

Immunological risk identified by single-cell multi-omics and cardiovascular outcomes

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Abstract

Background and Aims

While inflammation and (auto-)immunity are modifiers of atherosclerosis, immunological determinants of cardiovascular risk beyond the established inflammatory markers, high-sensitivity C-reactive protein (hsCRP) and interleukin-6 (IL-6) remain incompletely defined in humans. This exploratory proof-of-concept study aimed to detect immunological signals enriched in patients with increased cardiovascular risk.

Methods

A nested case–control study was performed within the Ludwigshafen Risk and Cardiovascular Health (LURIC) cohort, a single-centre prospective study of 3316 patients. Forty-four individuals with and without

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angiographically confirmed coronary artery disease (CAD) and defined survival status were selected for comprehensive immunophenotyping employing bulk RNA, single-cell RNA (scRNA), single-cell T-cell receptor sequencing, mass cytometry, spatial imaging, and cytokine secretion in blood immune cells. Multi-omics factor analysis was applied to identify shared transcriptional programmes. Readouts were tested for their association with CAD status and cardiovascular mortality and validated in two independent cohorts.

Results

The integrated analysis strategy revealed immunological signals not detectable with conventional biomarkers, including a prominent signature of cellular proliferation, linked to activation, memory formation, clonal expansion, interferon signalling, and cytokine secretion in T cells. Cell types and transcriptional programmes associated with CAD status and poor long-term survival were identified. These associations were independent of established inflammatory markers, hsCRP and IL-6 that originated from myeloid cells. Key findings were replicated across independent cohorts using 136 scRNA sequencing datasets. Cellular proliferation was confirmed by protein validation in atherosclerotic plaques, and signatures were evaluated as stratification tools to predict short-term outcomes in acute coronary syndromes.

Conclusions

Multi-omic profiling of blood immune cells revealed novel immune signatures associated with CAD, short- and long-term cardiovascular disease outcomes that are independent of systemic inflammation and may be used as risk stratification tools in the future.

Structured Graphical Abstract

Key Question

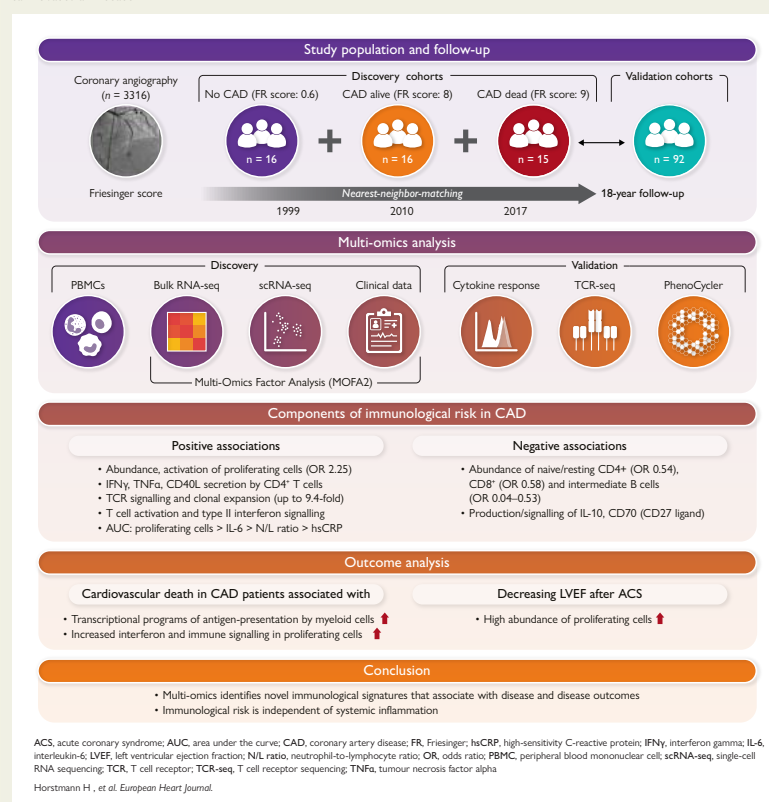
Can single-cell multi-omics identify determinants of immune risk relevant for cardiovascular disease beyond the established inflammatory biomarkers high-sensitivity C-reactive protein (hsCRP) and interleukin-6 (IL-6)?

Key Finding

In this nested case-control study, multi-omic integration of blood immune cells revealed a proliferative adaptive immune program characterized by T cell activation, clonal expansion, interferon signalling, and cytokine secretion. This signature was associated with coronary artery disease, cardiovascular mortality, and impaired cardiac function post-acute coronary syndrome, independent of hsCRP and IL-6.

Take Home Message

Proliferating lymphocytes define cardiovascular risk beyond systemic inflammation. Immune multi-omics may improve risk stratification in cardiovascular disease.



Multi-omic profiling of blood immune cells identifies a coronary artery disease-associated proliferative T-cell and cytokine signature. Defined by clonal expansion, interferon signalling, and cytokine secretion, this immune signature predicts cardiovascular mortality and post-acute coronary syndrome ejection fraction decline independently of hsCRP and IL-6.

Keywords

Atherosclerosis • Leukocytes • scRNA-seq • Transcriptome • Prediction • Immunity

Translational perspective

Chronic inflammation and immune dysregulation contribute to the development and progression of atherosclerosis. Yet, established biomarkers of inflammation lack specificity in identifying dysregulated immunity in patients. This study demonstrates that single-cell transcriptomic profiling of circulating immune cells can uncover excessive immune cell activation associated with coronary artery disease (CAD), long-term mortality, and short-term outcomes after acute coronary syndrome. Regulation of immune cell types and signatures was independent of the conventional inflammatory markers high-sensitivity C-reactive protein and interleukin-6. These findings support the novel concept of a residual immunological risk in patients with CAD, distinct from the general inflammatory response. Thus, with the future development of tools suitable for high-throughput screening of these signatures, immune cell transcriptomics may have considerable potential to improve cardiovascular risk stratification and identify patients who would benefit from future immune-modulatory therapies.

Introduction

Atherosclerosis and its clinical complications—coronary artery disease (CAD), myocardial infarction, and stroke—continue to be leading causes of death globally. Despite significant improvements in managing traditional risk factors, current strategies for modulating and monitoring atherosclerosis risk primarily focus on low-density lipoprotein cholesterol (LDL-C) and its therapeutic reduction. While it has long been understood as a cholesterol-storage disease, preclinical and clinical evidence emphasize the pivotal role of inflammation and immunity in the initiation, progression, and complications of atherosclerosis.¹ This is illustrated by inflammatory biomarkers, including high-sensitivity C-reactive protein (hsCRP) and interleukin (IL)-6, which may outperform LDL-C in predicting future cardiovascular events.² Similarly, anti-inflammatory treatments involving colchicine³ and anti-IL-1 β ⁴ have effectively reduced risk and improved cardiovascular outcomes in patients with persistent inflammation and otherwise controlled risk factors.

Atherosclerosis-related inflammation arises from a local immune response involving circulating leukocytes⁵ recruited into atherosclerotic plaques⁶ and from systemic inflammation starting in key metabolic organs such as visceral adipose tissue, the liver, and muscles. Although hsCRP is commonly used as an inflammatory biomarker, its levels can increase in response to a range of clinical conditions in a nonspecific manner. Consequently, hsCRP may not be the most accurate marker for detecting subclinical immunological risk, warranting the development of novel biomarkers of inflammation and cardiovascular immunity with increased specificity. For instance, it has been proposed that the proportions of specialized immune cell populations in peripheral and coronary blood correlate with the clinical course and complications of atherosclerosis, potentially independent of hsCRP.^{7,8} However, prediction of cardiovascular events based on circulating immune cells is currently limited to techniques that quantify broader haematopoietic lineages but lack cellular sublineage resolution.⁹ Traditional immune cell phenotyping methods, such as flow cytometry, used in these studies are susceptible to inherent biases introduced by marker preselection and therefore do not allow the identification of unknown cell types or disease-associated cellular states. In contrast, advanced phenotyping techniques, such as single-cell RNA sequencing (scRNA-seq), overcome this limitation and provide additional cell-specific transcripts. Unlike prior studies that examined

single modalities or short-term outcomes, this study integrates single-cell transcriptomics, T-cell receptor (TCR) repertoire sequencing, functional validation, and tissue-level imaging to identify immune programmes associated with long-term cardiovascular risk.

Methods

An expanded method section is available in the [Supplementary data online](#).

Code availability

In silico analysis was performed using custom R scripts based on the recommended pipelines from pre-existing packages listed below. No novel algorithms were developed for this study. Custom R scripts will be made available in a GitHub-Repository (<https://github.com/haukeh90/Immune-signatures-predicting-CAD-outcomes>).

