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Graphical abstract

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Single-cell profiling reveals comparatively inflammatory polarization of human carotid versus femoral plaque leukocytes

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Abstract

Rationale: Femoral atherosclerotic plaques are less inflammatory than carotid plaques histologically, but limited cell-level data exist regarding comparative immune landscapes and polarization at these sites.

Objectives: We investigated intraplaque leukocyte phenotypes and transcriptional polarization in 49 total patients undergoing femoral (N=23) or carotid (N=26) endarterectomy using single-cell ribonucleic acid sequencing (scRNA-seq; N=13), flow cytometry (N=24), and immunohistochemistry (N=12).

Findings: Comparative scRNA-seq of CD45 positive-selected leukocytes from femoral (N=9; 35265 cells) and carotid (N=4; 30655 cells) plaque revealed distinct transcriptional profiles. Inflammatory foam cell-like macrophages and monocytes comprised 2.5- to 4-fold higher proportions of myeloid cells in carotid plaques, whereas non-inflammatory foam cell-like macrophages and LYVE1-overexpressing resident-like macrophages comprised 3.5- to 9-fold higher proportions of myeloid cells in femoral plaque (p<0.001 for all). A significant comparative excess of CCR2⁺ macrophages in carotid versus femoral plaque was observed by flow cytometry in a separate validation cohort. B cells were more prevalent and exhibited a comparatively anti-inflammatory profile in femoral plaque, whereas cytotoxic CD8⁺ T cells were more prevalent in carotid plaque.
Conclusion: Human femoral plaques exhibit distinct macrophage profiles and diminished CD8+ T cell populations compared with carotid plaques. Experimental models elucidating determinants of plaque site-specific cell polarization cues are warranted.

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Graphical Abstract: Comparatively inflammatory macrophage profiles observed in carotid versus femoral plaque
Introduction

Vascular inflammation is a hallmark of atherosclerosis and is broadly characterized not only by the retention of cholesterol-laden lipoproteins, but also by dysregulation of innate and adaptive immune responses. (1, 2) Pharmaco-interventions broadly targeting inflammation in atherosclerosis have yielded mixed results depending on the biological and clinical target studied. (3-5) These trials focused primarily on coronary and carotid-cerebral atherosclerosis-related events; clinical trial data on inflammation modulation in peripheral arterial disease (PAD) are limited and consist primarily of pleotropic lipid-lowering and antithrombotic therapy effects. (6, 7) These mixed results demonstrate both the promise and challenges of broadly targeting systemic inflammation to treat and prevent atherosclerotic cardiovascular events in distinct vascular beds.

Given heterogeneity of human atherosclerosis, there is a need to move beyond singular, unified definitions of atherosclerosis. (8) Carotid plaques are prone to vulnerability and rupture leading to acute downstream effects such as acute ischemic stroke, (9-11) whereas femoral atherosclerosis and resultant peripheral arterial disease (PAD) progress gradually and generally without rupture; the majority of patients with symptomatic PAD have stable symptoms at 1 year. (12-14) Recent histologic analyses provided morphologic and bulk gene expression-related corollaries to these clinical observations: carotid plaques generally have more inflammatory cells and bulk expression of genes associated with inflammatory cytokines and cell proliferation compared with femoral plaques. (15-17)
The aforementioned studies have yielded key initial insights into phenotypic and functional differences between femoral vs. carotid arterial plaque, including distinct local immune milieus, yet several important questions remain unresolved. Whereas bulk sequencing assays provide high-level gene readouts across broad populations, single-cell analyses enable more granular definition of cell type-specific transcriptional programs and important functional heterogeneity. Recent single-cell analyses of leukocytes in human carotid atherosclerotic plaque obtained from surgical endarterectomy revealed important and previously under-appreciated features of innate and adaptive immune cells in plaques.(18, 19) Meanwhile, the only prior single-cell study of human femoral plaque to our knowledge was a recent study that compared scRNA-seq data generated from a single femoral plaque to a publicly available carotid scRNA-seq dataset.(20) Therefore, we compared the distinct, cell-specific transcriptional programs in human femoral vs. carotid plaques after endarterectomy using single-cell ribonucleic acid sequencing (scRNA-seq), and validated key plaque site-level differences in a separate validation cohorts by flow cytometry and immunohistochemistry.

**Results**

**Study Population**

We performed single-cell ribonucleic acid sequencing (scRNA-seq) on freshly excised plaque samples from 13 distinct patients who underwent femoral (N=9; 35265 CD45+ cells analyzed) or carotid (N=4; 30655 cells analyzed) endarterectomy; whole plaques were digested into single-cell suspensions to optimize cell yield for sequencing. To
validate key findings related to immune cell phenotypes in femoral versus carotid plaque, as well as correlates in blood, we enrolled 24 additional participants undergoing carotid (N=15) or femoral (N=9) endarterectomy to perform flow cytometry on plaque suspensions (prepared in the same manner as for scRNA-seq analyses) and pre-operatively obtained blood samples. We also investigated relative proportions of B and T cells in lymphoid aggregates in situ via immunohistochemistry from plaque specimens of an additional 12 patients who underwent femoral (N=5) or carotid (N=7) endarterectomy. This yielded a total of 49 distinct patients who underwent femoral (N=23) or carotid (N=26) endarterectomy (Supplemental Figure S1).

**Single Cell RNA sequencing of femoral and carotid plaques reveals 13 distinct leukocyte clusters**

After quality control (Table 1, Supplemental Figure S2, and Methods) 65,920 CD45+ cells (30655 carotid and 35265 femoral) were analyzed using the Seurat package. Clustering of total CD45+ cells revealed 13 leukocyte cell clusters (Figure 1; clusters with sample-level and gene-specific UMAP overlays in Supplemental Figures S3 and S4) and 1 cluster (Cluster 12) expressing CD34 and ACTA2. To validate manual cluster annotation, cell types were automatically annotated using the Celltypist package (21) to label cells against the "Immune_All_High" reference dataset, which includes immune population from 20 tissues derived from 18 studies; this validated our manual cluster annotation and observed overall similar proportions of cell subsets by plaque site (Supplemental Figure S5). The 13 leukocyte clusters included 5 myeloid clusters (Clusters 4, 5, 7, 10, and 11). Cluster 4 and 5 expressed upregulated CD14 and CD68,
consistent with macrophages. A small mast cell population (Cluster 10) was defined by upregulated expression of *KIT, HDC, CMA1, and TPSAB1*, while a distinct population (cluster 7) represented monocytes. Dendritic cells (cluster 11) expressed *CLEC4C*. The 8 lymphoid clusters (Clusters 0, 1, 2, 3, 6, 8, 9, and 13) consisted of a population of B cells (cluster 3, expressing *CD79A* and *CD22*), a smaller cluster of plasma cells (cluster 13, expressing *IGHM, JCHAIN, IGHG*), natural killer cells, and 5 T cell clusters. (22, 23) T cell clusters included two CD4+ T and three CD8+ T cell subsets (defined in more detail in lymphoid sub-clustering analyses below).

When comparing femoral versus carotid plaques, femoral plaques had lower proportions of macrophages and higher proportions of B cells than carotid plaque (Figure 2A, 2B). Our observed proportions of CD45+ cells in carotid plaque that were T cells vs. monocytes/macrophages were remarkably similar to those observed in two distinct scRNA-seq studies of carotid atherosclerotic plaque; (18, 19) no prior studies have performed such analyses in femoral plaque, precluding similar comparisons. Taken together, unique vascular bed-specific signatures between femoral and carotid plaques emerged. When investigating differential gene expression overall by plaque site for CD45+ cells (Figure 2C), inflammatory gene *IL1B* was the most comparatively over-expressed in carotid plaque, along with *CCL3*, which is a chemokine that recruits monocytes and T cells and triggers inflammatory macrophage polarization. Meanwhile, B cell-associated genes *IGHM* and *CD79A* were the most comparatively over-expressed for femoral versus carotid plaque (24). B cell-associated genes *IGHM* and *CD79A* were the most comparatively over-expressed for femoral versus carotid plaque.
To further delineate heterogeneous cell clusters and cluster-specific differences in proportion and transcriptomes in femoral and carotid plaque, subsequent analyses and interpretation were based on separate myeloid- and lymphoid-specific re-clustering.

