
Foam cell

ABSTRACT

Objective: Macrophages are decisive in the chronic inflammatory processes that drive atherogenesis. The purpose of this study was to explore the presence and spatial distribution of polarized macrophage populations in human atherosclerosis.
Methods & results: We used transcriptomics and immunohistochemistry to analyze macrophage subset dynamics in successive stages of atherogenesis. Developing lesions progressively accumulated both M1 and M2 cells, as was signified by the enhanced expression of associated markers at the transcriptional and protein level. Histologically, these markers were confined to overlapping, but spatially distinct $CD68^+$ areas of the intima. We subsequently quantified the presence of these markers in relation to morphological determinants of plaque stability. In line with their pro-inflammatory characteristics, M1 macrophages dominated the rupture-prone shoulder regions of the plaque over M2 polarized cells, while the fibrous caps of lesions showed no significant differences between subsets. In contrast, vascular adventitial tissue displayed a pronounced M2 activation profile. As expected, areas of intraplaque hemorrhage clearly associated with CD163 staining. Rather than being limited to complicated lesions, this M2 marker was also readily detectable in stable plaques. Finally, foamy macrophages displayed an ambiguous repertoire that incorporates individual M1 and M2 markers.
Conclusion: M1 and M2 macrophage populations are present throughout atherogenesis. These subsets display disparity when it comes to their prevalence in morphological compartments of the vessel wall. Our current findings warrant continued investigation into the functional implications of polarized macrophage populations in human atherosclerosis.

1. Introduction

Atherosclerosis is a chronic inflammatory disorder of the arterial wall that can give rise to acute atherothrombotic events (e.g. myocardial infarction, stroke) via plaque rupture or erosion [1]. Hereby, it represents the main cause of cardiovascular morbidity and mortality [2]. A pivotal step in atherogenesis involves the subendothelial accumulation of monocyte-derived macrophages at predisposed sites of endothelial dysfunction and intimal lipoprotein retention. In nascent lesions, these cells orchestrate the scavenging of lipids and cellular debris, as well as the local inflammatory equilibrium to ultimately define the likelihood of plaque complications [3,4].

Macrophages are hallmarked by phenotypic heterogeneity and express a spectrum of activational programs that exist as a function of their immediate surroundings. Amongst a plethora of micro-environmental signals, certain cytokines and microbial moieties have been demonstrated *in vitro* to profoundly skew macrophage expression patterns and functions [5]. The current paradigm includes interferon-γ (IFNγ) and lipopolysaccharide (LPS)-driven M1 polarization that supports the production of pro-inflammatory stimuli (e.g. TNF, interleukin (IL)-1β, IL-6, IL-12 and reactive nitrogen intermediates) and may cause tissue disruption when sustained; conversely, (IL)-4, IL-13 and IL-10 elicit macrophages under the M2 moniker that act to restrict these inflammatory responses through IL-10 secretion and mediate tissue repair and angiogenesis. Phenotypical distinction between these polarized macrophages involves differential expression of cell surface receptors. Among these, CD86, MHC-II and the macrophage receptor with collagenous structure (MARCO) are typically

* Corresponding author. Tel.: +31 20 5666762; fax: +31 20 695119.
E-mail address: m.dewinther@amc.uva.nl (M.P.J. de Winther).

0021-9150/
http://dx.doi.org/10.1016/j.atherosclerosis.2012.09.013

associated with M1 activation, whereas the mannose receptor (MR), dectin-1 (CLEC7A) and CD163 fit an M2 signature [5,6].

These dissimilarities have permitted the identification of macrophage subsets in several *in vivo* settings [7,8]. Indeed, the presence of M1- and M2-like populations was demonstrated in experimental and human atherosclerotic disease [9–11], while additional plaque-specific macrophage phenotypes were adopted into the existing paradigm only recently [12–14]. Given the contrasting properties of pro-inflammatory M1 and anti-inflammatory M2 macrophages in homeostasis and disease, the balance between these populations has been deemed crucial in determining plaque outcome (recently reviewed in Refs. [15,16]).

In the present study, we examined a panel of established M1- and M2-associated macrophage markers for their presence in stable and unstable human atherosclerosis and subsequently assessed their distribution relative to plaque morphology.

2. Methods

2.1. Plaque transcriptomics

Gene expression data were taken from an existing database [17]. Briefly, microarray analysis was performed on RNA from ruptured and paired stable control segments ($n = 22$, respectively) from human endarterectomy specimens that were obtained from the Maastricht Pathology Tissue Collection (MPTC). All use of tissue and patient data was in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (http://www.fmwv.nl). Illumina Human Sentrix-8 V2.0 BeadChip technology was used to detect differential mRNA expression (see Online Appendix for details).

2.2. Immunohistochemistry

For the purpose of immunohistochemistry (IHC), additional human atherosclerotic lesions were again collected from the Maastricht Pathology Tissue Collection (MPTC). All proceedings were in agreement with the Dutch Code of Conduct for Observational Research with Personal Data (2004) and Tissue (2001, www.fmwv.nl). Plaques were classified according to Virmani by their appearance on H&E staining [18]. We selected 6 early (intimal xanthoma, IX), 6 advanced (fibrous cap atheroma, FCA) and 6 hemorrhaged (FCA with intraplaque hemorrhage, IPH) lesions from 18 individuals (mean age 78 years, IQR 70–83 years with no statistically significant differences between groups; 12 males/6 females), who were referred for surgery or had died ($n = 9$, respectively) at Maastricht University Medical Center. Hemorrhaged lesions were carotid specimens from surgery, advanced plaques were post-mortem aortic tissues and early lesions were of mixed origin.