Clinical studies

We conducted a nested case-control multi-omic study within the Ludwigshafen Risk and Cardiovascular Health (LURIC) study, a prospective biomarker cohort of 3316 patients enrolled in Germany between 1997 and 2003 (median follow-up 9.9 years). The study follows a sequential design (see [Supplementary data online, Figure S1](#)): (i) cross-sectional CAD vs no-CAD comparison ([Figs 1–5](#)), (ii) replication of CAD-associated signals in external cohorts ([Figure 2](#)), (iii) a nested outcome analysis within CAD patients ([Figure 6](#)), and (iv) functional and tissue validation ([Figure 7](#)). To address these aims, the following three groups were selected from LURIC: patients without evidence of CAD based on coronary angiography, patients with CAD who experienced a fatal cardiovascular event before 2010, and patients with CAD who survived follow-up to 2010. The two CAD groups were matched to equal proportions for hsCRP, N-terminal pro-B-type natriuretic peptide (NT-proBNP), troponin T, LDL-C, and cystatin C using the matchControls function (R package e1071). CAD was defined angiographically (Friesinger score > 3), and mortality was ascertained from local public health records. After exclusion of three patients who failed quality control (QC), baseline characteristics of the final study population ($n = 44$) are listed in [Table 1](#). All participants provided written informed consent; the study was approved by the local institutional review board (approval no. 837.255.97). For external validation, two independent scRNA-seq cohorts were used: the Munich acute coronary syndrome (ACS) cohort¹⁰ comprised 55 samples (CAD $n = 38$, no CAD $n = 17$) from patients with ACS.

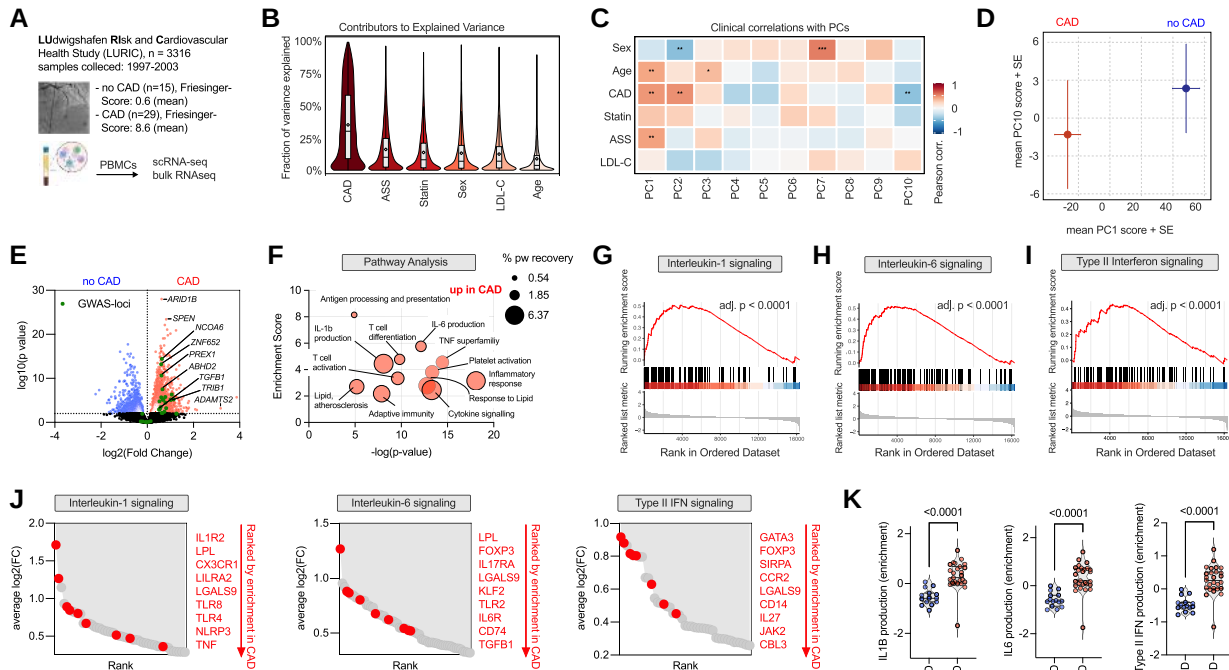


Figure 1 RNA sequencing of peripheral blood mononuclear cells identifies systemic inflammatory transcriptional programmes in coronary artery disease. (A) Study overview: A nested case-control study was conducted within the LURIC study ($n = 3316$) with patients with (coronary artery disease) and without (no coronary artery disease) angiographically confirmed coronary artery disease. Immunophenotyping was performed using single-cell RNA sequencing and bulk RNA sequencing. (B) Fraction of transcriptomic variance in bulk RNA sequencing explained by clinical variables across principal components, shown as violin plots, in the entire sub-study ($n = 44$). (C) Heatmap showing Pearson correlations between clinical covariates and principal components (PC1–PC10). Colour scale indicates correlation strength and direction; asterisks denote significance ($*P < .05$, $**P < .01$, $***P < .001$). (D) Mean PC1 and PC10 scores $\pm$ SEM stratified by coronary artery disease status. (E) Differential gene expression analysis between coronary artery disease and no-coronary artery disease individuals from bulk RNA sequencing data using DESeq2. Volcano plot displays genes with significant regulation (adjusted $P < .05$ after Benjamini–Hochberg correction). The dotted line indicates the significance threshold ($P = .05$). (F) Pathway enrichment analysis of genes significantly up-regulated in coronary artery disease was performed using Metascape. The bubble plot shows selected enriched pathways, with bubble size representing the percentage of pathway gene recovery, the y-axis indicating enrichment score, and the x-axis showing $-\log(\text{adjusted } P\text{-value})$. Gene set enrichment analysis probing enrichment of the following pathways in coronary artery disease samples: interleukin-1 production (G), interleukin-6 production (H), and Type II interferon production (I). (J) Ranked gene contribution plots for the indicated inflammatory pathways, displaying average $\log_2$ fold changes of leading-edge genes enriched in coronary artery disease. Selected genes are highlighted. (K) Comparison of pathway enrichment scores for interleukin-1 β production, interleukin-6 production, and Type II interferon production between no-coronary artery disease and coronary artery disease individuals. Each dot represents one individual; centre lines indicate medians, and whiskers the interquartile range. P -values are shown above comparisons. The schematic in A was created with biorender.com under agreement number ID29XN9LIH.

The Groningen cohort¹¹ contributed 37 donors with available scRNA-seq data (CAD $n = 37$).

Peripheral blood mononuclear cell processing and sorting

Cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed and washed as previously described. Viability was assessed by flow cytometry and by automated cell counting using a TC20™ cell counter (Bio-Rad). For scRNA-seq, viable CD45⁺ cells were sorted following staining with eFluor 780 and anti-CD45 and loaded onto the Chromium Controller (10 $\times$ Genomics) to generate single-cell gel bead emulsions. Libraries were prepared using the Chromium Single Cell 5' Library and

Gel Bead Kit and sequenced on an Illumina NovaSeq platform (BioCenter Core Facility, Vienna, Austria).

Bulk RNA sequencing and analysis

Total RNA was extracted from thawed PBMCs using the RNeasy kit (Qiagen). RNA integrity was assessed prior to library preparation (RIN range 6.8–7.2). Libraries were prepared with the NEBNext Ultra II Directional RNA Library Prep Kit and sequenced on an Illumina NovaSeq S2. Raw FASTQ files were aligned to the human reference genome, and gene-level count matrices were generated using standard pipelines. Data were processed using DESeq2 (v1.44.0) for normalization and differential expression analysis. Differentially expressed genes (DEGs) were defined by adjusted P -values $< .05$ using the Benjamini–Hochberg method.