*Myeloid-specific re-clustering elucidates distinct macrophage profiles in femoral versus carotid plaque*

Myeloid cell sub-cluster analyses revealed 8 distinct populations (My.0-My.7, Figure 3, Supplemental Figure S6). Macrophage clusters My.0 and My.5 highly expressed genes involved in lipid uptake and metabolism (*APOC1* and *APOE*), suggesting foamy macrophages; however, these clusters exhibited markedly distinct gene expression profiles. My.0 (*IL1B*+*APOE*+ inflammatory foam cell-like macrophages) was enriched for inflammatory cytokine activation, with >10-fold higher relative expression of *IL1B*, *CXCR4*,(26) and *CCL20* than My.5 (*IL1B*-*APOE*+ non-inflammatory foam cell-like macrophages), which was comparatively enriched for genes involved in repair and antigen presentation. Notably, My.5 had >4-fold higher expression of complement-related gene *C1QB* (relevant to complement's pro-survival, efferocytosis-boosting activities in foam cells(27) and context-dependent activity in atherosclerotic inflammation regulation(28)) and >4-fold higher expression of *RNASE1*, a regulator of vascular homeostasis that protects endothelial cells from damage-associated molecular protein effects in acute inflammation.(29) Proportions of these cells comprising carotid vs. femoral plaque myeloid cells differed significantly (Figure 4A, 4B). Inflammatory foam cell-like macrophages (My.0) comprised a significantly higher proportion of overall myeloid cells in the carotid versus femoral plaque [37.9% vs. 8.2%; log odds ratio (OR)]
6.8 (6.1-7.6), p<0.001], whereas non-inflammatory foam cell-like macrophages (My.5) were far less common in carotid versus femoral plaque [3.2% vs. 11.3%; log OR 0.26 (0.22-0.30), p<0.001].

We also identified a separate population of LYVE1+ macrophages (My.6: Figure 3) also expressing high levels of TREM2, a gene previously implicated in anti-inflammatory macrophages in murine atherosclerosis.(30-32) This LYVE1+ macrophage cluster comprised a roughly 10-fold higher proportion of the myeloid cells in femoral vs. carotid plaque [8.3% vs. 0.9%, log OR 10.1 (7.7-12.5, p<0.001]; this represented 26.7% of femoral plaque macrophages versus 2.1% of carotid plaque macrophages (p<0.001) suggesting a greater inflammatory potential of macrophages enriched in carotid plaques (Figure 4A, 4B). Carotid plaques were also comparatively enriched for macrophages with high IL1B but low APOE or APOC1 expression (My.1; IL1B+APOE- inflammatory non foam-cell-like macrophages; 34.0% of total myeloid cells in carotid plaque versus 14.1% in femoral plaque; p<0.001).

The enrichment of less inflammatory macrophage phenotypes in femoral versus carotid plaque and comparatively inflammatory nature of foam cell-like macrophages largely corresponded to distinct gene expression profiles (Figure 5). The My.0 cluster in femoral plaques had significantly higher expression of genes involved in cell-mediated immune response and monocyte recruitment (HLA-DPA1, HLA-DQA1, CCL2), as well as homeostatic (RNASE1) and complement-related (C1QA) genes, reflected in antigen processing and presentation enrichment identified by gene ontology (GO). In contrast,
the carotid My.0 cluster significantly exhibited a potent inflammatory profile, with GO analyses demonstrating enrichment of inflammatory cytokine and chemokine responses as well as over-expression of individual genes including \textit{MMP9}, which is associated with coronary and carotid plaque instability and plaque neo-angiogenesis(33-38), and \textit{CCL20}, a potent chemokine-encoding gene previously named macrophage inflammatory protein 3α which is highly induced by inflammatory stimuli and exerts context-dependent inflammatory effects.(39)

A separate myeloid population (My.2; Figure 3) minimally expressing \textit{CD14} and \textit{CD68} (unlike My.0, 1, 5, and 6), nor \textit{APOC1} and \textit{APOE} (unlike foam cell-like macrophage clusters My.0 and 5) was marked by high \textit{CSF3R}, \textit{S100A8}, and \textit{S100A9} expression and exhibited a gene expression profile most consistent with monocytes (21). In this cluster, two of the most over-expressed genes in carotid versus femoral plaque (both >6-fold higher expression) were \textit{MIF}, a potent acute inflammatory signaling molecule implicated in endotoxinemia and sepsis,(40) and \textit{SPP1} (osteopontin), a broadly activating cytokine with roles in acute and chronic inflammation,(41, 42) In contrast, genes involved in inflammation-regulating, pro-apoptotic functions were comparatively upregulated in femoral plaque My.2 (Figure 5B): the 3 most comparatively over-expressed genes (\textit{MNDA}, \textit{S100A8}, and \textit{S100A9}) were inflammation-response genes with context-dependent regulatory functions. \textit{MNDA} is an interferon-induced gene with regulatory effects including regulation of myeloid differentiation (43) and induction of apoptosis (44, 45) as a means of decreasing acute inflammation in conditions including sepsis.(43, 46)
S100A8/9 are likewise stress-induced polyfunctional genes with context-dependent functions including regulation of inflammation, apoptosis, and tissue repair. (47-49)

To determine whether the general differences in monocyte/macrophage and T cell transcriptional phenotypes corresponded to changes in cell types based on canonical protein markers, we prospectively enrolled an additional 24 patients undergoing femoral (N=9) or carotid (N=15) endarterectomy for flow cytometry analysis of plaque and paired blood (see Table 2 and Methods). Patient clinical and demographic characteristics were similar between those undergoing femoral vs. carotid endarterectomy – including similar age and sex distribution as well as prevalence of diabetes, hypertension, and statin use between femoral and carotid endarterectomy groups. The exception was that current smoking was more common in femoral endarterectomy patients, consistent with the higher prevalence of smoking among patients with PAD versus CAD or stroke. (50, 51)

Plaque specimens were digested into single-cell suspensions using the same digestion methods as for scRNA-seq analyses, gated on macrophages (CD11b+CD14+CD64+HLA-DR^{hi} cells), and further sub-classified based on expression of CCR2 (Figure 4D-F), a chemokine receptor expressed on monocyte-derived inflammatory macrophages (52-54). Consistent with our overall comparisons of myeloid phenotypes in the scRNA-seq data (with carotid plaques demonstrating a comparatively inflammatory myeloid signature), we observed a significantly higher proportion of CCR2^{+} macrophages in carotid (71.4% of total plaque macrophages) versus femoral (56.9%) plaque (p=0.03). Circulating classical (CD14^{++}CD16^{-}) monocytes from carotid patients also expressed high levels of CCR2 [mean fluorescence intensity (MFI) = 12.8]
relative fluorescence units (RFU)] versus blood classical monocytes from patients undergoing femoral endarterectomy (MFI = 7.0 ± 1.0 RFU) (p=0.008).

