BASIC SCIENCES

Nonpreferential but Detrimental Accumulation of Macrophages With Clonal Hematopoiesis-Driver Mutations in Cardiovascular Tissues—Brief Report

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BACKGROUND: Clonal hematopoiesis of indeterminate potential (CHIP) is an acquired genetic risk factor for both leukemia and cardiovascular disease. It results in proinflammatory myeloid cells in the bone marrow and blood; however, how these cells behave in the cardiovascular tissue remains unclear. Our study aimed at investigating whether CHIP-mutated macrophages accumulate preferentially in cardiovascular tissues and examining the transcriptome of tissue macrophages from *DNMT3A* (DNA methyltransferase 3 alpha) or *TET2* (Tet methylcytosine dioxygenase 2) mutation carriers.

METHODS: We recruited patients undergoing carotid endarterectomy or heart surgeries to screen for CHIP mutation carriers using targeted genomic sequencing. Myeloid and lymphoid cells were isolated from blood and cardiovascular tissue collected during surgeries using flow cytometry. DNA and RNA extracted from these sorted cells were subjected to variant allele frequency measurement using droplet digital polymerase chain reaction and transcriptomic profiling using bulk RNA sequencing, respectively.

RESULTS: Using droplet digital polymerase chain reaction, we detected similar variant allele frequency of CHIP in monocytes from blood and macrophages from atheromas and heart tissues, even among heart macrophages with and without CCR2 (C-C motif chemokine receptor 2) expression. Bulk RNA sequencing revealed a proinflammatory gene profile of myeloid cells from *DNMT3A* or *TET2* mutation carriers compared with those from noncarriers.

CONCLUSIONS: Quantitatively, CHIP-mutated myeloid cells did not preferentially accumulate in cardiovascular tissues, but qualitatively, they expressed a more disease-prone phenotype.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: clonal hematopoiesis ■ heart disease risk factors ■ inflammation ■ macrophages

Clonal hematopoiesis of indeterminate potential (CHIP), defined by somatic mutations in leukemia-associated driver genes, infers a precursor state of hematologic malignancies.[1,2] While CHIP-associated mutations might contribute to genomic instability and skewed clonality,[3,4] they are only mildly leukemogenic.[5] In spite of that, while lingering at the precursor state, CHIP significantly precipitates cardiovascular diseases and death.[6–10] Harboring CHIP is associated with higher risk of coronary artery disease, worse prognosis of congestive heart failure, ischemic stroke, and death.[10–13] Inflammation was revealed in both mouse and human studies as a potential mechanism, by which CHIP transmits cardiovascular risk.[9,10,14,15] While the presence of CHIP mutations in human atheroma plaque was confirmed,[16] to what extent mutated monocyte-derived macrophages accumulate in cardiovascular tissues remains unknown. First, we aimed to quantify the frequencies of mutated

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Nonstandard Abbreviations and Acronyms	
bp	base pair
CHIP	clonal hematopoiesis of indeterminate potential
ddPCR	droplet digital polymerase chain reaction
DNMT3A	DNA methyltransferase 3 alpha
HLA-DR	human leukocyte antigen-DR isotype
MHCII	major histocompatibility complex class II
RNA-seq	RNA sequencing
TET2	Tet methylcytosine dioxygenase 2
VAF	variant allele frequency

Highlights

- Clonal hematopoiesis of indeterminate potential–mutated monocytes and macrophages do not preferentially accumulate in human cardiovascular tissue.
- Atheromatous plaque and heart macrophages from *DNMT3A* (DNA methyltransferase 3 alpha) or *TET2* (Tet methylcytosine dioxygenase 2) mutation carriers are more proinflammatory than those from the noncarriers.
- Lack of CCR2 (C-C motif chemokine receptor 2) expression in heart macrophages does not preclude monocyte contribution in the adult human heart.

macrophages in human carotid artery plaque and heart tissues relative to those of mutated monocytes in the patients' blood. Second, we compared the gene expression profiles of atheromatous plaque and heart macrophages from CHIP carriers with those from noncarriers.