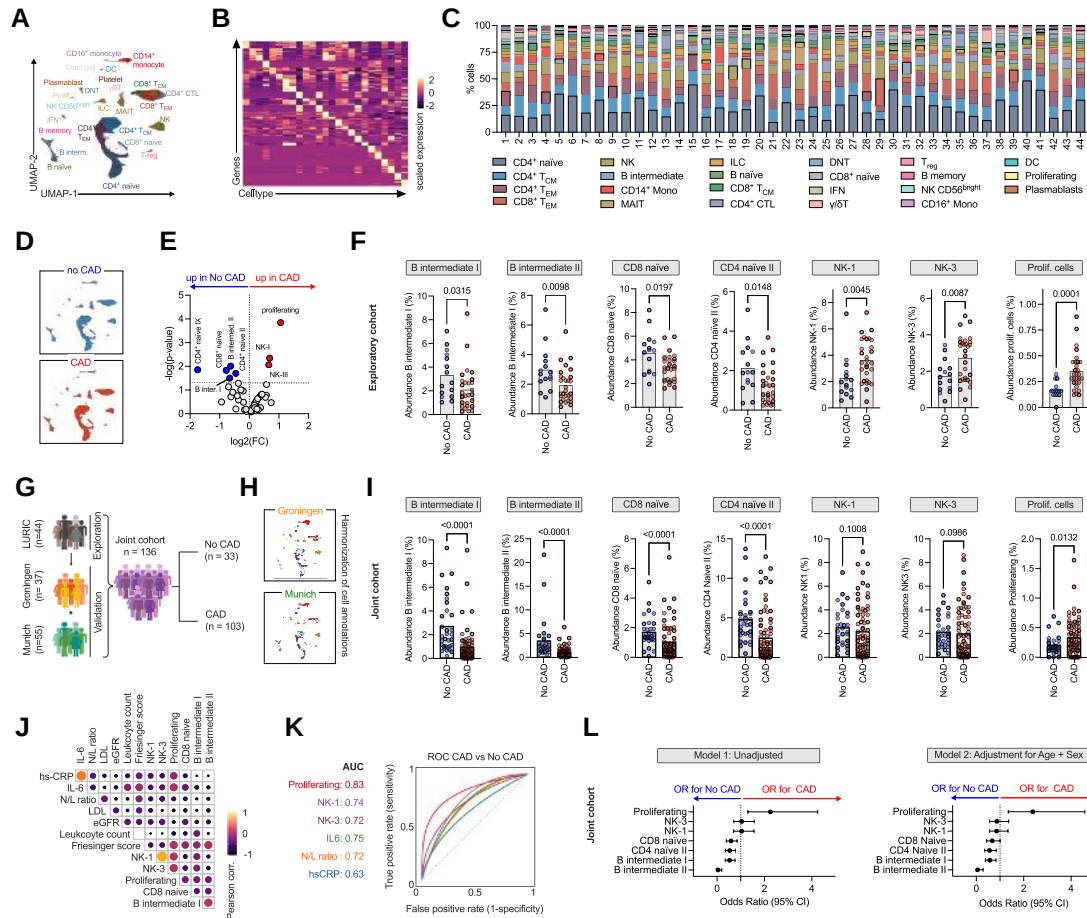


Figure 2 Coronary artery disease is marked by an expansion of proliferating leukocytes and a contraction of naive adaptive immune cells in peripheral blood. (A) Uniform manifold approximation and projection of single-cell RNA sequencing data from peripheral blood mononuclear cells of no-coronary artery disease and coronary artery disease patients, showing major immune cell populations annotated based on canonical marker gene expression. (B) Louvain clustering (resolution = 2.9) identified transcriptionally distinct cell clusters. Cluster-specific up-regulated genes were determined using a Wilcoxon rank-sum test and used to guide manual cell-type annotation. Heatmap of scaled expression of selected marker genes across annotated immune cell populations. (C) Relative abundance of immune cell populations across individual samples ($n = 44$), displayed as stacked bar plots showing the percentage of cells per population per sample. (D) Uniform manifold approximation and projection projections split by coronary artery disease status following batch correction using reciprocal principal component analysis and anchor-based integration. (E) Differential abundance analysis of immune cell clusters defined in (A). Cluster abundance was calculated as the percentage of cells per cluster relative to the total number of cells per sample. Clusters with a mean abundance $\geq 1\%$ of total leukocytes were tested for differences between coronary artery disease and no-coronary artery disease individuals using the Mann-Whitney U test. The volcano plot displays significantly enriched clusters in coronary artery disease and no coronary artery disease. (F) Quantification of selected immune cell populations. Each dot represents one individual; bars indicate the mean. P -values are shown above comparisons. (G) Validation study design schematic illustrating the independent validation using the 'Groningen' and 'Munich' cohorts. A total of 136 patients were analysed. (H) Uniform manifold approximation and projection projections demonstrating harmonization of cell-type annotations across validation cohorts. (I) Validation of immune cell abundance in the joint cohort (exploratory and validation). Relative abundances of selected immune cell populations are shown for no-coronary artery disease and coronary artery disease individuals. Each dot represents one individual; bars indicate the mean. (J) Correlation matrix showing pairwise Spearman correlations between immune cell abundances and clinical variables in the exploratory cohort. Colours indicate the direction and strength of correlation, with intensity proportional to the correlation coefficient (ρ). (K) Receiver operating characteristic analysis for the indicated variables discriminating coronary artery disease. Area under the curve values are indicated. Smoothed receiver operating characteristic curves (binormal method) are shown for visualization purposes only; area under the curve estimates and 95% confidence intervals were calculated exclusively from the empirical (non-smoothed) receiver operating characteristic curves using the nonparametric DeLong method. (L) Forest plots displaying the odds ratio and 95% confidence intervals for associations between immune cell abundances and coronary artery disease in the joint cohort calculated using logistic regression. Model 1 shows unadjusted estimates; Model 2 includes adjustment for age and sex. Directionality indicates coronary artery disease or no coronary artery disease. The schematic in G was created with biorender.com under agreement number ID29XN9LIH.

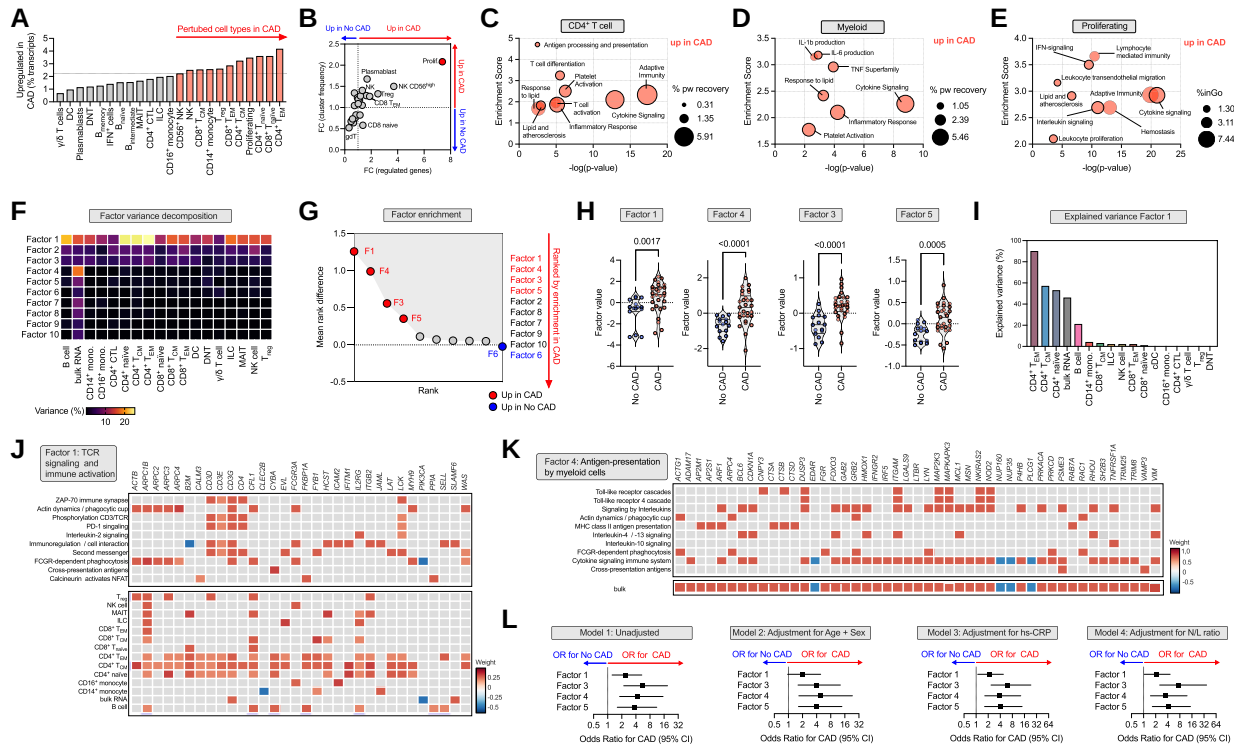


Figure 3 Multi-omic factor analysis identifies pan-cellular programmes of antigen recognition, memory formation, and tissue homing in CD4⁺ T-cell populations from coronary artery disease patients. (A) Fractions of differentially up-regulated genes in coronary artery disease were calculated as the percentage of all expressed genes per cell type, based on pseudo-bulk differential expression analysis using the LIBRA framework with edgeR and likelihood ratio testing across immune cell subsets. Cell types showing increased perturbation in coronary artery disease are highlighted (defined by % regulated genes > mean). (B) Comparison of fold changes in up-regulated genes and cluster abundance changes across the same immune cell populations. (C–E) Cell-type-resolved pathway enrichment analysis of genes up-regulated in coronary artery disease. Bubble plots show enriched pathways in CD4⁺ T cells (C), myeloid cells (D), and proliferating cells (E). Bubble size represents the percentage of pathway gene recovery, and axes indicate enrichment score and $-\log(P)$ value. (F) Variance decomposition heatmap showing the percentage of variance explained by multi-omics factor analysis-derived factors across individual views, including immune cell populations and bulk RNA sequencing data. (G) Ranking of factors based on differential enrichment between coronary artery disease and no-coronary artery disease individuals. Factors are ordered by mean rank difference, with factors enriched in coronary artery disease highlighted. (H) Patient-level distribution of selected multi-omics factor analysis factor values (Factors 1, 3, 4, and 5) stratified by coronary artery disease status. Data are shown as violin plots, and statistical significance was assessed using a Mann–Whitney *U* test. (I) Explained variance of Factor 1 across immune cell types and bulk RNA sequencing, highlighting dominant contributors to coronary artery disease-associated transcriptional variation. REACTOME pathway enrichment heatmap for genes defining Factor 1 (J) and Factor 4 (K) across all views. Pathways are shown only if at least 50% of genes overlapped with the feature set. Genes contributing to Factor 1 were required to be among the top 5% highest-ranking features. Upper heatmaps display averaged feature factor weights across views, while lower heatmaps show view-specific weights. Colour scale indicates factor weight direction and magnitude (red, positive; white, zero; blue, negative). (L) Forest plots showing odds ratios and 95% confidence intervals for associations between multi-omics factor analysis-derived Factors 1, 3, 4, and 5 and coronary artery disease in the LURIC cohort calculated by logistic regression. Model 1 shows unadjusted estimates; Model 2 includes adjustment for age and sex; Model 3 includes adjustment for high-sensitivity C-reactive protein; Model 4 includes adjustment for neutrophil-to-lymphocyte ratio. Directionality indicates enrichment in coronary artery disease or no coronary artery disease. All 10 factors shown in (L) were tested irrespective of their coronary artery disease enrichment rank in (G)

Principal component analysis and variance decomposition

Raw counts were normalized using TMM (edgeR) and transformed to $\log_2$ -CPM using the voom method (limma). Principal component analysis (PCA) was performed on the 1500 most variable genes (PCATools; centre = TRUE, scale = TRUE), and Pearson correlations between PC1–10 scores and six clinical covariates (sex, age, CAD status, statin use, aspirin use, LDL-C) were

computed to assess their contribution to transcriptional variance. Genome-wide variance decomposition was performed using the variancePartition package.