Immediately after resection, atherosclerotic tissues were divided into parallel segments of 4 mm. Alternate segments were designated for histology, fixed overnight in 4% paraformaldehyde and subsequently embedded in paraffin. For immunostaining, paraffin sections were deparaffinized before endogenous peroxidase quenching and heat-induced epitope retrieval (HIER, ChemMate citrate buffer for Antigen Retrieval, Dako, Glostrup, DK) or pepsin treatment. After blocking, slides were incubated with α-CD68, α-HLA-DP/Q/R (Dako, Glostrup, DK), α-iNOS, α-CD86 (Abcam, Cambridge, UK), α-MARCO (Hycult Biotechnology, Uden, NL), α-MRC1 (Sigma–Aldrich, St. Louis, MO), α-CD163 (Novocastra, Newcastle on Tyne, UK) and α-Dectin-1 (R&D, Abingdon, UK). Antibody binding preceded incubation with appropriate biotinylated secondary antibodies and alkaline phosphatase for most antibodies. Staining was visualized using the VectaStain® ABC-AP kit (Vector Labs, Burlingame, CA). Notably, CD68 staining was developed using horseradish peroxidase and DAB. In all cases, hematoxylin was used as a counterstain. Replacement of the primary antibodies by PBS served as a negative control. Full details regarding all antibodies and reagents are presented in Table S1.

2.3. Analysis of immunohistochemistry

Immunostainings were assessed semi-quantitatively to reflect the overall degree of positivity; + indicated slight positivity that adhered to a disseminated staining pattern, ++ was given to modest staining with additional focal accumulation, +++ signified widespread positivity throughout the intimal space. We scored individual plaques in a manner proportionate to other slides stained for that particular antibody, but irrespective of the degree of staining from other primary antibodies.

Immunohistochemical quantification for CD68, iNOS, CD86, HLA-DP/Q/R, MARCO, dectin-1, MR and CD163 in plaque shoulders, fibrous cap and adventitial tissue was performed using ImageJ image processing software (NIH, Bethesda, Maryland U.S.A, http://imagej.nih.gov/ij/). Photomicrographs (100× magnification, approximately 400 μm^2) of regions of interest (ROI) were assessed for the presence of the various markers and reported as the percentage positive counts out of $CD68^+$ cells. For shoulder and fibrous cap tissue all available ROIs were quantified. If an area spanned more than one image, values from those images were averaged and presented as a single measurement. For adventitial tissue, 3 separate areas encompassing advanced plaques ($n = 6$) were counted and averaged to yield one value per lesion. We used one-way analysis of variance (ANOVA) for statistical comparisons between multiple groups.

2.4. Spectral imaging

For detection of colocalization, we sequentially combined a double and a single IHC procedure [19]. Briefly, routine workup (including HIER) and antibody binding for CD68 and MR were followed by visualization of AP activity in blue with VectorBlue (Vector Labs) and HRP activity in brown with enhanced DAB (DAB+, Dako), respectively. A subsequent HIER step ensured removal of all previous immunoreagents, but left staining intact. Double-stained specimens were then incubated with an iNOS primary antibody, which was enzymatically visualized in red through VectorRed (Vector Labs). Sections were mounted in VectaMount medium (Vector Labs) without counterstaining.

Following triple staining of the aforementioned plaque specimens, we used a Nuance VIS-FL Multispectral Imaging System (Cambridge Research Instrumentation; Woburn, MA) to acquire spectral data (420–720 nm at 20-nm intervals). The unique spectral signature of each chromogen determined with Nuance software v3.0 allowed unmixing of spectral data cubes into fluorescent-like images that use pseudo-colors to accentuate the distribution of each staining. Once appropriately superimposed, these images enabled quantification of pixels showing colocalization vs. singular $CD68^+$ pixels, rather than cell-based counts.

3. Results

3.1. Ruptured plaque specimens overexpress M1 and M2 macrophage marker genes

To investigate whether M1 and M2 macrophage subsets are dynamically present in human atherosclerosis, we first assessed gene expression levels for known polarization markers using an existing plaque transcriptomics database [17]. We compared data

taken from ruptured and adjacent stable control segments of 22 endarterectomy specimens, which had been categorized according to the Virmani classification [18]. Many macrophage-related genes were highly expressed in ruptured plaque segments, indicating increased macrophage burden [20] and/or a greater level of cellular activation in these areas relative to stable cross-sections. However, decreased lesion stability in these specimens was not associated to a preferential polarized state, as multiple markers representative of either M1 or M2 skewing were significantly upregulated in ruptured plaque sections (Table 1). For M1 activation specifically, surface receptors such as CD86, HLA-DP/Q/R and MARCO showed enhanced expression, whereas relevant M2 markers such as DC-SIGN and PPARγ also displayed increased mRNA levels. Likewise, CD163 and HO-1, M2 markers regulated by heme-induced oxidative stress, showed remarkable upregulation upon plaque rupture. Other polarization markers did not show differential expression (e.g. MR) or were unavailable in this particular dataset (e.g. dectin-1, iNOS). Thus, the transcriptional profile of M1- and M2-related genes revealed that both populations exist and likely accumulate in ruptured plaque segments.

3.2. M1 and M2 macrophages amass in human atherosclerosis with advancing plaque severity

Next, we examined whether protein expression for M1 and M2 markers would mimic the overall transcriptional pattern. To this end, we selected human atherosclerotic samples that were defined as early (intimal xanthoma), advanced (fibrous cap atheroma) or hemorrhaged (fibrous cap atheroma with intraplaque hemorrhage), $n = 6$ per group. Where both early and advanced stages represent stable plaque phenotypes, intraplaque hemorrhage is characteristic of lesional instability [21]. We performed IHC for several established markers of macrophage polarization on these plaque cross-sections. Subsequent semi-quantitative analysis revealed that advancing lesion severity triggers incremental expression of all investigated markers (Fig. 1, see Fig. S1 for supporting photomicrographs). Thereby, overall protein expression of polarization markers was in line with the transcriptional signature outlined above.