METHODS

Data Availability

The bulk RNA-sequencing (RNA-seq) data have been made publicly available at the Gene Expression Omnibus data sets and can be accessed under the accession number GSE226642.

Study Cohorts and Workflow

We conducted an observational study in patients undergoing carotid endarterectomy and heart surgery at University Hospital Freiburg. The study complies with the Declaration of Helsinki and was approved by the local ethics committee (EK 249/14, 393/16, 6/20). With written informed consent, patients' blood and cardiovascular tissue retrieved during the surgeries were immediately processed for fluorescence-activated cell sorting of lymphocytes and myeloid cells (Figures S1 and S2). An aliquot of whole blood was subjected to targeted genomic sequencing for CHIP mutation screening. To compare the transcriptome of tissue macrophages from *DNMT3A* (DNA methyltransferase 3 alpha) or *TET2* (Tet methylcytosine dioxygenase 2) mutation carriers with those from noncarriers, we adjusted biological variables known to be associated with cardiovascular diseases and immune responses (Tables S1 and S2). We included and pooled both sexes for the final analysis. Given the relatively small sample size (n=24, separated into 2 groups by surgery type) for this study objective, we did not perform further subgroup analysis to determine whether these variables interact with the studied end points.

Droplet Digital Polymerase Chain Reaction

Isolated DNA of sorted cells from the CHIP mutation carriers was subjected to droplet digital polymerase chain reaction (ddPCR) to measure the CHIP clone size in each sorted cell type. Individual primers were designed with Primer3 (v4.1.0) and ordered from Eurofins. The primers were first validated with

whole blood samples to confirm the annealing temperatures. The ideal DNA product size should be <200 base pairs (bp) to have an optimized amplification efficiency. However, to identify adequate primer pairs and probes for specific targeted loci, the amplicon size of most patients was between 150 and 190 bp while the amplicon size of carriers 1 and 4 was 95 and 388 bp, respectively. The validated primers were then used to design ddPCR probes with BeaconDesigner (v7.0). When feasible, we chose probes containing locked nucleic acids for a higher target specificity (Figure S3A). The ddPCR probes were ordered from Integrated DNA Technologies. Genomic DNA isolated from sorted cells was preamplified using the high-fidelity Platinum SuperFi DNA Polymerase (Thermo Fisher) and subsequently purified using the Agencourt AMPure XP beads (Beckman Coulter). An input of 6 to 24 billion number of copies was used for ddPCR. The copy number was estimated using the formula. The ddPCR was performed following the instructor's guidance of the QX200 ddPCR system. In brief, the preamplified DNA samples were compartmented into oil droplets together with ddPCR Supermix (Bio-Rad), as well as customized primers and probes using QX200 Droplet Generator (Bio-Rad) and underwent a 40-cycle polymerase chain reaction with a ramp rate of 2.5 °C per second to enhance the fluorophore signal in the droplets. The samples were read and analyzed using QX200 ddPCR Droplet Reader and QuantaSoft Software (v1.7; Bio-Rad). An adequate result contained 3 droplet populations, namely double negative cluster and single positive cluster for both channel 1 and channel 2, as presented in Figure S3B. While it was not completely avoidable, we accepted readouts with minimal number of double positive droplets, which did not interfere distinguishing single positive clusters. The variant allele frequency (VAF) was calculated using the number of droplets in each cluster as illustrated in Figure S3B.

Bulk RNA-seq Library Preparation

RNA of sorted myeloid cells from the carriers and noncarriers was constructed into transcriptome libraries using NEBNext Single Cell/Low Input RNA Library Prep Kit (New England BioLabs). The number of cycles for amplifying the transcriptome of each sample was determined based on the sorted cell counts. The finished library was sequenced using the NextSeq2000 (Illumina) instrument with 50-bp paired-end reads.