Single-cell RNA sequencing analysis

Sequencing reads were processed with Cell Ranger and analysed using the Seurat package. Granulocytes were excluded from the PBMC preparation, and residual granulocyte transcriptomes

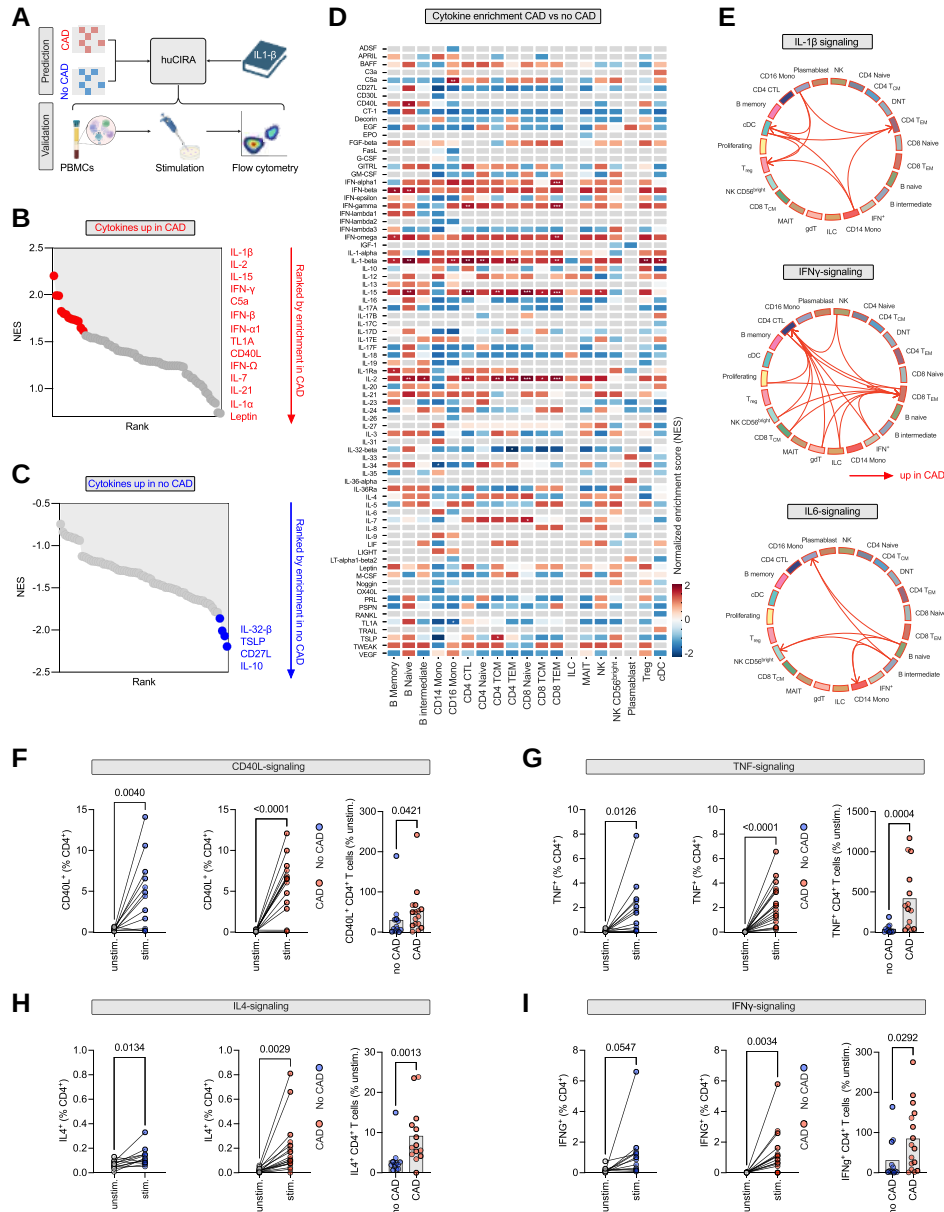


Figure 4 T cells from coronary artery disease patients are characterized by enhanced secretion of pro-inflammatory cytokines. (A) Overview of the integrative strategy that combines computational cytokine inference using huCIRA with experimental validation in peripheral blood mononuclear cells from coronary artery disease and no-coronary artery disease individuals. Ranked enrichment plot showing cytokines predicted to be up-regulated in coronary artery disease (B) and no coronary artery disease (C) based on normalized enrichment scores in the single-cell RNA sequencing dataset. Cytokines are ordered by enrichment strength, with the top-ranking cytokines highlighted. (D) Heatmap summarizing cytokine enrichment scores across immune cell populations comparing coronary artery disease vs no-coronary artery disease individuals. Colours indicate normalized enrichment scores, with red representing enrichment in no coronary artery disease. Asterisks denote significance (* $P < .05$, ** $P < .01$, *** $P < .001$). (E) Circos plots depicting cell-type-specific cytokine signalling networks for interleukin-1 β , interleukin-6, and IFN- γ signalling. Nodes represent immune cell populations, and connecting edges indicate cytokine signalling predicted to be enriched in coronary artery disease compared to no coronary artery disease. (F–I) Flow cytometry-based validation of cytokine secretion from CD4 $^{+}$ T cells following staphylococcal enterotoxin B stimulation of peripheral blood mononuclear cells for 6 h. Left panels show paired comparisons of unstimulated vs staphylococcal enterotoxin B-stimulated conditions within individuals without coronary artery disease and with coronary artery disease; right bar charts display the relative increase in cytokine-positive CD4 $^{+}$ T cells (expressed as % unstimulated). For (F)–(I), each dot represents one individual; paired samples are connected by lines where applicable. Bars indicate the mean. P-values are shown above the comparisons and were calculated using a paired t-test or an unpaired non-parametric Mann–Whitney U test, as appropriate. The schematic in A was created with biorender.com under agreement number EY29XN9HT2.