*Mast cells and dendritic cells exhibit inflammatory transcriptional bias in carotid versus femoral plaque*

Myeloid clusters My.3 and My.4 were defined Kit+ mast cells and dendritic cells (DCs, marked by CLEC4C and IRF8 expression), respectively. (18, 55, 56) Mast cells comprised a lower proportion of myeloid cells in femoral plaque, whereas DCs proportions were similar in carotid and femoral plaques (Figure 4A,4B). However, we observed striking transcriptional differences for these cells in femoral versus carotid plaque. Mast cells exhibited a comparatively potent inflammatory transcriptional signature in carotid vs. femoral plaque, with >6-fold higher expression of IL1B and CCL5 in carotid vs. femoral mast cells (Figure 5C). Amphiregulin (AREG), a master regulator of tissue homeostasis and repair, (57) was the most highly over-expressed gene in femoral versus carotid mast cells and DCs (Figure 5D). Femoral DCs cells also expressed high levels of HLA-DRB1 and CD74 compared with carotid DCs, suggestive of an enhanced antigen-presenting signature in femoral plaque.

*Lymphoid re-clustering suggests comparatively inflammatory and cytotoxic T cell bias in carotid plaque versus B cell bias in femoral plaque*

Lymphoid cell-specific re-clustering revealed 9 distinct lymphoid populations (Ly. 0-8, Figure 6). Clusters were determined using a combination of previously defined cluster-specific genes, reference datasets of combined human protein and transcript single cell
transcriptomes (58) (21), and gene set enrichment analyses. Clusters Ly.0-2, 4, and 7 all highly expressed CD3D and represent T cell clusters, with Ly.0 and Ly.7 expressing CD4, representing CD4+ T cells (and Ly.7 expressing TIGIT and FOXP3 as a T\textsubscript{reg} cluster). Ly.1 and 2 highly expressed CD8 as CD8+ T cells, most highly expressing cytotoxicity markers GZMK and GZMH, respectively. Gene set enrichment analyses suggested high composition of T helper type 1 (T\textsubscript{h}1) cells in Ly.0, which also had high IL7R expression (previously observed in T\textsubscript{h}1 effector cells (59)). Conversely, Ly.7 highly expressed FOXP3 and other T\textsubscript{reg}-related genes and without inflammatory or cytotoxicity-associated gene expression profiles. Cluster 4 represented a group of CD3-expressing T cells that did not have clear CD4 or CD8 expression but had comparatively high expression of CXCL8 (60, 61). Cluster 3 highly expressed NKG7 and did not express CD3D (while also having minimal CD4 and CD8 expression), thus representing natural killer (NK) cells. Clusters 5 and 6 were CD79-expressing B cells, with distinctions in gene expression between these two B cell clusters suggesting Ly.5 is type 1 B cells (B1) and Ly.6 is type 2 B cells (B2). B1-b cells have been implicated in the secretion of athero-protective IgM,(62) whereas B2 cells follow more conventional paradigms driven by interaction with T cells in germinal centers to form high-affinity antibodies orchestrating humoral responses.(63) Ly.5 overexpressed BHLHE41 and ZBTB32 – two transcriptome markers of B1 cells, and Ly.6 overexpressed Fcer2, which has recently been established as a B2 gene marker.(64)

The vast majority of lymphoid cells in both femoral and carotid plaque were T cells (Figure 7A, 7B), with approximately equal proportions of CD4+ (Ly.0 + Ly.7) and CD8+
T cells (Ly.1 + Ly.2) in carotid plaque as observed previously in a separate cohort (19). When comparing cell proportions in femoral versus carotid plaque, the most notable difference in T cell subsets was a comparatively higher proportion of cytotoxic CD8+ T cells in carotid versus femoral plaque (Ly.1 + Ly.2: 40.1% versus 27.8%; p<0.001). The CD8+ T cell enrichment in carotid plaque was validated by flow cytometry in the prospective cohort of patients undergoing carotid (N=15) or femoral (N=9) endarterectomy (Figure 7C, Supplemental Figure S8 for T cell gating); interestingly, the higher CD8+ T cell proportion was also observed in the blood of carotid patients. Meanwhile, femoral plaques were highly enriched in B cells compared with carotid plaque (9.0% versus 0.02% for Ly.5 and 7.9% versus 1.1% for Ly.6; p<0.001). To explore lymphoid cell clustering in situ, we quantified T cell and B cell aggregates in 12 separate patients who underwent carotid (N=7) or femoral (N=5) endarterectomy and observed that T cells were the most populous cell type in these aggregates in femoral and carotid plaques, although one femoral plaque had a marked B cell aggregate (278 CD20+ B cells and 143 CD3+ T cells per high powered field; Figure 7D-E).

Differential gene expression analysis suggested an inflammatory bias in several carotid plaque clusters (Figure 8). All four of the largest lymphoid clusters [Ly.0-Ly.3, representing a heterogeneous cluster of IL7R+ CD4+ T cells (Ly.0), two cytotoxic CD8+ T cell clusters (Ly.1-2), and NK cells (Ly.3)] over-expressed the antigen presentation-associated gene HLA-B in femoral plaque. Ly.2, one of two CD8+ T cell clusters (two-fold higher in carotid plaques), over-expressed several stress-sensing genes with regulatory functions. These included TXNIP, a metabolic stress sensor with context-
dependent actions including inhibition of cell proliferation (65) and suppression of inflammation, (66) and S100B (discussed above). The novel longevity-associated gene c1orf56 (67) was also overexpressed in Ly.2 femoral versus carotid plaque. In Ly.4 (NKG7+ T cells), CXCL8, a potent and broadly inflammatory cytokine-encoding gene, (68) was upregulated in carotid plaque. B2 cells had a more inflammatory signature in carotid than femoral plaque, with significant over-expression (in carotid plaque) of IL1β and SPP1, the latter encoding for a potent inflammatory cytokine (41) also overexpressed in carotid myeloid cluster 2.

DISCUSSION
In this study, we observed important differences in the immune cell landscape and cell-specific divergence in gene expression and phenotype for femoral versus carotid atheroma obtained at endarterectomy. Our findings provide insights into cellular composition and heterogeneity of femoral and carotid plaques, informing on transcriptional reprogramming that may underlie histopathologic (15-17) and clinical (12-14) stability of femoral versus carotid plaque. (9-11)

Femoral plaques largely exhibited homeostatic gene expression patterns signatures compared to more broadly inflammatory signatures of carotid plaques, a distinction that was especially apparent in myeloid clusters. Polarization of macrophages highly expressing APOC1 and APOE, suggestive of foamy macrophages, was starkly different by site. Inflammatory IL1β+ foam cell-like macrophages comprised over one-third of carotid myeloid cells, outnumbering carotid non-inflammatory (IL1β-) foam cell-like macrophages more than 10-fold. Conversely, non-inflammatory foam cell-like
macrophages actually outnumbered inflammatory foam cell-like macrophages in femoral plaques. These findings were mirrored in proportions of other monocyte and macrophage subtypes. \( \text{LYVE1}^+ \) macrophages, which play important roles in maintaining homeostasis including via regulation and resolution of inflammation, were approximately 10-fold higher in femoral plaques.\(^{(69)} \) We confirmed macrophage plaque site-divergent changes in cell frequency and phenotype via flow cytometry in a separate validation cohort of 18 patients undergoing endarterectomy, observing that \( \text{CCR2}^+ \) macrophages, generally reflecting activated monocyte origin, comprised a significantly higher proportion of macrophages in carotid versus femoral plaque. Interestingly, we also observed significantly higher \( \text{CCR2} \) expression in blood \( \text{CD14}^+\text{CD16}^- \) monocytes of patients undergoing carotid endarterectomy. Taken together, our observations in carotid plaque of comparative monocyte excess, macrophage inflammatory bias, and divergent plaque and blood \( \text{CCR2} \) expression on macrophages and monocytes raise the possibility of distinct monocyte/macrophage polarization profiles by plaque site.