Immunostaining for designated macrophage markers was confined to larger $CD68^+$ (a pan-macrophage marker) parts of the intima. Areas of tissue expression typically overlapped between markers, but were ultimately non-identical in appearance (Fig. S1). Specifically, iNOS, HLA-DP/Q/R and CD86 presented with considerable positivity in advanced and hemorrhaged plaques, while LPS-inducible MARCO expression was less pronounced. Dectin-1 embodied the most widespread M2 marker in these settings. Although positivity for MR was abundant in unstable lesions, staining adhered to a disseminated pattern in early and advanced plaques. Moreover, CD163 staining displayed steady increments with plaque progression and was typically expressed in hemorrhaged plaques (Fig. 1, Fig. S1). Yet even in earlier stages without indication of lesional bleeding we detected convincing positivity for this marker (Fig. S2a). Together, our findings provide clear evidence for the presence of M1 and M2 macrophage subsets in human atherosclerotic lesions. Importantly, the incremental accumulation of both these populations corresponded with plaque progression.

Table 1
Differential gene expression for established macrophage polarization markers in ruptured vs. stable human carotid plaque segments.

Protein	Gene symbol		Log fold change	Fold change ruptured/stable	Corrected *p*-value
M1 Mφ markers					
TNF	*TNF*	Cytokine	0.383	1.30	3.47E-04
IL-1β	*IL1B*	Cytokine	0.929	1.90	5.59E-04
IL-6	*IL6*	Cytokine	0.670	1.59	1.63E-03
IL-12p40	*IL12B*	Cytokine	0.019	1.01	3.12E-01
IL-23	*IL23*	Cytokine	0.056	1.04	1.28E-02
MARCO	*MARCO*	Surface receptor	1.406	2.65	7.50E-04
MHC-II	*HLA-DPB1*	Surface receptor	0.488	1.40	1.33E-03
CD80	*CD80*	Surface receptor	0.354	1.28	1.59E-04
CD86	*CD86*	Surface receptor	0.597	1.51	3.11E-04
iNOS	*NOS2*	Enzyme	n/a	n/a	n/a
M2 Mφ markers					
IL-1RA	*IL1RN*	Cytokine	0.065	1.05	1.58E-01
IL-10	*IL10*	Cytokine	0.096	1.07	8.57E-05
TGFβ	*TGFB1*	Cytokine	−0.050	0.97	7.02E-03
MR	*MRC1*	Surface receptor	0.034	1.02	2.31E-01
CD163	*CD163*	Surface receptor	1.712	3.28	2.46E-05
DC-SIGN	*CD209*	Surface receptor	0.970	1.96	1.31E-04
Dectin-1	*CLEC7A*	Surface receptor	n/a	n/a	n/a
Arg-1	*ARG1*	Enzyme	n/a	n/a	n/a
HO-1	*HMOX1*	Enzyme	3.061	8.35	5.09E-07
PPARγ	*PPARG*	Transcription factor	1.098	2.14	4.13E-05

3.3. Differential distribution of polarized macrophages within atherosclerotic lesions links M1 activation to rupture-prone plaque areas

We subsequently considered the spatial distribution of M1 and M2 markers in plaques by assessing their affiliation to key morphological aspects of the atherosclerotic intima known to influence lesion stability (e.g. plaque shoulders, fibrous cap, hemorrhage) [4,22]. In appropriate areas, positive cell counts per marker were expressed as a percentage of $CD68^+$ cells.

Plaque shoulders were defined as area between adjacent normal intima and the outer edges of the necrotic core. Here, the amount of iNOS, HLA-DP/Q/R and CD86 positively offset the presence of dectin-1, MR and $CD163^+$ cells in a statistically significant manner, whereas the selective induction pattern of MARCO was reflected in its limited expression (Fig. 2A,B and Fig. S3). Of note, while macrophages within shoulder regions expressed independent iNOS or MR positivity, we found negligible colocalization of iNOS and MR in $CD68^+$ macrophages (Fig. 2C and Fig. S4). This upheld the standing of these macrophages as a genuine M1 population that exists as the principal subset in the inflammatory plaque shoulder.

Similar analyses for cellular staining in the fibrous cap regions (i.e. a thin tissue layer covering the necrotic core) failed to show clear differences in M1 and M2 macrophage numbers. Only iNOS and HLA-DP/Q/R staining showed an upward trend in comparison to the other markers (Fig. 2D,E). As expected, the presence of intraplaque iron deposits (microscopically identifiable as brown pigmented granules dispersed in the tissue) within hemorrhaged atherosclerotic plaques coincided with pronounced $CD163^+$ staining (Fig. S2b). Although IPH was not a requirement for the occurrence of this marker in plaques, CD163 was the only marker to consistently display this particular association in our dataset.

Hereby, we demonstrated disparity in the distribution of polarized macrophage subsets in human atherosclerotic lesions, notably within its shoulder regions. Since the plaque shoulders are important predilection sites for plaque rupture, this finding supports the hypothesis that M1 macrophages mediate deleterious effects on plaque stability.

3.4. Macrophages situated in the perivascular adventitial tissue exhibit pronounced M2 activation

The adventitia is home to resident populations of immune cells, including macrophages [23]. Upon investigation, we observed that