RESULTS

Patient Cohorts and Immune Cells

We screened a total of 28 patients undergoing carotid endarterectomy and 22 patients undergoing heart surgery, among whom we identified 7 and 4 CHIP mutation carriers, respectively, by targeted whole blood DNA sequencing (Table S3). The average age of the patient group with carotid atherosclerosis was 71.5±11.4 (mean±SD). More than 80% of them had a carotid stenosis above 70%. The average age of the patient group undergoing heart surgery was 67.1±10.6 (mean±SD). Nearly 70% of them underwent surgery for heart valve repair with or without additional procedures. Five patients were subjected to coronary artery bypass graft surgery alone, and 2 patients received a left ventricular assist device. We primarily aimed for comparing the VAF of CHIP in blood monocytes with that in cardiovascular tissue macrophages, while we also explored the VAF of CHIP in other cell types, such as lymphocytes in circulation.

From the patients undergoing carotid endarterectomy, we acquired living CD14+ CD64+ MerTK+ macrophages from the plaque and CD4+ T cells, CD8+ T cells, CD19+ B cells, and CD14+ monocytes from the blood. From the patients undergoing heart surgery, we acquired living CD14+ CD64+ HLA-DR− (human leukocyte antigen-DR isotype) monocytes and CD14+ CD64+ HLA-DR+ CCR2+ (C-C motif chemokine receptor 2) and CCR2− macrophages from right atrial appendage or left ventricle, as well as CD3+ T cells, neutrophils, and CD14+ monocytes from the blood (Figures S1 and S2). DNA and RNA were isolated from the sorted cells for VAF measurement with ddPCR and transcriptomic profiling with bulk RNA-seq, respectively.

Comparison of CHIP VAF in Blood and Cardiovascular Tissue

As the amount of DNA extracted from sorted macrophages per cardiovascular tissue was not sufficient for performing targeted genomic sequencing, we embarked on deploying ddPCR technology, which measures an absolute VAF of predefined targets. For each CHIP carrier, we designed specific primers and probes to target their mutated locus. The primers and probes were first validated using the patients' whole blood DNA. Owing to the low cell counts we could acquire from the cardiovascular tissues, a preamplification of the DNA was required to achieve a sufficient number of copies for ddPCR. We, therefore, first validated the adequacy of the preamplification approach using whole blood samples and assured a comparable VAF obtained from targeted genomic sequencing versus ddPCR with or without DNA preamplification (Figure S3). Fragment analysis was performed to quantify VAF of the 6-bp insertion in carrier 7 because ddPCR was not feasible for this mutation (Figure S4).

The results of ddPCR showed that the CHIP clone size of monocytes in the blood strongly correlated with that of macrophages in both the plaque and the heart with Pearson correlation coefficients (r) of 0.98 and 0.89, respectively. Both regression lines had a slope close to 1, indicating similar VAF in the blood and tissue myeloid cells (Figure 1A and 1B). The CHIP frequency of monocytes and T cells was weakly correlated with the r values below 0.30. VAF in monocytes and B cells moderately correlated (r value, 0.62), albeit the slope of the regression line was as low as 0.48 (Figure S5). All in all, our data indicated that CHIP-mutated and non-mutated monocytes had similar capabilities to give rise to macrophages in cardiovascular tissues. Low VAF in blood lymphocytes reflected myeloid skewing driven by some CHIP mutations. In 1 patient (carrier 11), we did not detect mutated alleles in the 148 CCR2+ heart macrophages. We presume that the absence of mutated cells resulted from low cell counts rather than the inability of the CHIP-mutated monocytes to develop into CCR2+ macrophages in the heart, given that we identified a sizable CHIP-mutated clone among CCR2− macrophages (575 cells) in the same sample.

Surprisingly, we detected similar VAF in CCR2+ and CCR2− heart macrophages (Figure 1B), challenging the concept that CCR2− heart macrophages represent a resident population with minimal monocyte contribution.[17] To inspect the distinct gene expression profiles of CCR2+ and CCR2− macrophages in the human heart, we explored the gene signatures published by Bajpai et al[17] and confirmed the upregulation of proinflammatory genes in our sorted CCR2+ macrophages (eg, *NLRP3* and *IL1B*), as well as the upregulation of negative immunomodulators and tissue-resident macrophage markers in our sorted CCR2− macrophages (eg, *LILRB5* and *LYVE1*; Figure S6). Our finding thus suggests that in the elderly, CCR2+ and CCR2− heart macrophages have been repopulated by blood monocytes at comparable rates.