We likewise observed a comparatively inflammatory T cell bias in carotid versus femoral plaques. Our observed proportions of \( \text{CD4}^+ \) and \( \text{CD8}^+ \) T cells in carotid plaque mirrored those observed previously in a separate cohort\(^{(19)} \), underscoring reproducibility. Our sequencing and flow data in femoral plaque add substantially to this existing carotid plaque-focused literature on intraplaque T cell phenotypes, as we observed consistently lower proportions of \( \text{CD8}^+ \) T cells, which highly expressed cytotoxicity markers, in femoral versus carotid plaque. Furthermore, similar to our macrophage findings, we observed comparative over-expression in femoral plaques of inflammation-regulating
genes (including $TXNIP$) versus carotid plaque over-expression of inflammatory genes such as $CXCL8$.

Our B cell-related findings were unanticipated. Although T cells have been reported to be the predominant lymphoid cell population in coronary, aortic, and carotid plaques,(19, 70, 71) there is evidence from experimental models and human data that B cells play a complex role in atherosclerosis, with two over-arching B cell subsets that have divergent functions.(70, 72, 73) These subtype-specific roles of B cells may explain inconsistent experimental and clinical findings related to effects of broader B cell modulation on atherosclerosis:(74-76) net effects of nonspecific B cell-targeted interventions may depend on the extent to which (putatively atheroprotective) B1 versus (atherogenic) B2 cells are preferentially targeted.(70) In this context, our findings related to specific B cell subsets in carotid vs. femoral plaque are interesting. There were >60x more B2 cells compared to B1 cells in carotid plaque, whereas there were actually fewer B2 cells than B1 cells in femoral plaque. Our findings raise the possibility that net effects of B cells in different plaque microenvironments may vary considerably, with carotid plaques having an atherogenic, inflammatory B2 bias and femoral plaques having a more balanced B1 and B2 cell presence, with a resulting stronger signature of regulatory B1-type functions. These observations, if replicated in future studies of diverse human plaque microenvironments, would have important implications for atherosclerosis location-specific interventions targeting broad and specific B cell niches.
Taken together, our findings suggest a comparative homeostatic non-inflammatory bias in femoral versus carotid plaque that exists across several leukocyte phenotypes. An important resulting question is why these stark differences may exist in femoral versus carotid plaque sites, despite both being large arteries – and how these distinctions may inform more site-specific therapeutic approaches in addition to currently-indicated lipid lowering. Several possibilities exist and elicit hypotheses warranting future study. One possibility is that distinct metabolic microenvironments contribute to epigenetic programs favoring upregulation of pro-homeostatic genes in femoral plaques, versus a pro-inflammatory bias in inflammatory macrophages in carotid plaque. This question warrants further study in future analyses of plaque site-specific functional metabolism. Other possibilities include differences in site-specific mechanical and shear forces priming the immune microenviroments in different ways, with potential mechano-immunologic effects on myeloid and lymphoid cell activation and hematopoietic niche reconstruction. Differential femoral versus carotid plaque endothelial permeability to immune cell migration from the vascular lumen into plaque is another possible explanation for our findings, which revealed relative predominance of monocytes and inflammatory foam cell-like macrophages in carotid plaque versus a comparative anti-inflammatory and resident macrophage signature in femoral plaques. These hypotheses generated highlight the need for future in models that trace cell lineage, pair simultaneously-collected blood samples with plaque from these diverse vascular beds, and recapitulate plaque site differences in migration and margination of immune cells in plaque. Such studies could also investigate mechanisms of immune priming and cell-cell interactions, detailed investigation into inflammation-resolving
biology in unique atheroma microenvironments, and chemo-atraction of cells to diverse plaque sites.

Limitations

Our study has important limitations. To investigate plaque from live humans, we analyzed endarterectomy samples from people undergoing femoral or carotid endarterectomy for clinical indications, with potential resulting sources of confounding. Surgical indications differ widely for femoral versus carotid endarterectomy given distinct underlying physiologies and clinical sequelae, and differences in comorbidities between femoral and carotid endarterectomy groups may further contribute to confounding. Although our femoral and carotid endarterectomy groups were similar in age, sex, statin use, and presence of hypertension or diabetes, smoking was far more common among patients undergoing femoral endarterectomy. While this reflects a potential confounder (e.g., if smoking contributes to the distinct immune profiles of femoral versus carotid plaque), it also reflects the clinical reality of smoking being more prevalent among patients with PAD than those with other athero-thrombotic sequelae such as CAD or stroke.(50, 51) Heterogeneity within groups also may have also affected the results; for instance, among patients undergoing femoral endarterectomy, surgical indications varied from chronic claudication to ulcer or gangrene, and presence of occlusive disease at or near the endarterectomy site likewise varied among patients. While these differences in surgical indication and clinical characteristics such as smoking may reflect common precipitants and sequelae of femoral versus carotid plaque biology, they are nevertheless important potential sources of confounding, as
are the lack of data regarding potentially relevant variables such as circulating inflammatory markers that may differ between groups. A separate concern relates to potential batch effects from samples being harvested and undergoing sequencing reactions at different times, with potential differential effects on early response genes. Although we aimed to correct for these with Harmony, a software package used for batch-effect correction of scRNA-seq data (90), and observed overall good sample- and plaque site-level integration, residual confounding related to processing and batch effects remains possible.

As with many single-cell analyses of relatively rare plaque specimens obtained from humans in vivo,(18, 19, 91) our sample size was limited, with the potential to adversely affect generalizability of our plaque site-specific conclusions. We profiled 35,265 femoral plaque CD45+ cells derived from 9 patient specimens and 30,655 carotid CD45+ cells derived from 4 patient specimens. However, our number of individual cells analyzed is larger than those from recent single-cell analyses of carotid plaque (scRNA-seq on 3,282 and 7,169 cells(18, 19)) and the only prior scRNA-seq study of femoral plaque to our knowledge, which compared scRNA-seq data from a single femoral plaque specimen to data from an existing carotid plaque scRNA-seq dataset.(20) The potential generalizability of our findings is further supported by (1) confirmatory flow cytometry and immunohistochemistry in distinct validation cohorts, and (2) similarity of our observed T cell and myeloid cell proportions in carotid plaques to those observed in prior scRNA-seq studies.(18, 19) Nevertheless, an important limitation is low CCR2 expression throughout our scRNA-seq dataset (although five-fold higher among carotid
than femoral myeloid cells), meaning that our flow-based validation using CCR2 reflects a broad validation of comparatively inflammatory, activated monocyte and macrophage profiles in carotid versus femoral plaque. Additional markers such as LYVE-1 would have been useful to further validate macrophage populations and should be used in future studies. Still, our plaque site-specific differences are consistent with recent histologic studies, which observed comparatively more inflammatory cells and higher bulk expression of inflammation-potentiating genes in carotid plaque than femoral plaque. (15-17) A separate, major limitation is that while our investigation surveys specific immune phenotypes and transcriptomes in femoral versus carotid plaque, causal experiments and functional assays will be critical to validate and extend these findings in future studies.