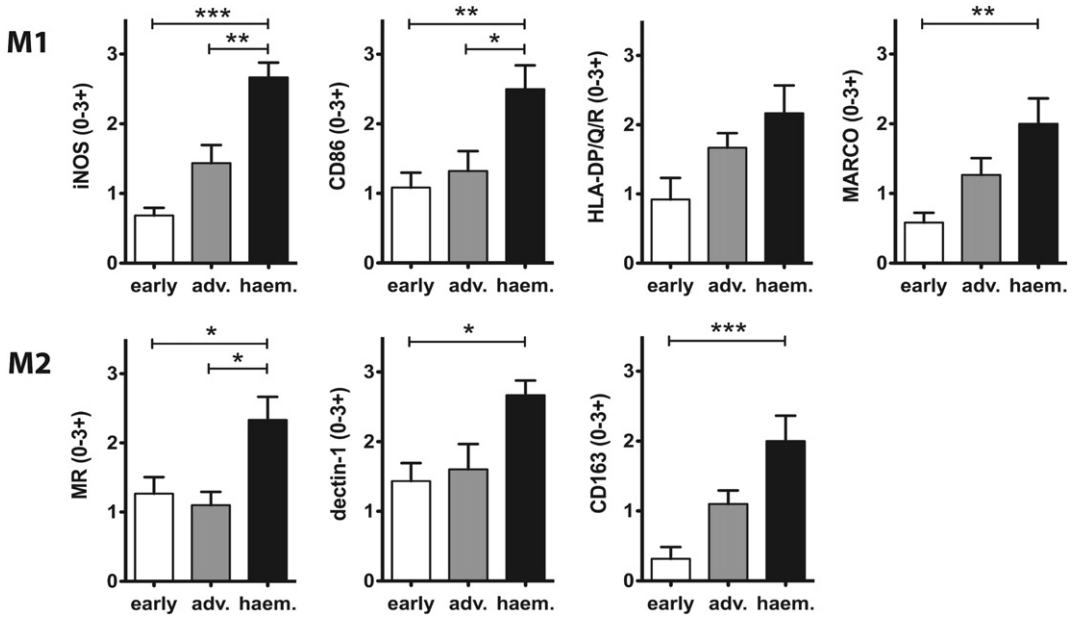

Fig. 1. M1 and M2 macrophages accumulate incrementally with plaque progression. Semi-quantitative analysis of immunostainings for M1 and M2 markers in early, advanced (adv.) and hemorrhaged (heme.) human plaques. Scores of 0 to 3+ reflect the degree of intimal positivity. One-way ANOVA was used to test statistical significance. Error bars indicate SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

adventitial macrophages presented as a loosely disseminated population that characteristically expressed high levels of MR and CD163 and to a lesser extent, dectin-1 (Fig. 3A, Fig. S5). These adventitial M2 macrophages outnumbered their M1 counterparts by 2–3 fold (Fig. 3B). Thus, adventitial macrophages are selectively skewed towards an M2 polarized phenotype.

3.5. Foam cells ambiguously express macrophage polarization markers

Recent studies reported that plaque-associated macrophages expressing certain M2 markers display altered foam cell formation [12,24]. We questioned whether the expression of other M1 and M2 macrophage markers would be dissimilar between foamy and non-foamy plaque-associated macrophages. Indeed, we too observed that $CD68^{+}$ macrophages with a foamy appearance typically expressed low levels of MR (Fig. 4) and CD163 (not shown), whereas non-foamy macrophages often stained positive for these receptors. By contrast, dectin-1, like MR induced by IL-4, was widely expressed by lesional foam cells. Additionally, the M1 markers examined here were characterized by comparable ambiguity (Fig. 4). HLA-DP/Q/R and iNOS were readily found on foamy macrophages, whereas staining for MARCO showed little positivity. Overall, these findings verify and add nuance to earlier reports by demonstrating an inconsistent M1/M2 expression profile of lesional foam cells.

4. Discussion

In this study, we investigated the presence and spatial distribution of polarized M1 and M2 macrophages in human atherosclerotic plaques. We found that gene expression signatures for both activational states were enhanced in ruptured vs. stable plaque segments. Subsequent histopathological analysis revealed that M1 and M2 macrophages persistently accumulate in plaques with progressing lesion severity. Pro-atherogenic M1 macrophages surpassed their M2 counterparts in a key rupture-prone area of the intima (i.e. plaque shoulder), but did not display such predominance in fibrous cap regions. Remarkably, the macrophage content in the vascular adventitia conformed strongly to an alternative M2 phenotype. Thus, M1 and M2 macrophages both characterize diverse stages of human plaque development but localize to distinct morphological features of the lesions.

Monocyte-derived macrophages are instrumental to the atherogenic process and contribute to its initiation, progression and symptomatology. As plaque development may originate not only from persistent inflammation, but also from inadequate anti-inflammatory responses, the macrophage polarization balance holds clear implications for lesion formation [15,25]. The atherosclerotic plaque provides a unique environment for intimal macrophages to adopt divergent activational states. Consequently, their phenotypic heterogeneity in atherosclerosis was recognized early on [26].

Bouhlel et al. first documented the presence of $CD68^{+}MR^{+}$ macrophages in human carotid plaques [10]. Notably, this M2 subset was typically situated far from the lipid core and distinct from $CCL2^{+}$ M1 macrophages in the lesion. Further reports by the same group expanded on these observations by identifying MR^{+} macrophages as resistant to cholesterol loading [24]. Our findings corroborate that foam cells express low levels of MR, whereas overall positivity for this marker was increasingly detected with plaque progression. Complementary to earlier reports that external differentiation factors can profoundly affect macrophage lipid handling [11], a recent publication by Finn et al. provided additional insight into this phenotype by revealing that hemoglobin elicits $CD163^{+}MR^{+}$ macrophages that exhibit enhanced reversed cholesterol transport capabilities [12]. In turn, we observed that lesional foam cells express an ambivalent repertoire that incorporates some M1 and M2 markers, while excluding others. This marker promiscuity likely originates from the fact that multiple additional triggers (e.g. heme, oxLDL) interact with plaque macrophages *in vivo*, beyond the traditional array of cytokine stimuli. Identifying which factors can specifically impede foam cell formation could prove clinically beneficial, provided they are free from harmful properties.

Preceding the findings by Finn et al., Boyle and co-workers reported that CD163 defines a subset of atheroprotective macrophages exclusive to hemorrhaged lesions [21]. Here, we indeed find CD163 is strongly expressed in case of IPH, but can also detect