Gene Expression of Myeloid Cells From *DNMT3A* or *TET2* Mutation Carriers

Although the *DNMT3A*- or *TET2*-mutated macrophages did not dominate accumulation in the cardiovascular tissue, we suppose that dysregulated immune activities of these cells still play a role in enforcing the associated cardiovascular risks. To evaluate whether tissue macrophages from the *DNMT3A* or *TET2* mutation carriers present distinctive gene profiles, we selected 16 noncarriers, in whom no CHIP mutations were detected (8 from each patient group) as controls, matched for sex, age, body mass index, disease severity, blood work, and medical histories (Tables S1 and S2). Bulk RNA-seq revealed an upregulation of genes associated with active immune responses in myeloid cells from the carriers, especially
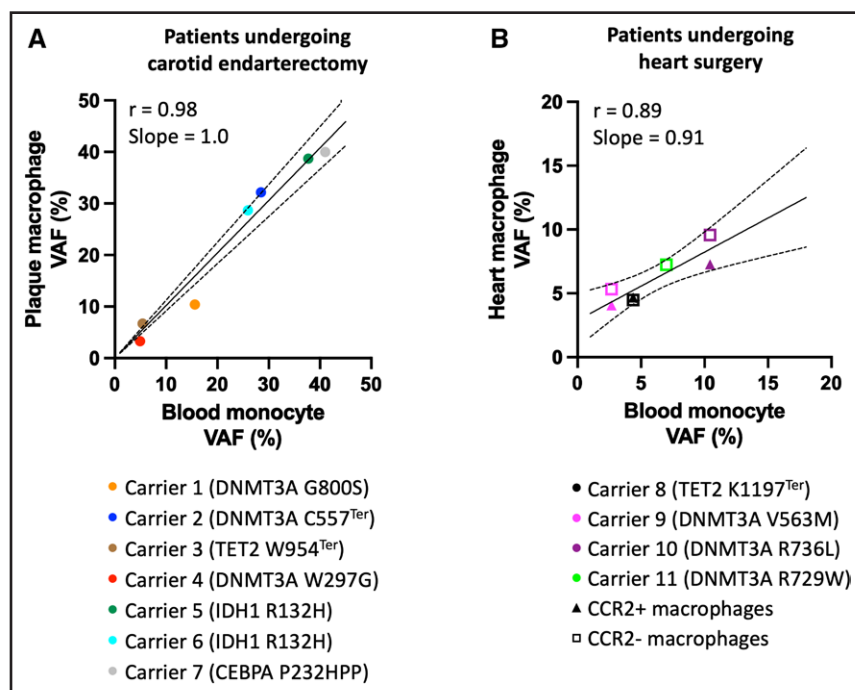
Figure 1. Correlation of clonal hematopoiesis of indeterminate potential clone size between blood monocytes and tissue macrophages. Dot plots depicting variant allele frequency (VAF) of blood monocytes vs that of macrophages in (**A**) carotid plaques and (**B**) hearts. Simple linear regression was performed to compute a fit line (solid line) and the 95% confidence bands (dotted lines) for each plot. The Pearson correlation coefficient (r) was computed to evaluate correlation. Ter indicates translation termination codon, that is, stop codon.

in tissue macrophages (Figure 2A through 2C). Some of these differentially expressed genes promote inflammation and chemotaxis. Interestingly, although CCR2+ macrophages generally exhibited a more proinflammatory profile compared with their CCR2− counterparts (Figure S6), our data revealed that both macrophage subsets from the carriers displayed an overall higher expression of inflammatory genes (eg, IL1B, BIRC3, and CCL3), as opposed to the corresponding macrophages from the noncarriers (Figure 2C and 2D). These results suggest a widespread activation of myeloid cells resulting from *DNMT3A* and *TET2* mutations.[18]