Conclusion

In conclusion, we observed several plaque site-specific single-cell immune cell gene expression profiles in human femoral versus carotid plaque. These included comparatively increased inflammatory macrophage activity and CD8+ T cell bias in carotid versus femoral plaque. Other notable findings included a comparative B cell bias and over-expression of inflammation-regulating genes in several leukocyte clusters in femoral versus carotid plaque. These findings inform on the immunology underlying distinct clinical courses of femoral versus carotid atherosclerosis and suggest targets for future experimental models of atherosclerotic inflammation resolution.
Methods

Overview of Study Design and Participants

All analyses involved freshly excised plaque samples obtained intra-operatively from patients undergoing clinically indicated femoral or carotid endarterectomy at Northwestern Medicine (total N=49; Supplemental Figure S1). These samples were obtained in accordance with Northwestern University Institutional Review Board (IRB)-approved studies 205451 (cohort 1 of femoral and carotid endarterectomy patients: collection of de-identified fresh endarterectomy specimens for scRNA-seq) and 211811 (cohort 2 of femoral and carotid endarterectomy patients: preoperative collection of blood and collection of fresh endarterectomy specimens for flow cytometry and histology, with detailed clinical data). Our primary analyses compared scRNA-seq-derived transcriptional programs of leukocytes in femoral versus carotid plaque from different patients undergoing femoral or carotid endarterectomy. Plaques were also analyzed by flow cytometry and in situ immunohistochemistry was performed on plaque obtained from different patients undergoing femoral or carotid endarterectomy. The most common surgical indication for carotid endarterectomy was severe carotid stenosis without stroke and the most common surgical indication for femoral endarterectomy was claudication (Tables 1 and 2). The first protocol involved single-cell ribonucleic acid sequencing (scRNA-seq) of freshly excised, de-identified plaque samples from 13 distinct patients who underwent femoral (N=9; 35265 CD45+ cells analyzed) or carotid (N=4; 30655 cells analyzed) endarterectomy. To validate key findings related to immune cell phenotypes in femoral versus carotid plaque, as well as correlates in blood from the same patients, we then prospectively enrolled 24 additional
patients undergoing carotid (N=15) or femoral (N=9) endarterectomy to perform flow
cytometry on plaque suspensions (prepared in the same manner as for scRNA-seq
analyses) and pre-operatively obtained blood samples. We also investigated relative
proportions of B and T cells in lymphoid aggregates in situ via immunohistochemistry
from plaque specimens of an additional 12 patients who underwent femoral (N=5) or
carotid (N=7) endarterectomy. This yielded a total of 49 distinct patients who underwent
femoral (N=23) or carotid (N=26) endarterectomy.

**Plaque processing into single-cell suspensions for scRNA-seq or flow cytometry**

Immediately following excision in the operating room, femoral and carotid plaque
samples were placed in saline and transported to the lab for processing into single-cell
suspensions (ensuring time from plaque excision in the operating room to in-lab cell
processing of <30 minutes). We used a protocol previously validated for carotid
endarterectomy specimens (19) which involves initial tissue processing on ice until the
enzymatic digestion step. The protocol includes washing the plaque thoroughly in
dulbecco’s modified Eagle medium (DMEM, Corning, 10-013-CV), digesting at 37°C for
1 hour in: 10 milliliters of DMEM with 10% FBS; type IV collagenase (Sigma, C5138) at
1 mg/ml final concentration; and DNase (Sigma, DN25), hyaluronidase (Sigma, H3506),
collagenase type XI (Sigma, C7657) and collagenase type II (Sigma, C6885) each at
0.3 mg/ml final concentration. The mixture was filtered consecutively through 70 then 40
micrometer strainers, washed twice in PBS, and centrifuged at 300g for 8 minutes. Cell
counts from the resulting single-cell suspensions were overall comparable for femoral
versus carotid plaques, with femoral plaques exhibiting more variation in cell numbers
isolated from individual plaques (Supplemental Figure S9). For scRNA-seq analyses, immediately following generation of single-cell suspensions (the generation of which took <2.5 hours after excision of plaques from operating rooms given initiation of specimen processing <30 minutes after plaque excision and <2 hours’ time required to generate single-cell suspensions), we then enriched suspensions for live cells and CD45+ cells. Dead cells were removed with a dead cell removal kit (Miltenyi Biotec 130-090-101 or Stem Cell Technologies 17899) according to manufacturer’s instructions. Suspensions were then enriched for immune cells with CD45 positive selection using a CD45+ enrichment kit (Miltenyi, 130-045-801). These CD45+ enriched single cell suspensions of plaque were then immediately transported on ice (within the same building) to load for sequencing reactions, ensuring <3 hours’ time from plaque excision to sequencing reaction. For analyses of pooled samples (carotid samples 1 and 2, femoral samples 1-3, the same steps were performed at the same intervals, but CD45+ enriched specimens were cryopreserved immediately in liquid nitrogen and then thawed together and loaded for sequencing reactions within <30 minutes of thawing. For flow cytometry analyses, CD45+ selection was not performed and suspensions were analyzed (or frozen on liquid nitrogen and then analyzed immediately after thaw) following the dead cell removal step.

**Sequencing**

For sequencing, the CD45+ enriched single cell suspensions were converted to barcoded scRNA-seq libraries using the Chromium Single Cell 3’ Library, Gel Bead, and Chip Kit from 10X Genomics. The Chromium Single Cell 3’ V3.1 Reagent (10x
Genomics, PN-1000286) kit was used to prepare scRNA-seq libraries. Reverse transcription, barcoding, complementary DNA amplification and purification for library preparation were performed according to the manufacturer's instructions. Sequencing was performed on a NovaSeq 6000 platform with Read 1 of 28nt and Read 2 of 90bp (Illumina). Sequencing reads were demultiplexed and aligned to the human GRCh38 transcriptome using the CellRanger V3 software (10X Genomics).(92) Filtering, unsupervised clustering, differential expression and additional analysis were completed using Seurat V4 and ClusterProfiler packages for R.(93-96)

**Quality control, filtering, integration, and clustering**

For analyses, femoral plaque sample matrices were imported into the Seurat v4 R package (93-96) and combined into a “femoral plaque” Seurat object. Likewise, carotid plaque sample matrices were imported and combined into a “carotid plaque” Seurat object. For both Seurat objects, cells were filtered for mitochondrial reads <10%, 200 < nCount_RNA < 10 000, and 200 < nFeature_RNA < 10 000. Each Seurat object was then filtered to remove mitochondrial (MT-) and ribosomal (RP-) genes. Each Seurat object was also normalized and scaled and filtered to keep the top variable features (greatest standardized variance; n = 3000) across the datasets. The objects were then merged using the Seurat merge command and integrated using the R package Harmony.(97) The RunHarmony command was used to calculate harmonized dimension reduction components using the samples as the grouping variable, and doublet discrimination was performed. Principle components were then calculated, and an elbow plot was generated to select principal components to use for downstream
analysis; here, 30 principal components explained most of the variation. UMAP dimensional reduction was then computed followed by unsupervised clustering using the `FindNeighbors` and `FindClusters` Seurat functions, using the number of principle components mentioned above and a resolution of 0.3 (`FindClusters`), which captured distinct cell types empirically.

Myeloid and Lymphoid Sub-clustering and Batch Correction

Sub-clustering of myeloid cells was completed by first extracting the raw expression matrix from all myeloid cells for each sample using the `GetAssayData` function from the Seurat package. All carotid myeloid matrices were then combined into a carotid myeloid Seurat object, whereas all femoral myeloid matrices were combined into a femoral myeloid Seurat object. The carotid and femoral myeloid objects were then merged and, to correct for batch effects, integrated with Harmony (90) using sample as the grouping variable. UMAP dimensional reduction, downstream differentially expressed genes (DEG) and pathway analyses was then performed in the same manner as described above. This process was repeated with lymphoid cell data to create sub-clusters for lymphoid cells.