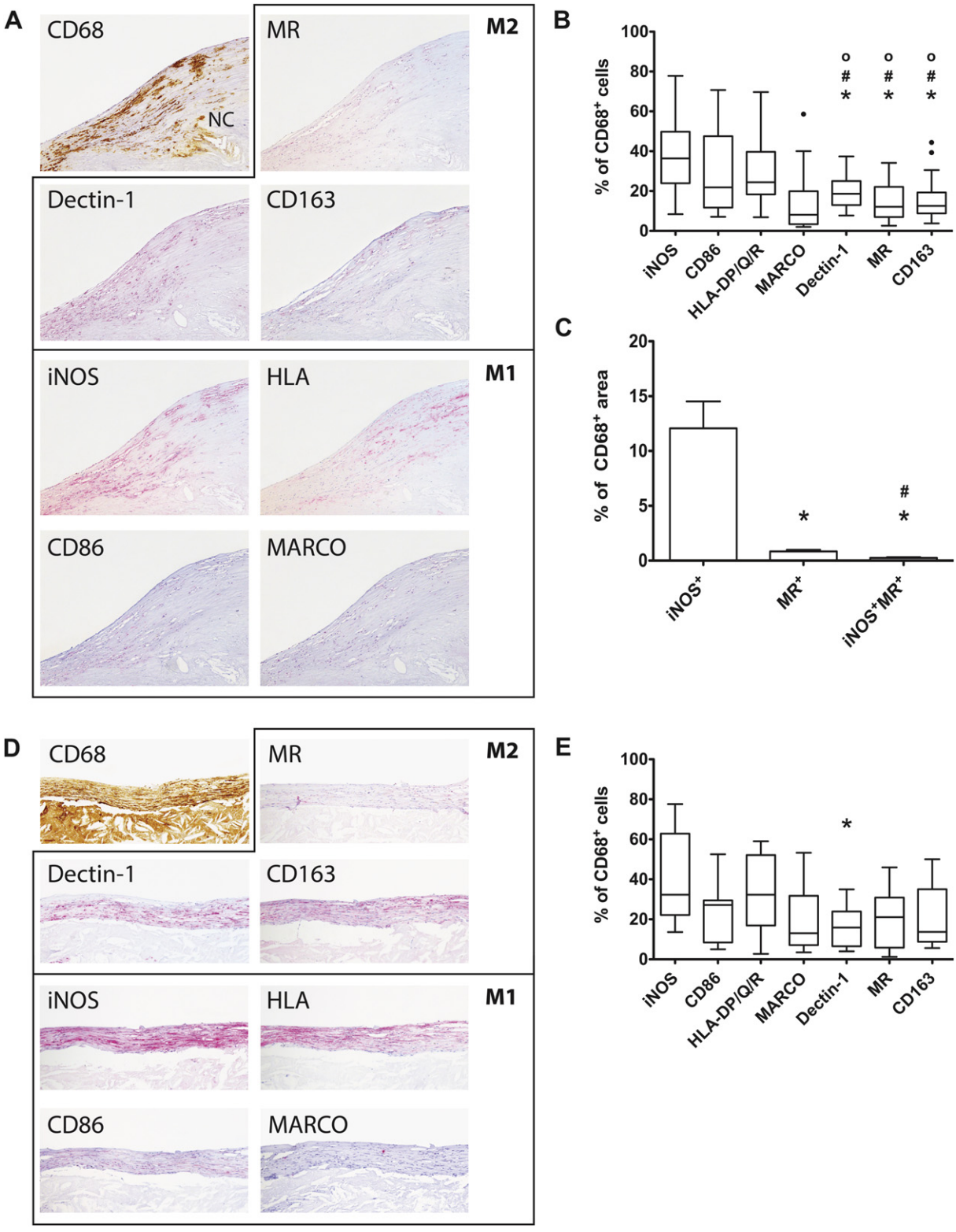

Fig. 2. M1 macrophages dominate plaque shoulders, but not fibrous caps of human atherosclerotic plaques. Representative photomicrographs showing occurrence (red) of a panel of M1 (bottom) and M2 markers (top) in plaque shoulders (A) and fibrous cap regions (D). CD68 indicates macrophages (brown), NC = necrotic core. Magnification 100×. Subsequent quantitative analysis demonstrates M1 predominance over M2 markers in the former (B), but not in fibrous cap tissue (E). *, # and ° represent statistical significance with one-way ANOVA vs. iNOS, CD86 and HLA-DP/Q/R respectively. $CD68^+$ macrophages in plaque shoulders showed negligible colocalization of iNOS and MR (C). *$p < 0.001$ vs. $iNOS^+$, #$p < 0.05$ vs. MR^+ with one-way ANOVA.