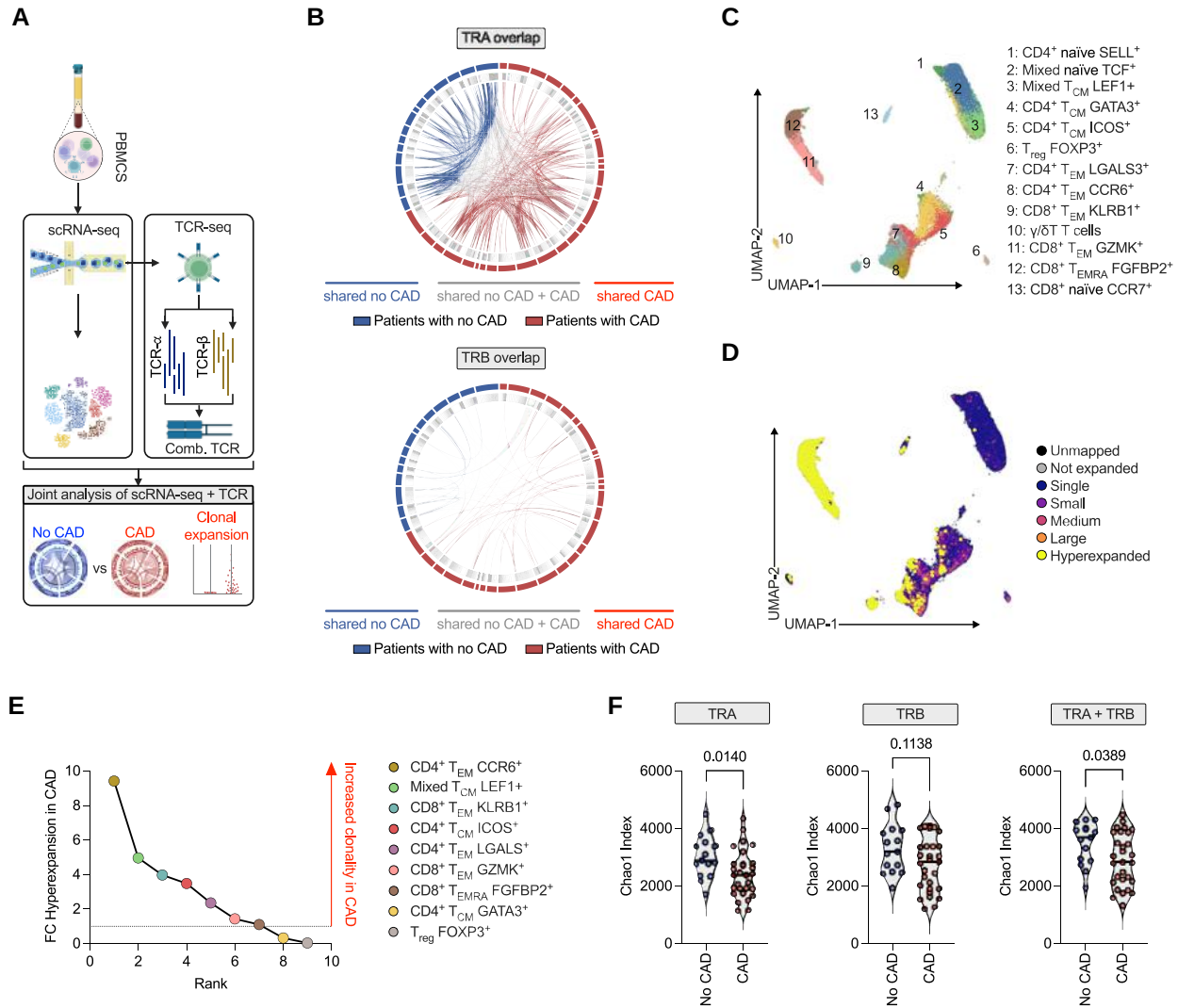


Figure 5 Paired single-cell and T-cell receptor repertoire analysis reveals increased clonal expansion of circulating T cells in coronary artery disease. (A) Experimental workflow of paired single-cell RNA sequencing and T-cell receptor sequencing of peripheral blood mononuclear cells: T cells were subclustered based on transcriptional profiles, and paired T-cell receptor alpha and T-cell receptor beta sequences were used to assess clonal relationships and expansion in individuals with coronary artery disease and those without coronary artery disease. (B) Circos plots depicting clonal overlap based on shared T-cell receptor alpha (top) and T-cell receptor beta (bottom) sequences. Blue arcs indicate clones shared exclusively among no-coronary artery disease individuals, red arcs indicate clones shared exclusively among coronary artery disease individuals, and grey arcs indicate clones shared between both groups. (C) Uniform manifold approximation and projection representation of transcriptionally defined T-cell subclusters annotated based on marker gene expression. Identified populations include naïve, memory, effector memory, regulatory, and cytotoxic T-cell subsets. (D) Uniform manifold approximation and projection coloured by clonal expansion status, categorized as unmapped, not expanded, single, small, medium, large, or hyperexpanded clones. (E) Ranking of T-cell subclusters based on fold change in hyperexpanded clones in coronary artery disease relative to no-coronary artery disease individuals, highlighting subsets preferentially undergoing clonal expansion in coronary artery disease. (F) Comparison of T-cell receptor repertoire diversity between no-coronary artery disease and coronary artery disease individuals quantified using the Chao1 diversity index for T-cell receptor alpha, T-cell receptor beta, and combined T-cell receptor alpha + T-cell receptor beta repertoires. Each dot represents one individual; violin plots show distribution and median values. *P*-values are indicated above comparisons. Statistical significance was assessed using a Mann–Whitney *U* test. The schematic in A was created with biorender.com under agreement number OE29XN9PP5.

were removed using standard Cell Ranger settings. Doublets were identified and removed using DoubletFinder. Of the 47 sequenced samples, three were excluded a priori based on predefined, analysis-independent QC criteria: (i) <2000 high-quality cells after filtering (mitochondrial content, ≥ 200 genes/cell, doublet

removal) or (ii) markedly reduced transcript complexity (median genes/cell below threshold). Excluded sample IDs and QC metrics are reported in [Supplementary data online, Tables S3–S5](#). Cells were filtered, log-normalized, and integrated using reciprocal PCA (RPCA). Clustering was performed using the Louvain algorithm,

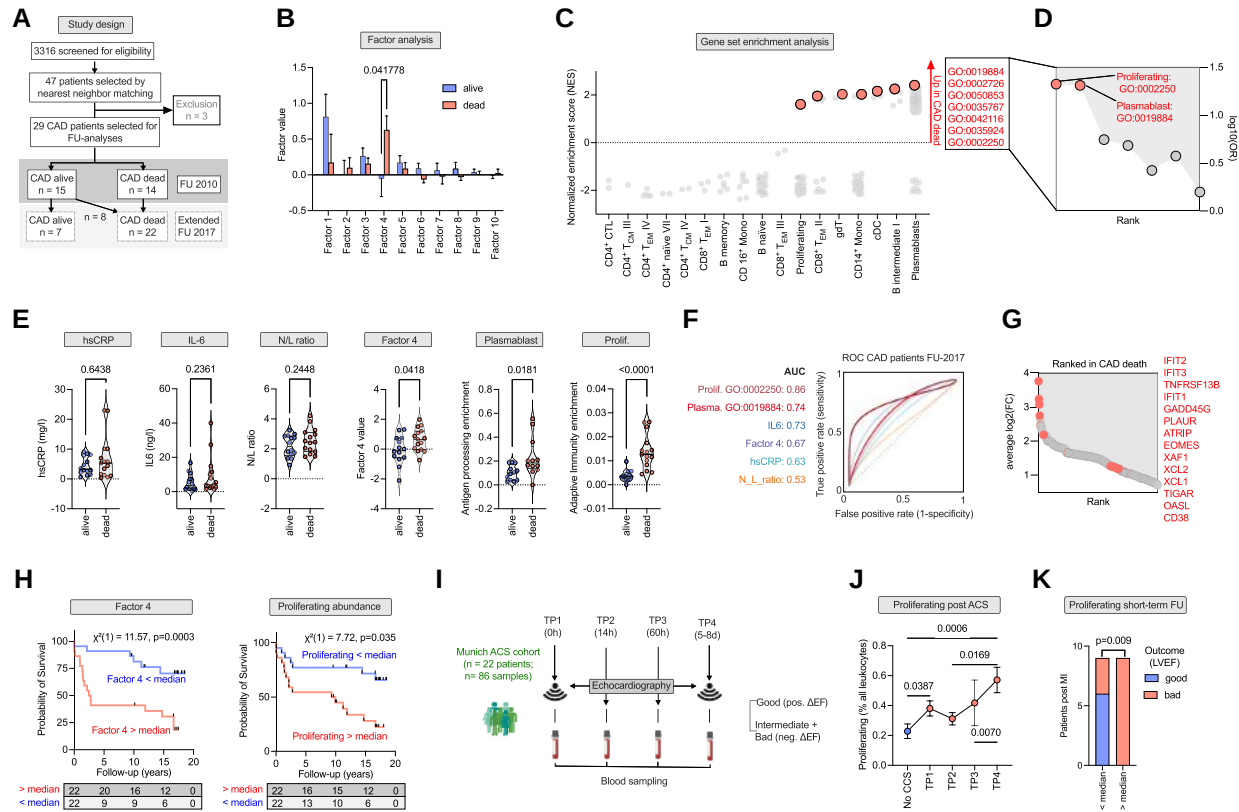


Figure 6 Detection of transcriptional programmes and cell states that associate with cardiovascular death and poor outcomes after acute coronary syndrome. (A) Study design: For outcome-focused analyses, a nested case-control design was applied within the coronary artery disease cohort. Twenty-nine coronary artery disease patients with available follow-up data were included and stratified according to cardiovascular survival status (alive vs deceased). Both groups were matched for high-sensitivity C-reactive protein, N-terminal pro-B-type natriuretic peptide, troponin T, low-density lipoprotein cholesterol, and cystatin C. (B) Multi-omics factor analysis-derived factor values between both groups. Bars represent mean factor values $\pm$ SEM. Statistical significance was assessed using an unpaired t-test. (C) Gene set enrichment analysis of immune cell-specific gene signatures ranked by normalized enrichment score, highlighting cell populations with the strongest transcriptional perturbation in patients who died during follow-up. (D) Ranking of gene ontology terms associated with mortality was performed using Firth's penalized logistic regression after adjustment for age, sex, acetylsalicylic acid, and statin use. Log odds ratios were calculated at the per-patient level. (E) Comparison of systemic inflammatory markers (high-sensitivity C-reactive protein, interleukin-6, neutrophil-to-lymphocyte ratio) and immune-derived pathways (GO:0002250 in proliferating cells, GO:0019884 in plasmablasts) between survivors and deceased coronary artery disease patients. Violin plots display distributions with the median and interquartile range; *P* values are shown above each comparison. Statistical significance was assessed using a Mann-Whitney *U* test. (F) Receiver operating characteristic analysis for the indicated variables at follow-up (2017). Area under the curve values are indicated. Smoothed receiver operating characteristic curves (binormal method) are shown for visualization purposes only; area under the curve estimates and 95% confidence intervals were calculated exclusively from the empirical (non-smoothed) receiver operating characteristic curves using the nonparametric DeLong method. (G) Ranking of genes derived from proliferating cells that were up-regulated in deceased vs alive coronary artery disease patients. (H) Kaplan-Meier survival analysis stratified by Factor 4 (left) and proliferating cells abundance (right). Patients above and below the median are shown as separate survival curves. Log-rank test statistics: Factor 4, $\chi^2(1) = 11.57, P = .0003$; proliferating cells, $\chi^2(1) = 7.72, P = .035$. Numbers at risk are shown below each panel. (I) Schematic representation of longitudinal blood sampling and parallel echocardiographic assessment in the Munich cohort in patients with acute coronary syndrome (*n* = 22). Patients were stratified by the recovery of left ventricular ejection fraction (Δ EF at TP4 vs TP1). (J) Proliferating immune cell abundance at the indicated time points post-acute coronary syndrome. Data are shown as mean $\pm$ SEM; *P*-values indicate pairwise comparisons between time points. (K) Stratification of patients based on proliferating cells abundance (below or above the median) and fractions of patients with intermediate/bad vs good Δ EF outcomes. Statistical significance was assessed using Fisher's exact test. The schematic in I was created with biorender.com under agreement number XN29XNB71E.