Detection of differentially expressed genes

Detection of DEGs between clusters was performed using the `FindAllMarkers` Seurat function, specifying return of significantly (Bonferroni p adjusted < 0.05) upregulated genes with a log₂ fold change (log₂FC) threshold of 0.25. Cell types were assigned to clusters by evaluating gene expression of individual clusters using
differential gene expression. For individual clusters, detection of DEGs between carotid and femoral plaque location was performed using the FindAllMarkers command specifying to return both positively and negatively changed genes, and no log$_2$FC or P value cutoffs. Genes with positive and negative log$_2$FC values were used to identify upregulated genes in the carotid or femoral plaque location, respectively. For all DEG calculations, the “RNA” assay and data slot were used and performed using the default Wilcoxon rank-sum method.

**Pathway analyses**

Pathway analysis was completed using the ClusterProfiler R package (93-96). For comparison of enriched pathways between clusters, the compareCluster function was utilized on a matrix derived from the Seurat DEG analysis filtered for the top 100 positive, p adjusted (< 0.05) genes that contained a column that indicated in which cluster each gene was upregulated. This analysis utilized the enrichGO database from ClusterProfiler (95) to return a table with enriched GO pathways in each cluster. For comparison of enriched pathways between carotid and femoral plaque locations within a specific cluster, the top 100 positive and negative genes from the Seurat DEG analysis by plaque location were separated to identify enrichment in either the carotid or femoral location, respectively.

**Gene Set Enrichment Analyses**

To identify subtypes of CD4+ T cells in our dataset, we performed gene set enrichment analysis (GSEA) on clusters that were identified as CD4+ T cell clusters using DEG.
GSEA was conducted using GSEA desktop software (98, 99) and our group’s previously curated gene sets of CD4+ T cell subtypes. (100) Normalized enrichment scores were acquired using gene set permutations 1000 times, and a cutoff p value of 0.05 was used to filter the significant enrichment results.

**Descriptive Statistics**

Comparisons of cell clusters as a proportion of total CD45-positive cells, myeloid cells, and lymphoid cells were performed using logistic regression; log odds ratios (ORs) were determined using Fishers exact test and used to indicate comparatively higher or lower proportions in carotid versus femoral plaque. Based on 13 comparisons of proportions for overall clusters, 8 for myeloid clusters, and 9 for lymphoid clusters (a total of 30), we incorporated a conservative Bonferroni correction of p<0.0017 (=0.05/30) to determine statistically significant differences in cell cluster proportions in carotid versus femoral plaque. For post-hoc analyses, a p<0.05 was considered statistically significant.

*Flow cytometry of myeloid cells and T cells in plaque and blood of femoral and carotid endarterectomy patients*

To externally validate plaque site-specific findings related to macrophage, monocyte, and T cell phenotype observed in plaque from scRNA-seq analyses, we prospectively enrolled 24 patients undergoing carotid (N=15) or femoral (N=9) endarterectomy. In these patients, we obtained blood preoperatively for processing into peripheral blood mononuclear cells (PBMCs) and flow cytometry of myeloid cells and T cells, as well as surgically excised plaque which was processed using the same procedures as for
scRNA-seq analyses (with the exception of sorting out live cells and non-leukocytes by flow cytometry rather than positive selection). For myeloid-focused flow cytometry of digested plaque specimens, cells were first gated on live, single cells and macrophages were identified as CD11b+CD14+CD64+HLA-DRhi cells, then further distinguished based on CCR2 expression. For PBMCs, monocytes were determined based on CD16 and CD14 expression, which was used to sort into classical, nonclassical, and intermediate monocyte populations. Subsequently, monocyte sub-populations were further distinguished based on CCR2 expression. T cell gating was performed on PBMCs and plaque first by identifying T cells as CD3+ and further distinguishing them by CD4 or CD8 expression. Of note, because T cells were the most common cell type in plaque in scRNA-seq analyses, followed by macrophages and monocytes, we performed T cell phenotype-focused flow cytometry on samples from all 24 patients and additional myeloid-focused flow cytometry on a sub-cohort of patients with plaque with sufficient cell numbers (N=11 who underwent carotid endarterectomy, N=8 who underwent femoral endarterectomy; clinical characteristics in Table 2). To determine differences by plaque site, we performed pairwise t-tests at an alpha of 0.05 comparing patients undergoing carotid vs. femoral endarterectomy regarding: (1) proportions of macrophages in plaque that were CCR2+ for carotid versus femoral plaque, (2) blood monocyte CCR2 expression (as mean fluorescent intensity), and (3) proportions of T cells in plaque and blood that were CD4+ and CD8+ (as a proportion of total T cells in that specimen). Flow cytometry reagents are included in Supplemental Table T1.

Histological staging of plaque
Plaques used for flow cytometry or scRNA-seq analyses with sufficient tissue available following generation of single-cell suspensions were fixed in formalin and embedded in paraffin by the Pathology Core Facility at Northwestern University as above, then sectioned into 5 micrometer sections and mounted onto slides to undergo Masson’s Trichrome staining for histological grading. Two trained pathologists performed the grading (shown in Supplemental Figure S10) blinded to plaque site and surgical indication, according to American Heart Association Classification (101) as done previously (19).

In situ histologic exploration of leukocyte aggregates in femoral and carotid plaques

For immunohistochemistry analyses, post-surgical femoral and carotid plaque tissue from patients was prepared through the Pathology Core Facility at Northwestern University. Each tissue was fixed in formalin and embedded in paraffin, and 5μm-thick slices were cut from each paraffin block and stained with hematoxylin and eosin (H&E). Histology was reviewed by a trained pathologist blinded to plaque location. The pathologist screened whole H&E-stained slides cut from these formalin-fixed, paraffin embedded blocks of atheroma from 42 additional patients who underwent carotid or femoral endarterectomy, for the purpose of determining whether 5 or more cells with lymphoid appearance were present in any high-powered field; 12 out of 42 samples (7 carotid, 5 femoral) met these criteria. Notably, none of the 42 samples contained adventitial tissue per the reviewing pathologist, consistent with endarterectomy technique of avoiding adventitial tissue.(102) Contiguous slides from the same blocks of these 12 plaque samples subsequently underwent immunohistochemistry. This included
processing for 3, 3-diaminobenzidine (DAB-HRP) staining, counterstaining with 699 hematoxylin, then immunostaining for four separate surface markers (CD45, CD20, and CD3). Antigen retrieval and antibody staining was optimized at the Northwestern University Pathology Core Facility. The stained sections were imaged utilizing 4x and 40x objectives on the brightfield mode via the Vectra 3 Automated Quantitative Pathology Imaging System (PerkinElmer) at the Immunotherapy Assessment Core at Northwestern University. The field of interest for each sample was chosen by a trained pathologist based on the available cellularity within each tissue. From there, the same cell cluster population within each sample was located under 40x for each staining available per sample (CD45, CD20, and CD3). In this 40x view, the DAB staining quantification was carried out blinded by counting the number of cells of interest within this cell cluster population. We quantified cell cluster compositions individually to determine whether T cells and/or B cells were clustering in large aggregates.

**Study Approval**

Human subjects research, including written informed consent, was approved by the Northwestern University IRB (study numbers 205451 and 211811 used for this investigation).