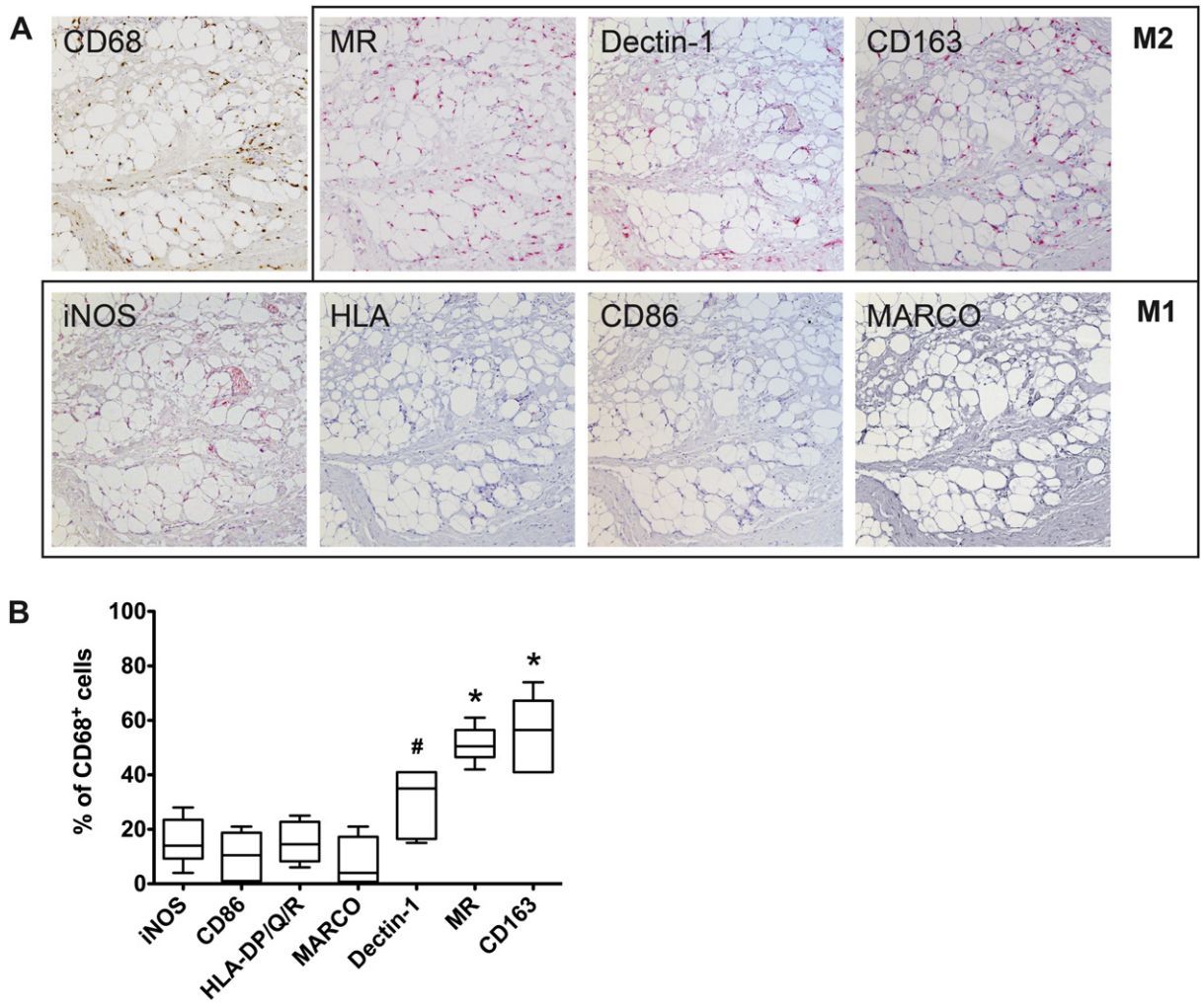

Fig. 3. Adventitial macrophage content is profoundly skewed to an M2 phenotype. Representative microscopic images showing M1 (bottom) and M2 (top) macrophages in perivascular tissue (red). CD68 denotes macrophages (brown). Magnification 100×. (A) Quantitative analysis of adventitial tissue revealed an abundance of M2 macrophages over M1 cells. (B) #$p < 0.05$, *$p < 0.001$ vs. all investigated M1 markers using one-way ANOVA.

a modest presence in earlier stages of atherosclerosis (i.e. in absence of hemoglobin). Although in our view this does not negate CD163's ability to signify an unstable plaque phenotype [20], these results contribute to the ongoing debate concerning the function of $CD163^+$ macrophages in *in vivo* disease models [27].

To our knowledge, we are the first to report a robust M2 population in the adventitia underlying advanced plaques. In mice, the normal aortic wall houses macrophages as part of a local immune compartment that considerably expands upon hyperlipidemia [23]. Intriguingly, the inflammatory nature of this influx [28] challenges the dogma that M2 macrophages are atheroprotective. One could argue that perivascular M2 macrophages support leukocyte recruitment by enhancing adventitial and plaque neovascularization [29]. Thus opposing the anti-inflammatory and profibrotic functions commonly attributed to M2 subsets, this would advocate a deleterious role for adventitial M2 cells in atherogenesis. While we lacked appropriate specimens to settle these matters at present, continued investigation could potentially draw from other fields of study. Notably, adipose tissue macrophages assist healthy metabolism under lean conditions through their anti-inflammatory M2 properties [30]. Ensuing obesity however triggers CCL2-dependent recruitment of M1 macrophages that aggravates metabolic disturbance [7,31]. An analogous phenotypic switch may occur in the adventitia with changing plaque morphology; alternatively, a local shift in adventitial macrophage polarization balance could even precede, rather than just reflect, disease progression. Thus, our studies set the stage for more detailed investigation of these fascinating issues.

In this context, the macrophage polarization balance in adipose tissues creates a platform for cardiovascular and metabolic research alike. Interesting in this respect is a yet to be explained phenomenon termed the 'obesity paradox'. Whereas obesity augments mortality risk in the general population, certain subpopulations of patients with established cardiovascular disease (e.g. coronary artery disease, heart failure) and disproportionate bodyweight show improved survival vs. normal weight individuals, irrespective of health status or gender [32–34]. Although the interdependence of macrophage and adipocyte function is of major importance to adipose tissue pathology, ongoing studies have yet to consider the relation of leukocyte biology with all-cause mortality in overweight and obese subjects. Doing so will require a multidisciplinary approach, but may yield considerable insight.

Here, we demonstrated a strong and continued presence of both M1 and M2 macrophages during human atherosclerotic plaque development. Markers belonging to either subset were observed in overlaying, but spatially distinct areas of early and more advanced atherosclerotic lesions. These findings are unlike macrophage subset dynamics recently described in a study of murine