and clusters were annotated by cluster-specific genes and reference mapping. Only clusters with $\geq 1\%$ mean abundance were retained for downstream analysis. Cluster-specific DEGs were identified using Wilcoxon rank sum testing, and gene module scores were computed using Seurat and UCell. To account for

potential cryopreservation-related biases, subtype-level QC and benchmarking analyses were performed, including assessment of relative subset representation. Pseudo-bulk DEGs between conditions of interest were determined using the LIBRA framework and EdgeR.

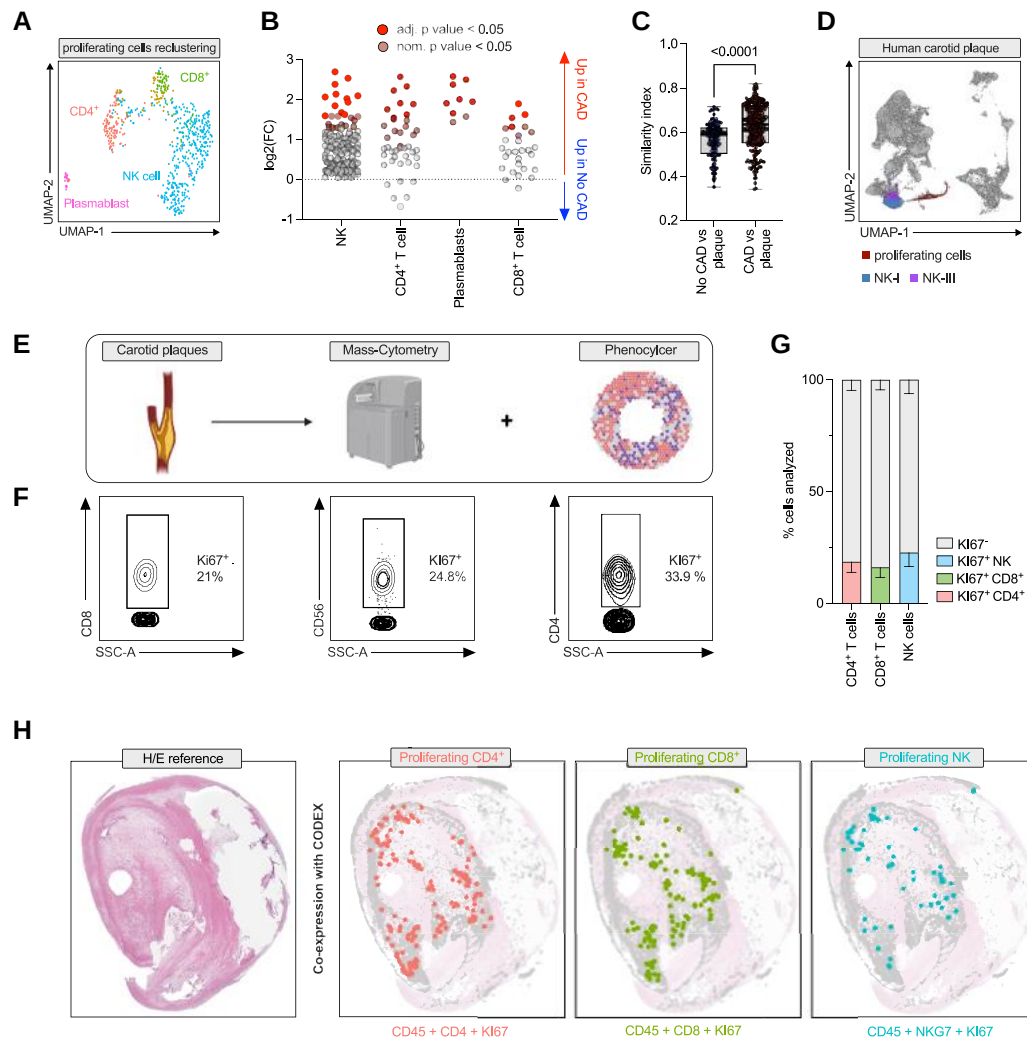


Figure 7 Local and peripheral cellular proliferation is a feature of coronary atherosclerosis. (A) Reclustering of proliferating immune cells after regression of cell cycle-associated genes. Cell identities were assigned manually. (B) Differential abundance analysis by MiloR showing regulation of the indicated proliferating cell subsets between coronary artery disease and no-coronary artery disease individuals. (C) Similarity index comparing transcriptional overlap of proliferating immune cells from peripheral blood and carotid plaques in coronary artery disease and no-coronary artery disease individuals. Higher similarity indicates convergence of proliferative immune programmes between circulation and plaque tissue. *P*-value is shown above the comparison. (D) Mapping of immune cell clusters (proliferating cells, NK-I, and NK-III) onto human carotid plaque leukocyte transcriptomes using Seurat's anchor-based reference mapping strategy. (E) The validation workflows for mass cytometry (CyTOF) and spatial imaging with the PhenoCycler (CODEX) are shown. (F and G) Quantification of proliferating (Ki67⁺) immune cell fractions in CD4⁺ T, CD8⁺ T, and natural killer cells in human carotid plaques. Bars indicate mean $\pm$ SEM. (H), Representative haematoxylin and eosin-stained section of a human carotid plaque (left) and spatial localization of proliferating immune cells detected by CODEX (right). Proliferating CD4⁺ T cells (CD45⁺CD4⁺Ki67⁺), CD8⁺ T cells (CD45⁺CD8⁺Ki67⁺), and natural killer cells (CD45⁺NKG7⁺Ki67⁺) are overlaid on the plaque architecture. The schematic in E was created in biorender.com under agreement number ZZ29XN9UC7.

Pathway and interactome analysis

Pathway enrichment was performed with MetaScape and gene set enrichment analysis (GSEA) using MSigDB gene sets. Enrichment scores were visualized using bubble plots and Z-score-scaled heat-maps. Cell-cell communication was inferred with CellChat (v2.1.2) using ligand-receptor interactions and network analyses.

Multi-omics factor analysis

We utilized MOFA2 (version 1.2.2) to integrate scRNA-seq, bulk RNA-seq, and clinical data. Pseudo-bulk gene expression was

calculated for each scRNA-seq cluster. After gene filtering, 28 516 features across 18 data views were included. Model training was performed with 10 factors and 50 000 iterations. Pathway enrichment on multi-omics factor analysis (MOFA) factors used REACTOME immune system annotations.

Cytokine response inference (huCIRA)

Cytokine response activity was inferred from scRNA-seq data using the huCIRA framework, which applies GSEA to a curated human cytokine response signature dictionary across 19

Table 1 Baseline characteristics of the study population

Parameter	No coronary artery disease (n = 15)	Coronary artery disease (n = 29)	P-value
Age (years)	58.3 ± 9.4	66.8 ± 9.5	.004
Female sex	6 (40.0)	4 (13.8)	.066
Diabetes	3 (20.0)	11 (37.9)	.314
Hypertension	9 (60.0)	25 (86.2)	.066
Friesinger score	0.6 ± 0.9	8.6 ± 1.7	<.001
Smoking	4 (26.7)	9 (31.0)	1
Body mass index (kg/m ²)	28.0 ± 3.1	26.5 ± 3.8	.183
Systolic blood pressure (mmHg)	138.5 ± 21.2	150.7 ± 25.8	.147
Diastolic blood pressure (mmHg)	80.5 ± 9.4	84.9 ± 13.9	.207
N-terminal pro-B-type natriuretic peptide (pg/mL)	657 ± 725	1220 ± 1680	.243
Estimated glomerular filtration rate (units)	85.0 ± 16.3	74.4 ± 22.7	.114
Glycated haemoglobin (%)	6.0 ± 0.6	6.51 ± 1.4	.234
Total cholesterol (mg/dL)	208.9 ± 31.2	191.5 ± 28.6	.113
Low-density lipoprotein cholesterol (mg/dL)	131.9 ± 24.5	116.0 ± 22.4	.112
High-density lipoprotein cholesterol (mg/dL)	43.1 ± 10.8	42.8 ± 11.5	.629
High-sensitivity C-reactive protein (mg/L)	3.2 ± 2.7	5.7 ± 5.6	.113
Cystatin C (mg/dL)	0.9 ± 0.2	1.1 ± 0.4	.116
Angiotensin-converting enzyme inhibitor use	5 (33.3)	11 (37.9)	1
Aspirin/other antiplatelet use	5 (33.3)	19 (65.5)	.058
Statin use	1 (6.7)	14 (48.3)	.007

Data are presented as mean ± SD, or n (%). Differences in baseline characteristics were examined by Student's *t*-test or Wilcoxon rank-sum test for normally and non-normally distributed continuous variables, respectively, and χ^2 test or Fisher's exact test for categorical variables.

matched immune cell populations. Enrichment was quantified for the CAD vs no CAD contrast and summarized as normalized enrichment score, filtered for robustness across multiple significance thresholds. Key predicted cytokine responses were experimentally validated by *ex vivo* stimulation of PBMCs and quantification of cytokine-producing CD4⁺ T-cell fractions in flow cytometry.