While in general inflammation was a prominent phenotype of both plaque and heart macrophages in the carriers, some enriched biological processes by the upregulated differentially expressed genes differed in the plaque and heart macrophages (Figure 2E). Module scores, which quantitatively summarized specific biological processes, demonstrated that macrophages from the carriers showed stronger inflammatory responses and phagocytosis activity, along with a positive regulation of cell cycle in both plaque and heart tissue. However, *DNMT3A* and *TET2* mutations were associated with an enhanced chemotaxis activity and antigen presentation of macrophages isolated from the heart, while gene expression associated with these immune functions was relatively reduced in plaque macrophages from the carriers. *DNMT3A* and *TET2* mutations also impacted genes linked to macrophage metabolism suggestive of enhanced energy production by fatty acid consumption in heart macrophages. On the contrary, plaque macrophage gene signatures of the carriers pointed toward more glycolysis and less fatty acid or glutamine metabolism (Figure 2F; Figure S7).

DISCUSSION

Accumulation of proinflammatory macrophages in the cardiovascular tissue is one of the key drivers of atherosclerosis and heart disease.[19] Plaque macrophages, in particular, derive from infiltrating monocytes that proliferate locally as the disease advances.[20,21] Thus, we hypothesized that CHIP-associated cardiovascular risk was linked to augmented infiltration or local proliferation of CHIP-mutated monocytes. While our research marks the initial evidence of CHIP clones' presence in cardiovascular tissue, the comparable VAF between blood monocytes and tissue macrophages suggested an equivalent net accumulation of mutated and nonmutated monocytes and their progeny in the cardiovascular tissue. The significantly smaller VAF in the lymphocyte population reinforces the phenomenon of myeloid skewing led by some CHIP driver mutations. As a noticeable frequency of mutated B cells was formerly reported in some patients carrying the *DNMT3A* and *TET2* mutations,[4,22] our data showed that the VAF of blood monocytes and B cells was moderately correlated. Of note, while we consider that the CEBPA[p.P232HPP] mutation is likely germline, as it has been once reported (rs762459325),[23] the VAF (37% by targeted genomic sequencing) does not indicate either a heterozygous or homozygous germline mutation and we lack stromal cells from this patient to perform a confirmatory experiment.