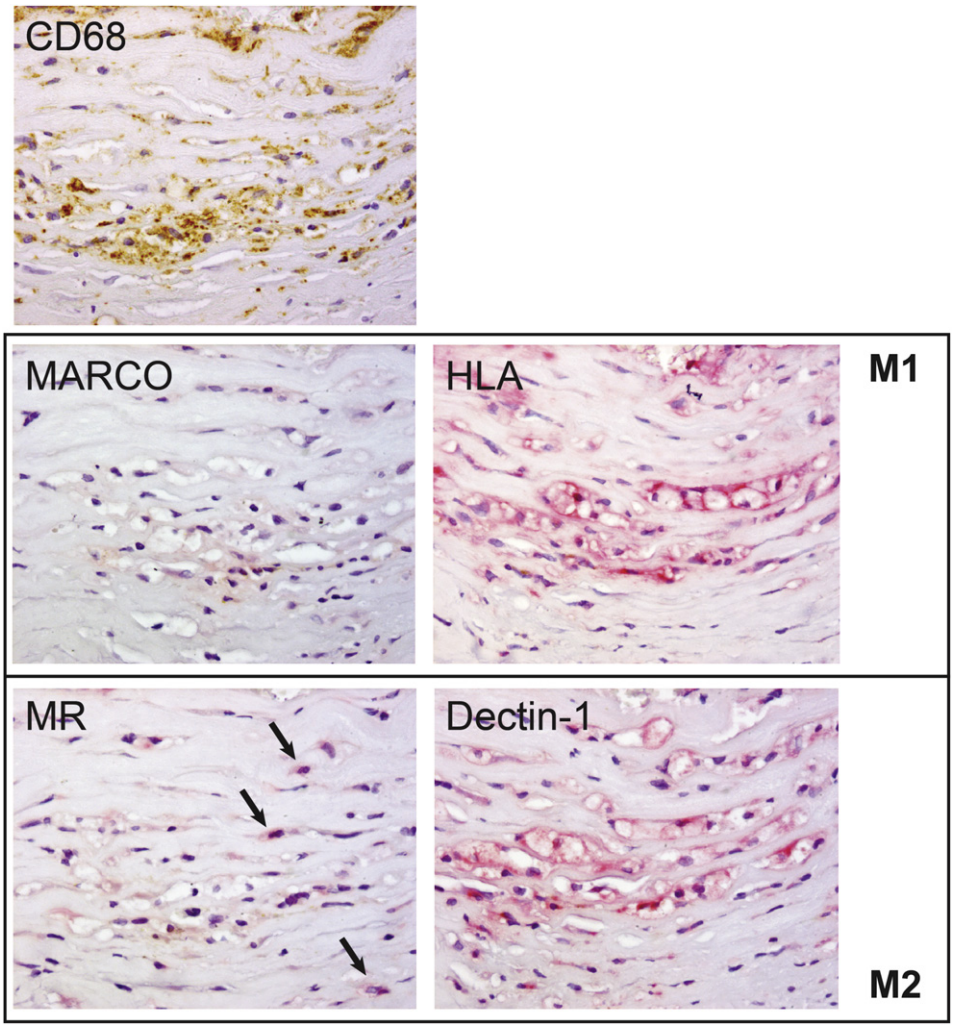

Fig. 4. Lesional foam cells ambiguously express macrophage polarization markers. Representative photomicrographs show ambivalent expression of MARCO, HLA-DP/Q/R (M1) and MR, dectin-1 (M2) on foamy intimal macrophages (red). CD68 indicates macrophages (brown). Arrows indicate small non-foamy MR^+ macrophages. Magnification 400×.

atherosclerosis, revealing an initial M2 predominance was ultimately overturned by M1 macrophages with ensuing plaque growth [9]. In man, we lacked evidence in support of such a phenotypical switch. Whereas this discrepancy could simply point out interspecies differences, others have made observations of M1/M2 co-localization in an elegant murine model of hyperlipidemia reversal that closer resemble our data, where changes in the M1/M2 balance were related to plaque stability [35].

The current paradigm regarding plaque complication incorporates inflammation as the driving force behind fibrous cap rupture [4,36]. Cap disruption most often occurs at the plaque shoulders, where its integrity is degraded by cellular infiltrates consisting of activated macrophages and other leukocytes [22]. This grants considerable significance to the distribution of polarized macrophage populations within the atherosclerotic lesion, as presented in this study. Fibrous cap macrophage composition was balanced, with no distinct differences in M1 and M2 numbers. This observation could imply that M1-derived deleterious effects are actively countered by the reparative, pro-fibrotic efforts of a sustained M2 presence in the fibrous cap. Macrophages residing in the inflammatory shoulder of the plaque displayed strong and clear-cut M1 polarization, with only limited amounts of M2 macrophages present. In our view, this excessive M1 activation fosters sustained inflammation and disruption of fibrous tissue in an intrinsic weak spot of the plaque by boosting production of pro-inflammatory cytokines and tissue-degrading enzymes such as MMPs [37]. Consequently, even a locally enhanced M1 profile could diminish lesion integrity as a whole and increase the likelihood of adverse clinical events. Reinforcing this notion are two recent reports indicating a positive relation between M1-derived pro-inflammatory cytokines and the manifestation of cardiovascular symptoms [38,39]. Collectively, these data therefore strengthen the premise that M1 macrophages are preferentially linked to plaque progression and subsequent thromboembolic complications.

4.1. Limitations

From this study it is apparent that each macrophage marker carries its own distribution pattern. Although this study was performed using several indices for each archetype, we therefore cannot rule out that other markers might behave dissimilarly to the ones presented here. Additionally, the relatively small sample size used in this study may limit the general applicability of our findings.

5. Conclusions

To conclude, we disclose prominent signatures for M1 and M2 polarized macrophages in human atherosclerosis development. As

M1 macrophages likely increase plaque susceptibility to atherothrombosis by virtue of their preferential allocation to plaque shoulders, our work supports the hypothesis that macrophage polarization balance is of critical importance to plaque stability. Novel agents that can either visualize macrophage subsets, or skew the M1/M2 balance towards a more preferable phenotype promise to help reduce (residual) cardiovascular risk. Our study may assist in guiding new avenues for development of such applications in human atherosclerosis.

Conflict of interest statement

All authors declare that they have no competing financial interests.

Author contributions

J.L.S., M.J.J.G. and M.P.J.d.W. conceived experiments, analyzed data and were involved in writing the paper. C.v.d.L and M.J.D. provided expertise for co-localization experiments and subsequent spectral imaging analysis. J.L.S. and S.v.d.V. carried out experiments. M.M. performed statistical analysis of microarray data. E.A.L.B. and E.L. provided scientific input. All authors had final approval of the submitted and published versions of this manuscript.

Acknowledgments

The authors would like to acknowledge Linda Beckers, Clairy Dinjens, Claire Mackaaij and Anique Janssen for their excellent technical assistance. This work was supported by AMC Fellowships to M.P.J.d.W. and E.L. and an Established Investigator grant to M.P.J.d.W. from the Netherlands Heart Foundation (grant-no. 2007-T067).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2012.09.013.