T-cell receptor repertoire analysis

TCR sequencing data were processed with Cell Ranger VDJ and integrated with transcriptomic data using the scRepertoire package. Clones were categorized by size and projected onto the T-cell uniform manifold approximation and projection (UMAP). Clonal diversity was quantified per patient using the Chao1 index for the T-cell receptor alpha (TRA) and beta (TRB) chains, and differences between CAD and no CAD patients were assessed by the Wilcoxon rank-sum test. Shared clonotypes across patients were displayed as circo plots stratified by CAD status.

Plaque mapping and tissue validation

Transcriptomes of proliferating PBMCs were isolated and re-clustered following cell cycle score regression, identifying CD4⁺ T cell, CD8⁺ T cell, natural killer (NK) cell, and Plasmablast sub-populations, with differential abundance between CAD and no CAD patients assessed by MiloR. Transcriptional similarity between circulating and

plaque-resident proliferating cells was quantified at the patient level using pseudobulk Spearman correlation. Transcriptomes of proliferating blood cells were projected onto a human carotid plaque scRNA-seq reference using Seurat's anchor-based transfer workflow. Protein-level validation was performed in human carotid plaques using (i) mass cytometry (CyTOF) to confirm the presence of Ki67⁺ proliferating CD4⁺ T cells, CD8⁺ T cells, and NK cells and (ii) using PhenoCycler spatial proteomics to localize triple-positive CD45⁺Ki67⁺ immune cell populations *in situ*.

Statistics

Baseline characteristics were presented as mean ± standard deviation (SD) or as median [interquartile range (IQR)], based on the distribution of each continuous variable, and as count (percentage) for categorical variables. Comparisons were made using Student's *t*-test or the Wilcoxon rank-sum test for continuous variables, and χ^2 or Fisher's exact test for categorical variables, as appropriate. Associations with CAD status (CAD vs no CAD) and with cardiovascular death (within the CAD cohort) were assessed using Firth's penalized logistic regression, which reduces small-sample bias in maximum likelihood estimation and is particularly relevant given the limited number of outcome events in this cohort. Each predictor was tested in a separate univariable model and in multivariable models adjusted for age, sex, statin use, aspirin use, and neutrophil-to-lymphocyte (N/L) ratio. Immune signatures were modelled as continuous variables (per-SD increases) and as quantiles, with odds ratios (ORs) and 95% confidence intervals (CIs)

reported for each approach. Discriminatory performance was assessed using receiver operating characteristic (ROC) analysis (pROC, R). Pairwise area under the curve (AUC) comparisons were performed using the DeLong nonparametric method for correlated ROC curves, with 95% CIs reported. DEGs in bulk RNA-seq were identified using DESeq2, which models count data with a negative binomial distribution to account for overdispersion in RNA-seq read counts. In scRNA-seq, differential expression was performed using the LIBRA framework with edgeR and likelihood ratio testing, comparing pseudo-bulk transcriptomes between the indicated groups; this pseudo-bulk approach aggregates counts per donor prior to testing to account for within-patient correlations and reduce the inflated false discovery rates inherent to single-cell-level testing. All statistical analyses were performed using R (v4.3.2) or Prism (v10.1.1). *P*-values < .05 were considered statistically significant. Numerical *P*-values are reported throughout. All analyses are exploratory in nature. *P*-values and 95% CIs should therefore be interpreted as descriptive measures of the observed data rather than as confirmatory evidence.

Results

RNA sequencing of peripheral blood mononuclear cells identifies systemic inflammatory transcriptional programmes in coronary artery disease

To assess differences in the abundance and transcriptional regulation of PBMCs from patients with and without CAD, we characterized cryopreserved PBMCs obtained from the LURIC trial. This clinical biomarker trial collected 3316 blood samples between 1997 and 2003.¹² We defined a subpopulation ($n = 47$; $n = 44$ after QC exclusion) comprising three groups: patients without evidence of CAD based on coronary angiography, patients with CAD who experienced a fatal cardiovascular event before 2010, and patients with CAD who survived the 2010 follow-up. Both CAD groups were designed for a nested case-control analysis, ensuring equal proportions of patients matched for hsCRP, NT-proBNP, troponin T, LDL-C, and cystatin C (Figure 1A; Supplementary data online, Figure S1). Patient characteristics are summarized in Table 1. Exclusion based on QC criteria did not affect the distribution of baseline characteristics (see Supplementary data online, Table S1). Mean Friesinger scores reflecting CAD complexity were 8.6 (CAD) and 0.6 (no CAD). Viable mononuclear leukocytes were sorted by flow cytometry using the leukocyte marker CD45 and a viability dye (see Supplementary data online, Figure S2). On average, we retrieved $0.89 \pm 0.35 \times 10^6$ viable PBMCs ($CD45^+ L/D^{neg}$), representing 89% of all frozen input cells, indicating adequate post-thaw viability and recovery. Sorted leukocytes underwent RNA-seq of bulk PBMC samples (bulk RNA-seq) and scRNA-seq. QC metrics were consistent with high-quality transcriptomes. Benchmarking single-cell-derived metrics against fresh and frozen PBMC reference datasets demonstrated recovery of major PBMC lineages comparable to those in reference datasets, without a significant cryopreservation bias (see Supplementary data online, Figure S3). To identify potential clinical contributors of transcriptional variation, we performed PCA across bulk PBMC transcriptomes: CAD status accounted for a substantially larger fraction of explained variance than measured covariates such as sex, age, statin use, aspirin use, or LDL-C (Figure 1B). Consistently, CAD status correlated with multiple principal components, whereas medication use and demographic variables

contributed only modestly to variance (Figure 1C). Mean PC scores demonstrated a clear separation between CAD and no-CAD samples, supporting the presence of a robust transcriptional signal in circulating immune cells linked to CAD (Figure 1D).

Bulk RNA-seq identified 1320 transcripts with a higher expression and 791 transcripts with a lower expression in samples from patients with CAD compared with patients without CAD, as determined by DESeq2 analysis (fold change > 0.5, adjusted *P* < .05). Only a few genes previously identified as risk loci in genome-wide association studies, such as *TGFB1*, *TRIB1*, and *ADAMTS*,¹³ exhibited significantly higher expression in patients with CAD. In contrast, most known risk genes were not significantly regulated (Figure 1E; Supplementary data online, Table S2). We identified enrichment of pathways linked to enhanced cytokine signalling by IL-6, IL-1 β , and TNF- α , as well as pathways related to inflammation, adaptive immunity, T-cell activation, and lipid metabolism in CAD patients (Figure 1F; Data File S1). We validated the upregulation of IL-1, IL-6, and type II interferon signalling programmes in CAD by gene set enrichment analysis (Figure 1G–I). Ranking genes by enrichment revealed that these pathways were driven by a coordinated regulation of multiple immune-related genes rather than isolated transcript-level effects (Figure 1J). Among the top-ranked genes contributing to these signatures were mediators with established relevance for atherosclerosis and vascular inflammation, including *IL1R2*, *TLR4*, *TLR8*, *NLRP3*, and *TNF* within the IL-1-associated programme; *IL6R*, *LPL*, *LGALS9*, and *TGFB1* within the IL-6-responsive programme; and interferon-regulated transcription factors and chemokine receptors such as *GATA3*, *CCR2*, *JAK2*, and *CBL3* within the type II interferon signature. At the patient level, enrichment scores for IL-1 β production, IL-6 signalling, and type II interferon signalling were significantly higher in CAD patients than in those without CAD (Figure 1K), indicating that pro-inflammatory cytokine-responsive programmes are activated in circulating blood cells in CAD patients. Notably, plasma IL-6 levels correlated with IL-6 signalling pathways in leukocytes, whereas *IL6* mRNA in circulating leukocytes did not. This suggests that functional module scores reliably capture leukocyte responses to systemic IL-6 plasma levels (see Supplementary data online, Figure S4).