Another distinctive aspect of our research lies in the identification of significant *DNMT3A*- or *TET2*-mutated clones within CCR2− heart macrophages. These macrophages are currently labeled as tissue-resident macrophages of prenatal origin. Circulating monocytes were
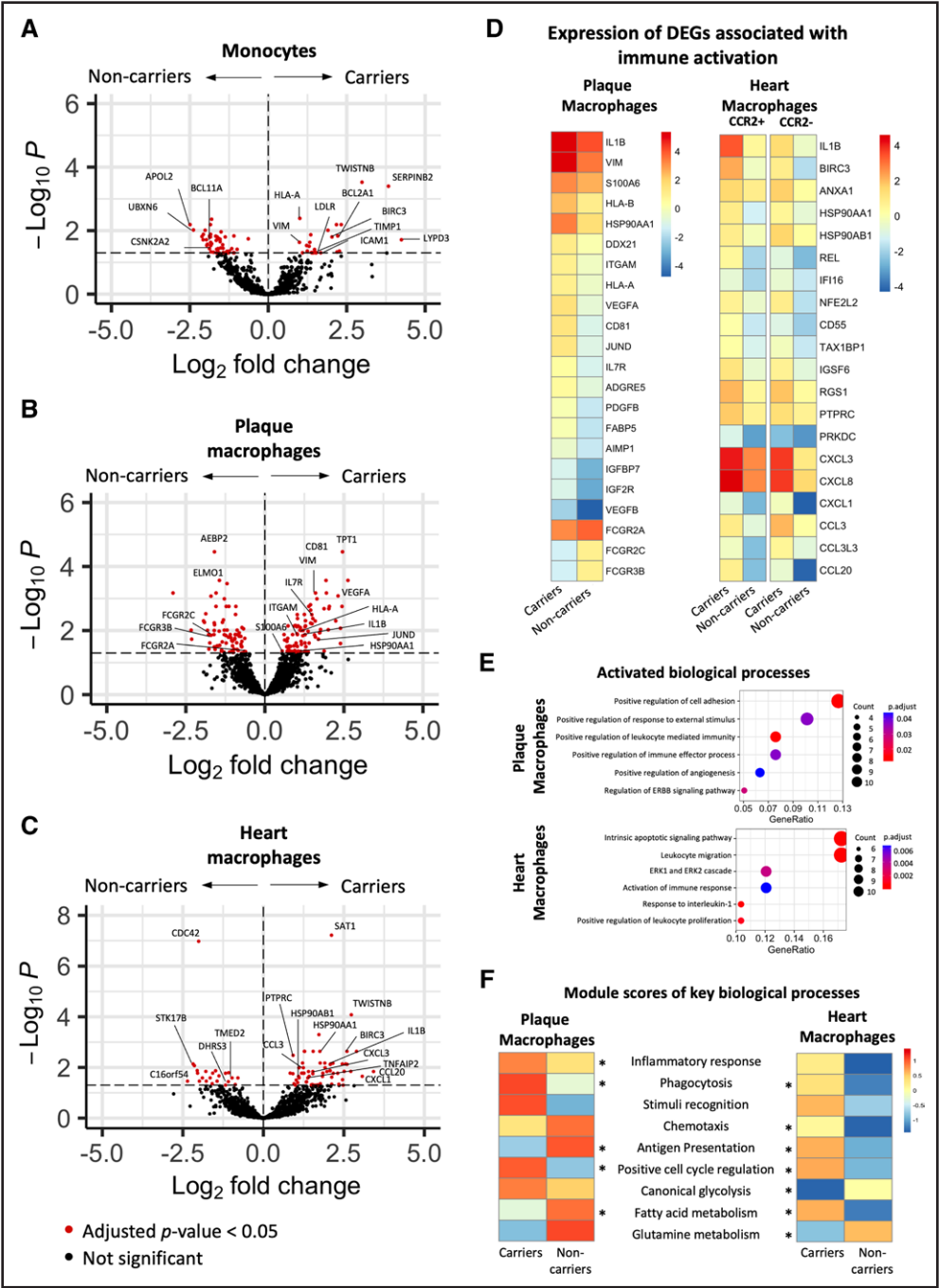
Figure 2. Activated immune responses of tissue macrophages from *DNMT3A* (DNA methyltransferase 3 alpha) or *TET2* (Tet methylcytosine dioxygenase 2) mutation carriers.
A through **C**, Volcano plots showing differentially expressed genes (DEGs; red dots) between carriers and noncarriers. **D**, Heatmaps of representative DEGs associated with activated immune responses. **E**, Dot plots of enriched gene ontology terms of upregulated DEGs in plaque and heart macrophages from clonal hematopoiesis of indeterminate potential mutation carriers. **F**, Heatmaps depicting the mean of module scores of plaque and heart macrophages from carriers and noncarriers. *$P<0.05$ of 1-tailed Mann-Whitney U test.

reported to contribute trivially to the CCR2– heart macrophage population in patients receiving sex-mismatched heart transplantation.[17] However, given that the majority of CHIP mutations are acquired at a relatively advanced age,[7,24] and the same frequencies of mutations were detected in the blood and tissue of our patients,

we suppose that these CHIP-mutated CCR2– heart macrophages were derived from infiltrating monocytes originated from the bone marrow. However, based on some recent evidence suggesting that *DNMT3A* and *TET2* mutations can emerge as early as in the in-utero period[25] and expand at a variable speed over lifetime,[26]

we cannot fully exclude the possibility that some mutated tissue macrophages might originate prenatally and self-renew in situ, independent of infiltrating monocytes. Our work supplements similar findings in the mouse, where Tet2-deficient bone marrow−derived hematopoietic cells infiltrate hearts and differentiate into CCR2−MHCII[high] (major histocompatibility complex class II) macrophages.[27] Furthermore, we confirmed that these CCR2− macrophages expressed a reparative, anti-inflammatory phenotype as shown previously.[17] Our data thus support using CCR2 to identify reparative tissue macrophages but cast doubt on using CCR2 as a single marker to distinguish the origins of tissue macrophages in humans. These data align with a recent report of CHIP mutations in brain microglia, which were also believed to maintain independent from monocytes.[28]