References

[1] Ross R. Atherosclerosis — an inflammatory disease. N Engl J Med 1999 Jan. 14;340(2):115–26.
[2] Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJL. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 2006 May 27;367(9524):1747–57.
[3] Moreno PR, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT. Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. Circulation 1994 Aug.;90(2):775–8.
[4] van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 1994 Jan.;89(1):36–44.
[5] Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci 2008;13:453–61.
[6] Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J Immunol 2006 Nov. 15;177(10):7303–11.
[7] Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007 Jan. 2;117(1):175–84.
[8] Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002 Nov.;23(11):549–55.
[9] Khallou-Laschet J, Varthaman A, Fornasa G, et al. Macrophage plasticity in experimental atherosclerosis. Fritz JH, editor. PLoS ONE 2010 Jan. 25;5(1):e8852.
[10] Bouhlel MA, Derudas B, Rigamonti E, et al. PPARγ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab 2007 Aug.;6(2):137–43.
[11] Waldo SW, Li Y, Buono C, et al. Heterogeneity of human macrophages in culture and in atherosclerotic plaques. Am J Pathol 2008 Apr.;172(4):1112–26.
[12] Finn AV, Nakano M, Polavarapu R, et al. Hemoglobin directs macrophage differentiation and prevents foam cell formation in human atherosclerotic plaques. J Am Coll Cardiol 2012 Jan. 10;59(2):166–77.
[13] Kadl A, Meher AK, Sharma PR, et al. Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. Circ Res 2010 Sep. 17;107(6):737–46.
[14] Gleissner CA, Shaked I, Little KM, Ley K. CXC chemokine ligand 4 induces a unique transcriptome in monocyte-derived macrophages. J Immunol 2010 Apr. 21;184(9):4810–8.
[15] Wolfs IMJ, Donners MMPC, de Winther MPJ. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost 2011;106(5):763–71.
[16] Mantovani A, Garlanda C, Locati M. Macrophage diversity and polarization in atherosclerosis: a question of balance. Arterioscler Thromb Vasc Biol 2009 Sep. 16;29(10):1419–23.
[17] Goossens P, Gijbels MJJ, Zernecke A, et al. Myeloid type i interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions. Cell Metab 2010 Aug. 4;12(2):142–53. Elsevier Ltd.
[18] Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2000 May;20(5):1262–75.
[19] van der Loos CM. Multiple immunoenzyme staining: methods and visualizations for the observation with spectral imaging. J Histochem Cytochem 2008 Apr.;56(4):313–28.
[20] Papaspyridonos M, Smith A, Burnand KG, et al. Novel candidate genes in unstable areas of human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 2006 Aug.;26(8):1837–44.
[21] Boyle JJ, Harrington HA, Piper E, et al. Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. Am J Pathol 2009 Mar.;174(3):1097–108.
[22] Pasterkamp G, Schoneveld AH, van der Wal AC, et al. Inflammation of the atherosclerotic cap and shoulder of the plaque is a common and locally observed feature in unruptured plaques of femoral and coronary arteries. Arterioscler Thromb Vasc Biol 1999 Jan. 1;19(1):54–8.
[23] Galkina E, Kadl A, Sanders J, Varughese D, Sarembock IJ, Ley K. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. J Exp Med 2006 May 15;203(5):1273–82.
[24] Chinetti-Gbaguidi G, Baron M, Bouhlel MA, et al. Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPAR and LXR pathways. Circ Res 2011 Apr. 14;108(8):985–95.
[25] Shalhoub J, Falck-Hansen MA, Davies AH, Monaco C. Innate immunity and monocyte-macrophage activation in atherosclerosis. J Inflamm 2011;8:9.
[26] Poston RN, Hussain IF. The immunohistochemical heterogeneity of atheroma macrophages: comparison with lymphoid tissues suggests that recently blood-derived macrophages can be distinguished from longer-resident cells. J Histochem Cytochem 1993 Oct.;41(10):1503–12.
[27] Sindrilaru A, Peters T, Wieschalka S, et al. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. J Clin Invest 2011 Mar. 1;121(3):985–97.
[28] Maiellaro K, Taylor WR. The role of the adventitia in vascular inflammation. Cardiovasc Res 2007 Sep. 1;75(4):640–8.
[29] Moulton KS, Vakili K, Zurakowski D, et al. Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. Proc Natl Acad Sci USA 2003 Apr. 15;100(8):4736–41.
[30] Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, et al. Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. Nature 2007 May 21;447(7148):1116–20.
[31] Morris DL, Singer K, Lumeng CN. Adipose tissue macrophages: phenotypic plasticity and diversity in lean and obese states. Curr Opin Clin Nutr Metab Care 2011 Jul.;14(4):341–6.
[32] Anyfanti P, Doumas M, Gavriilaki E, Triantafyllou A, Nikolaidou B. More fuel in the obesity paradox debate. Int J Obes 2012 [Epub ahead of print].
[33] Clark AL, Chyu J, Horwich TB. The obesity paradox in men versus women with systolic heart failure. Am J Cardiol 2012 Jul. 1;110(1):77–82.
[34] Younge JO, Damen NL, van Domburg RT, Pedersen SS. Obesity, health status, and 7-year mortality in percutaneous coronary intervention: in search of an explanation for the obesity paradox. Int J Cardiol 2012, in press.
[35] Feig JE, Parathath S, Rong JX, et al. Reversal of hyperlipidemia with a genetic switch favorably affects the content and inflammatory state of macrophages in atherosclerotic plaques. Circulation 2011 Mar. 7;123(9):989–98.
[36] Falk E, Shah PK, Fuster V. Coronary plaque disruption. Circulation 1995 Aug. 1;92(3):657–71.
[37] Duffield JS. The inflammatory macrophage: a story of Jekyll and Hyde. Clin Sci 2003 Jan.;104(1):27–38.
[38] Shalhoub J, Cross A, Allin D, Essex D, Davies A, Monaco C. BAS/BSCR36 cytokine profiling in culture reveals a predominance of M1 macrophage polarisation in symptomatic carotid plaques. Heart 2010 Aug. 26;96(17):e23–3 [Internet]. Available from: http://heart.bmj.com/cgi/doi/10.1136/hrt.2010.205781.49.
[39] Hirata Y, Tabata M, Kurobe H, et al. Coronary atherosclerosis is associated with macrophage polarization in epicardial adipose tissue. J Am Coll Cardiol 2011 Jul. 12;58(3):248–55.