**Data Availability**

Data underlying this publication are available in Gene Expression Omnibus (GEO; accession Number GSE234077) and underlying code is deposited in GitHub with interactive links (https://github.com/Feinstein-Lab).
**Author Contributions**

*JS:* designed study, acquired data, analyzed data, writing and revising the manuscript

*AS:* designed study, conducted experiments, acquired data, writing the manuscript

MD: acquired data, conducted experiments, analyzed data

SS: acquired data, conducted experiments, analyzed data

HA: analyzed data, writing the manuscript

IL: analyzed data, drafted figures, writing the manuscript

KG: acquired data, conducted experiments

RN: acquired data, conducted experiments

PD: acquired data, analyzed data, writing the manuscript

SA: analyzed data, writing the manuscript

CMW: acquired data, conducted experiments

RT: acquired data, conducted experiments

MV: acquired data, analyzed data

AS: acquired data, conducted experiments

XW: acquired data, conducted experiments

MS: analyzed data, conducted experiments

RS: analyzed data

RG: analyzed data

JV: analyzed data

RS: analyzed data

DML-J: analyzed data, writing the manuscript
JL: designed study, analyzed data
SW: analyzed data, writing the manuscript
KH: designed study, acquired data, writing the manuscript
KPL: designed study, analyzed data, writing the manuscript
CG: designed study, analyzed data, writing the manuscript
*EBT: designed study, analyzed data, writing the manuscript
**MJF: designed study, acquired data, analyzed data, writing and revising the manuscript

*Co-first authors. JS was assigned first between JS and AS based on his primary role in bioinformatics analyses and writing

**Co-senior/corresponding authors.
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Disclosure

C.G. is listed as an inventor on Tech 160808G PCT/US2022/017777 filed by the Icahn School of Medicine at Mount Sinai, which has no competing interest with this work.
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Cells in Coronary Culprit Plaques Causing Acute Coronary Syndrome. 


**Table 1. Individual carotid and femoral plaque sample cell count and quality metrics following CD45 positive selection for single-cell sequencing (N=13)**

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Plaque site</th>
<th>CD45+ cell viability (%)</th>
<th>Cells/uL after CD45 positive selection</th>
<th>Cells sequenced</th>
<th>Mean reads per cell</th>
<th>Median genes per cell</th>
<th>Valid barcodes</th>
<th>Reads mapped confidently to genome</th>
<th>Sex</th>
<th>Surgical Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS 1</td>
<td>Carotid</td>
<td>63.3%</td>
<td>754 cells/uL</td>
<td>12394</td>
<td>22688</td>
<td>804</td>
<td>99.0%</td>
<td>94.5%</td>
<td>M</td>
<td>Severe carotid stenosis without stroke</td>
</tr>
<tr>
<td>CS 2</td>
<td>Carotid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS 3</td>
<td>Carotid</td>
<td>89.2%</td>
<td>226 cells/uL</td>
<td>10379</td>
<td>33879</td>
<td>1437</td>
<td>97.9%</td>
<td>97.4%</td>
<td>F</td>
<td>Severe carotid stenosis without stroke</td>
</tr>
<tr>
<td>CS 4</td>
<td>Carotid</td>
<td>93.0%</td>
<td>549 cells/uL</td>
<td>15358</td>
<td>24105</td>
<td>1055</td>
<td>97.8%</td>
<td>97.6%</td>
<td>M</td>
<td>Severe carotid stenosis without stroke</td>
</tr>
<tr>
<td>FS 1</td>
<td>Femoral</td>
<td>66.8%</td>
<td>208 cells/uL</td>
<td>2444</td>
<td>124133</td>
<td>1224</td>
<td>99.0%</td>
<td>92.5%</td>
<td>M</td>
<td>Claudication</td>
</tr>
<tr>
<td>FS 2</td>
<td>Femoral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Claudication</td>
</tr>
<tr>
<td>FS 3</td>
<td>Femoral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Claudication</td>
</tr>
<tr>
<td>FS 4</td>
<td>Femoral</td>
<td>63.4%</td>
<td>682 cells/uL</td>
<td>4665</td>
<td>54761</td>
<td>1596</td>
<td>97.5%</td>
<td>94.3%</td>
<td>M</td>
<td>Claudication</td>
</tr>
<tr>
<td>FS 5</td>
<td>Femoral</td>
<td>42.1%</td>
<td>388 cells/uL</td>
<td>1734</td>
<td>156149</td>
<td>1260</td>
<td>97.7%</td>
<td>95.6%</td>
<td>M</td>
<td>Claudication</td>
</tr>
<tr>
<td>FS 6</td>
<td>Femoral</td>
<td>96.9%</td>
<td>676 cells/uL</td>
<td>9770</td>
<td>26022</td>
<td>1196</td>
<td>97.8%</td>
<td>95.7%</td>
<td>F</td>
<td>Claudication</td>
</tr>
<tr>
<td>FS 7</td>
<td>Femoral</td>
<td>93.5%</td>
<td>1930 cells/uL</td>
<td>8422</td>
<td>30417</td>
<td>1334</td>
<td>97.4%</td>
<td>94.5%</td>
<td>M</td>
<td>Ischemic Foot Ulcer</td>
</tr>
<tr>
<td>FS 8</td>
<td>Femoral</td>
<td>95.6%</td>
<td>623 cells/uL</td>
<td>11300</td>
<td>33611</td>
<td>1454</td>
<td>97.9%</td>
<td>97.8%</td>
<td>M</td>
<td>Claudication</td>
</tr>
<tr>
<td>FS 9</td>
<td>Femoral</td>
<td>85.3%</td>
<td>258 cells/uL</td>
<td>6655</td>
<td>47527</td>
<td>1643</td>
<td>98.2%</td>
<td>98.0%</td>
<td>M</td>
<td>Ischemic Rest Pain</td>
</tr>
</tbody>
</table>

*Carotid sequencing samples 1&2 (CS1&2) were pooled together for sequencing reactions, as were femoral samples 1, 2, & 3 (F1-3), due to low CD45+ cell counts. Therefore, cells sequenced, mean reads per cell, median genes per cell, valid barcodes, and reads mapped confidently to genome in the Table reflect the results from these pooled sample sequencing reactions.
Table 2. Flow Cytometry Validation Cohort Characteristics (N=24)