Although we did not observe an enrichment of CHIP-mutated myeloid cells in the cardiovascular tissue, we discovered that the circulating and tissue myeloid cells from *DNMT3A* or *TET2* mutation carriers present a distinctive transcriptome as opposed to those from the matched noncarriers. In the plaque, macrophages from the carriers upregulated genes promoting inflammation, immune responses, phagocytosis, cellular differentiation, and apoptosis. These features reflected the expected pathological behaviors of atheroma macrophages, which transform from infiltrating monocytes, induce inflammatory responses, and scavenge in the lesion. As carotid endarterectomy technique retrieves intima and media vessel layers, the plaque macrophages were largely derived from blood monocytes devoid of tissue-resident macrophages that settle in the adventitia.

Immune activation phenotypes were also shown in the heart macrophages with additional chemokines (Figure 2C and 2D) upregulated in the carriers. Some of these upregulated genes can function intracellularly to activate macrophages or be released to the extracellular space to amplify inflammatory activities through other cells.[29–31] We observed that these mutations casted similar impacts on both the CCR2+ and CCR2− macrophages, suggesting a pan-immune activation associated with the mutations.

In addition, our data also showed distinctive impacts of *DNMT3A* and *TET2* mutations on plaque and heart macrophage functions and metabolism. These distinctions could result from the tissue type where macrophages adapt to their local environment or the general disease context. The formation of carotid artery plaques involves excessive lipid deposition, endothelial damage, and inflammatory immunocyte infiltration at the lesions,[32–34] while heart diseases encompass malfunctioning cardiomyocytes, fibroblasts, and imbalanced immune activities in response to intrinsic or extrinsic (eg, ischemia, valvular defects) triggers.[35–37] Overall, our data manifest that the *DNMT3A* and *TET2* mutations

associate with inflammatory macrophages in both cardiovascular tissues.

A limitation of our study is that the bulk RNA-seq technique we used does not discriminate between mutated and nonmutated cells. Therefore, we cannot comment on whether the inflammatory signature of tissue macrophages in the carriers solely derived from the relatively small fraction of mutated clones or whether the majority of nonmutated macrophages contributed as well, for example, following paracrine activation. While deep sequencing techniques were applied to reveal heightened immune activities in *DNMT3A*-mutated blood monocytes[38] and a chimeric mouse model showed distinctive phenotype between *Dnmt3a*-deficient and wild-type atheroma macrophages,[18] how mutated macrophages differ from their nonmutated counterparts in human awaits investigation.

CONCLUSIONS

Our study quantitatively manifested the presence of proinflammatory myeloid cells in human plaque and heart tissues from *DNMT3A* or *TET2* mutation carriers. The equivalent VAF in the circulation and tissue indicates a comparable ability of mutated and nonmutated monocytes to accumulate and differentiate into macrophages in cardiovascular lesions. Similar mutation frequencies among circulating monocytes and both CCR2+ and CCR2− heart macrophages suggest that, contrary to the prevailing concept, CCR2− macrophages in the aged human heart derive from infiltrating monocytes. While the *DNMT3A*- and *TET2*-mutated macrophages did not enrich locally, their gene expression profile reinforced that inflammation contributes to *DNMT3A* mutation−associated or *TET2* mutation−associated cardiovascular disease and may represent a therapeutic target.

Sources of Funding

Disclosures

None.

Supplemental Material

Supplemental Materials and Methods
Figures S1–S7
Tables S1–S6
Major Resources Table

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