<table>
<thead>
<tr>
<th></th>
<th>Carotid plaque (N=15)</th>
<th>Femoral plaque (N=9)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± standard deviation)</td>
<td>70.3 ± 5.4</td>
<td>67.6 ± 7.8</td>
<td>0.32</td>
</tr>
<tr>
<td>Male sex (N, %)</td>
<td>10 (67%)</td>
<td>7 (78%)</td>
<td>0.56</td>
</tr>
<tr>
<td>Surgery performed during inpatient admission (N, %)</td>
<td>1 (6.7%)</td>
<td>2 (22%)</td>
<td>0.26</td>
</tr>
<tr>
<td>Hypertension (N, %)</td>
<td>14 (93%)</td>
<td>8 (89%)</td>
<td>0.70</td>
</tr>
<tr>
<td>Diabetes mellitus (N, %)</td>
<td>3 (20%)</td>
<td>2 (22%)</td>
<td>0.90</td>
</tr>
<tr>
<td>Smoking (N, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>2 (13%)</td>
<td>5 (56%)</td>
<td>0.057</td>
</tr>
<tr>
<td>Former</td>
<td>10 (67%)</td>
<td>4 (44%)</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>3 (20%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Statin use (N, %)</td>
<td>14 (93%)</td>
<td>8 (89%)</td>
<td>0.70</td>
</tr>
<tr>
<td>Indications for surgery (carotid)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe carotid stenosis without stroke</td>
<td>11 (79%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td>3 (21%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indications for surgery (femoral)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Claudication</td>
<td></td>
<td>5 (56%)</td>
<td></td>
</tr>
<tr>
<td>Critical limb ischemia</td>
<td></td>
<td>2 (22%)</td>
<td></td>
</tr>
<tr>
<td>Ulcer or gangrene</td>
<td></td>
<td>2 (22%)</td>
<td></td>
</tr>
<tr>
<td>Total arterial occlusion present (femoral)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any location within common or superficial femoral artery</td>
<td></td>
<td>5 (56%)</td>
<td></td>
</tr>
<tr>
<td>Proximal to endarterectomy site</td>
<td></td>
<td>1 (11%)</td>
<td></td>
</tr>
<tr>
<td>At endarterectomy site</td>
<td></td>
<td>3 (33%)</td>
<td></td>
</tr>
<tr>
<td>Distal to endarterectomy site</td>
<td></td>
<td>2 (22%)</td>
<td></td>
</tr>
<tr>
<td>Sub-cohort for which myeloid flow cytometry was also performed (N=19; excludes 5 patients from the above 24 who had lymphoid flow performed but insufficient cells for additional myeloid flow)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mean ± standard deviation)</td>
<td>70.2 ± 5.6</td>
<td>66.9 ± 6.8</td>
<td>0.32</td>
</tr>
<tr>
<td>Male sex (N, %)</td>
<td>7 (70%)</td>
<td>6 (75%)</td>
<td>0.81</td>
</tr>
<tr>
<td>Surgery performed during inpatient admission (N, %)</td>
<td>1 (10%)</td>
<td>0</td>
<td>0.39</td>
</tr>
<tr>
<td>Hypertension (N, %)</td>
<td>10 (91%)</td>
<td>7 (88%)</td>
<td>0.81</td>
</tr>
<tr>
<td>Diabetes mellitus (N, %)</td>
<td>3 (27%)</td>
<td>2 (25%)</td>
<td>0.91</td>
</tr>
<tr>
<td>End-stage renal disease (N, %)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Smoking (N, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>0</td>
<td>5 (63%)</td>
<td>0.007</td>
</tr>
<tr>
<td>Former</td>
<td>8 (73%)</td>
<td>3 (38%)</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>3 (27%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Statin use (N, %)</td>
<td>11 (100%)</td>
<td>7 (88%)</td>
<td>0.23</td>
</tr>
<tr>
<td>Indications for surgery (carotid)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe carotid stenosis without stroke</td>
<td>8 (73%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td>3 (27%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indications for surgery (femoral)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Claudication</td>
<td></td>
<td>4 (50%)</td>
<td></td>
</tr>
<tr>
<td>Critical limb ischemia</td>
<td></td>
<td>2 (25%)</td>
<td></td>
</tr>
<tr>
<td>Ulcer or gangrene</td>
<td></td>
<td>2 (25%)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. CCA Clustering of CD45 positive-selected cells derived from scRNA-seq of femoral (n=9; 35265 cells) and carotid (n=4; 30655 cells) atherosclerotic plaque reveals distinct immune cell types and populations. CCA Clustering and UMAP visualization of all femoral (N=9) and carotid (N=4) plaque samples for which scRNA-seq was performed, including (A) UMAP for overall samples colored by 14 cell types. (B) Dotplot of top marker genes per cluster. (C) Cell type identities were validated based on marker gene expression.
Figure 2. Quantitative comparison between femoral (n=9; 35265 cells) and carotid (n=4; 30655 cells) atherosclerotic plaque from CCA Clustering of CD45 positive-selected cells. (A) UMAP visualization of separated carotid and femoral samples. (B) Corresponding table and the stacked bar graph of the logistic regression comparing cell proportions in carotid vs. femoral plaque (Log odds ratios (OR), 95% confidence intervals expressed). (C) Volcano plot of highly expressed genes for carotid and femoral plaque samples.
Figure 3. Myeloid re-clustering of scRNA-seq data reveals inflammatory foam cell-like macrophage and monocyte predominance in carotid plaque (n=4; 8941 myeloid cells) and comparative anti-inflammatory and resident-like macrophage biases in femoral plaque (n=9; 4461 myeloid cells). (A) CCA Re-Clustering and UMAP visualization of carotid and femoral myeloid cell samples, including overlay by vascular bed revealed 8 distinct populations (B) Dotplot of top marker genes per cluster. (C) Top marker genes per cluster are shown by violin plot, and (D) Macrophages with high APOE and APOC1 expression suggestive of foam cell features consisted of two distinct clusters, labeled inflammatory and non-inflammatory based on differential gene expression.
Figure 4. Distinct macrophage gene expression and phenotypic profiles confirmed by scRNA-seq (8941 carotid myeloid cells, 4461 femoral myeloid cells) and in a validation cohort by flow cytometry. (A) Cell clusters as a proportion of myeloid cells were compared for carotid vs. femoral samples and are displayed in UMAP visualization of separated carotid and femoral samples with (B) stacked bar graph and corresponding table of the logistic regression comparing cell proportions in carotid vs. femoral plaque (Log odds ratios (OR), 95% confidence intervals expressed; *Asterisk depicts differences between carotid and femoral plaques that were significant at Bonferroni-corrected p<0.0017. (C) Volcano plot of highly expressed genes for carotid versus femoral plaque overall. (D) Flow cytometry of digested plaque macrophages, identified as CD11b<sup>+</sup>CD14<sup>+</sup>CD64<sup>+</sup>HLA-DR<sup>hi</sup> live cells and further distinguished based on CCR2 expression, revealed a significantly higher proportion of carotid plaque macrophages expressing CCR2 than carotid plaque macrophages (representative plot in E, comparison in F, left plot). Classical (CD14++CD16<sup>-</sup>) monocytes from blood also expressed CCR2 more highly in patients undergoing carotid versus femoral endarterectomy (F, right plot).
Figure 5. Differential gene expression analyses of femoral versus carotid myeloid clusters reveal comparative homeostatic, inflammation-regulating biases in femoral plaque. Volcano plots of differential gene expression and gene ontology (GO) analyses of biological processes in femoral versus carotid plaque for: (A) inflammatory foam cell-like macrophages, (B) monocytes, (C) mast cells, and (D) dendritic cells depict differential gene expression for carotid and femoral plaques.
Figure 6. Lymphoid re-clustering of scRNA-seq data reveals T cell predominance and cytotoxicity-associated gene expression in highly prevalent T cell clusters. (A) Visualization of carotid and femoral lymphoid cells revealed 9 distinct populations. (B) Highly expressed genes are visualized with the dotplot. (C) Top marker genes per cluster are shown by violin plot. (D) Related gene set enrichment shown by the dotplot.
Figure 7. Carotid plaque exhibits comparative cytotoxic CD8+ T cell bias, whereas B cells are more prevalent in femoral plaque. (A) UMAP visualization of separated carotid and femoral samples with (B) stacked bar graph and corresponding table of the logistic regression comparing cell proportions in carotid vs. femoral plaque (Log odds ratios (OR), 95% confidence intervals expressed). * Asterisk depicts differences between carotid and femoral plaques that were significant at Bonferroni-corrected p<0.0017. (C) Flow cytometry of T cells from paired plaque and blood samples revealed comparative excess in CD8+ T cells (as a proportion of overall T cells) in plaque and blood from carotid endarterectomy patients (** depicts differences significant at p<0.05). (D) In-situ determination and (E) numbers of B and T cells per high powered field in intraplaque leukocyte aggregates.
Figure 8. Differential gene expression analyses of femoral versus carotid lymphoid clusters demonstrate comparative inflammation-regulating bias in femoral plaque. Volcano plots of differential gene expression in femoral versus carotid plaque for (A) \textit{IL7R}+ CD4+ Effector T cells, (B) \textit{GZMK}+ CD8+ T cells, (C) \textit{GZMH}+ CD8+ T cells, (D) natural killer (NK) cells, (E) \textit{CXCL8}+ T cells, (F) CD79A+ B cells type 2, (G) \textit{TIGIT}+ CD4+ Treg cells, and (H) Plasma cells. CD79A+ B cells type 1 were not included due to the insufficient cell number in carotid plaques.