Coronary artery disease is marked by an expansion of proliferating leukocytes and a contraction of naive adaptive immune cells in peripheral blood

Due to the inherent limitations of bulk RNA-seq, which averages gene expression across mixed cell populations, we conducted scRNA-seq on the same PBMC samples using droplet sequencing (10 $\times$ genomics). We noted that three samples did not meet the pre-specified quality thresholds and were therefore excluded (see Supplementary data online, Tables S3 and S4). After pre-processing, doublet exclusion, and down-sampling to harmonize cell numbers across samples, we included an average of 2489 leukocyte transcriptomes per patient, with an average of 1070 unique genes expressed per cell (see Supplementary data online, Table S5). Datasets were integrated using Seurat's RPCA-based anchor-detection and integration algorithm¹⁴ without apparent batch effects (see Supplementary data online, Figure S5) and annotated by mapping on an established CITE-seq reference atlas.¹⁵ This strategy allowed us to detect 47 clusters of distinct cellular identities including rare cell populations (highest resolution, 'R3')

that we grouped into either states of activation and differentiation (intermediate resolution, 'R2') or principal lineages (lowest resolution, 'R1') (Figure 2A and B; Supplementary data online, Figs S6 and S7; Supplementary data online, Table S6; Data Files S2–S4). Immune cell states were consistently detected across all patients (Figure 2C). While none of the cell clusters across all resolutions were exclusive to either clinical group (Figure 2D), we identified significant CAD-associated shifts in the abundance of multiple immune cell populations (Figure 2E and F; Supplementary data online, Figure S8; Supplementary data online, Table S7): Two of the three populations of NK cells, which resemble the previously identified NK1C (NK-I) and NK1B (NK-III)¹⁶ (see Supplementary data online, Figure S9), and one cluster expressing cell cycle-associated genes, such as *STMN1*, *TYMS*, *MKI67* (termed 'proliferating cells'), were more abundant in the blood of patients with CAD. We replicated a modest increase in flow cytometry-defined NK-cell concentrations in CAD patients across the entire LURIC cohort ($n = 3316$; Supplementary data online, Figure S10). In contrast, naive and intermediate stages of adaptive immune cells, including B cells, CD4⁺ T cells, and CD8⁺ T cells, were less abundant in the blood of CAD patients than in those without CAD. We confirmed these findings in scRNA-seq data from two external cohorts (termed 'Munich'¹⁰ and 'Groningen'¹¹), which were harmonized at the level of cell annotations and integrated into a joint scRNA-seq dataset comprising 55 Munich samples (CAD $n = 38$, no CAD $n = 17$) and 37 Groningen donors (CAD $n = 37$) in addition to the initial set of samples ($n = 44$), for a total of 136 patients (Figure 2G–I; Supplementary data online, Figure S2). In this combined dataset, we validated increases in proliferating cells and reductions in naive CD4⁺ and CD8⁺ T-cell subsets in CAD patients. Interestingly, cell-type abundance changes correlated only weakly with the established inflammatory biomarkers hsCRP, IL-6, and the N/L ratio,⁹ a marker of cardiovascular inflammation (Figure 2J). Although N/L ratio was significantly elevated in our and the full LURIC cohort in CAD patients, its effect size was markedly smaller than that of cell-type abundance changes (see Supplementary data online, Figure S10), suggesting that N/L ratio is independent of other immune cell-derived parameters. In ROC analysis, proliferating cell abundance provided the best discrimination of CAD status among all tested predictors (AUC 0.83; Figure 2K), nominally outperforming hsCRP (AUC 0.63; $P = .028$, 95% CI -0.3821 , -0.0219), with higher point estimates than IL-6 and N/L ratio across all pairwise comparisons (Figure 2K; Supplementary data online, Tables S8 and S9). Finally, we adjusted for potential confounders in the logistic regression model. The abundance of proliferating cells showed a strong association with CAD in the joint cohort (OR 2.25, 95% CI 1.35–4.59) and remained consistent in different models adjusted for age, sex, hsCRP, N/L ratio, as well as aspirin and statin use (Figure 2L; Supplementary data online, Figure S11; Supplementary data online, Table S10; Data Files S5 and S6). Sensitivity analyses by variance partitioning demonstrated that statin and aspirin use accounted for only a minor proportion of transcriptional variance relative to disease status (see Supplementary data online, Figure S12). Consistent with contracting naive adaptive immune compartments, the memory-to-naive T-cell ratio increased by 87% in CD8⁺ T cells ($P = .026$) and showed a similar trend in CD4⁺ T cells in patients with CAD. In conclusion, we show that hallmarks of adaptive immune activation develop in the blood of patients with CAD, independent of systemic inflammation.

Multi-omics factor analysis identifies pan-cellular programmes of antigen recognition, memory formation, and tissue homing in CD4⁺ T-cell populations from coronary artery disease patients

Next, we aimed to identify DEGs per cell type. The cell types exhibiting the highest proportion of transcripts up-regulated in CAD samples were associated with adaptive and innate immunity, including CD4⁺ and CD8⁺, regulatory T (T_{reg}) cells and proliferating cells (Figure 3A). Likewise, T cells and NK cells showed the greatest regulation in interaction strength in cell interaction prediction by CellChat in CAD samples compared with no CAD samples (see Supplementary data online, Figure S13). When integrating cluster abundance and transcriptional perturbation, proliferating cells showed the highest regulation in CAD (Figure 3B). To resolve the functional context of these transcriptional changes, we performed pathway enrichment analysis per cell type: In CD4⁺ T cells, CAD-associated gene regulation was enriched for pathways related to antigen processing and presentation, T-cell activation, differentiation, platelet interaction, and adaptive immune responses (Figure 3C). In contrast, myeloid cells showed selective enrichment of inflammatory and innate immune pathways, including IL-1 β production, IL-6 signalling, TNF superfamily signalling, lipid response, and inflammatory response pathways (Figure 3D). We noticed that specific pathways discovered in bulk RNA-seq, particularly those related to IL-1 β and IL-6 cytokine signalling, were exclusively expressed and regulated in myeloid cells (see Supplementary data online, Figs S14 and S15). These findings are consistent with the notion that the inflammatory axis involving NLRP3-inflammasome activation, IL-1 β secretion, and IL-6 expression results from myeloid cell activation in the atherosclerotic plaque and blood cells.¹⁷ Conversely, pathways involved in adaptive immunity, T-cell activation, lipid response, and antigen processing were regulated only within T-cell clusters (Figure 3C). Interestingly, we did not observe profound changes in transcriptional signatures related to CD4⁺ T-helper types, such as T_H1, T_H17, or T_{reg} (see Supplementary data online, Figure S16). This indicates that distinct effector T_H programmes are detectable within atherosclerotic plaques or in the lymphoid system, but not in the periphery. In proliferating cells, CAD-associated transcriptional programmes were dominated by interferon signalling, leukocyte proliferation, leukocyte transendothelial migration, cytokine signalling, and pathways linked to lipid metabolism and atherosclerosis (Figure 3E). These findings position proliferating cells as a transcriptionally distinct compartment integrating inflammatory, metabolic, and migratory programmes in CAD.

A significant portion of transcripts and pathways regulated in CAD samples was shared across several lineages at R1 resolution and not confined to a specific cell type (see Supplementary data online, Figure S17), suggesting that transcriptional programmes associated with CAD reflect multicellular transcriptional programmes. To further investigate such shared programmes, we utilized MOFA, which employs inter-patient variability to identify key axes of variation across complex datasets, including omics and clinical markers.^{10,18} This framework captures coordinated immune responses, or multicellular

programmes, by reducing high-dimensional data into a limited set of interpretable latent factors, thereby mitigating challenges associated with high feature dimensionality relative to sample size. Overall, 792 observations from 44 individuals spanning 18 molecular and cellular modalities were included, comprising a feature space of 28 516 measured genes. MOFA effectively reduced this complexity and identified 10 latent factors that capture coordinated variation across modalities and immune compartments, with Factors 1–5 explaining, on average, 15.4%, 5.1%, 4.2%, 1.7%, and 1.5% of the total variance, respectively. Gene expression derived from bulk RNA-seq and from T- and B-cell single-cell transcriptomes contributed most to the captured variance across all patients, reaching up to 74.3% for bulk RNA-seq and 41.8% for CD4⁺ T_{EM} cells (Figure 3F; Supplementary data online, Figure S18A). We subsequently examined the correlation between clinical variables and factor values at the patient level and observed a significant enrichment in values of Factors 1, 3, 4, and 5 between patients with CAD. Factor 1 was mostly influenced by features expressed in effector memory (T_{EM}), central memory (T_{CM}), and naive CD4⁺ T cells, with some contribution from bulk RNA-seq (Figure 3G; Supplementary data online, Figure S18B and C). Factors 4 and 5 primarily captured patterns of features derived from bulk RNA sequencing. Features from CD4⁺ T_{EM}, naive CD4⁺, CD8⁺ T_{CM}, and monocytes dominated Factor 3. Factors 1 and 4 demonstrated the most considerable absolute differences in factor values between patients with and without CAD (Figure 3H and I).

Within Factor 1, features corresponding to genes associated with lymphocyte migration, cytoskeleton assembly, homing, and cellular defence responses, such as *LSP1*, *CD52*, *RAC2*, *CFL1*, and *ANXA6*, drove factor values in CAD patients across all four cell types (Figure 3J; Data File S7). Consistently, we found that features defining Factor 1 corresponded to genes that were essential constituents of pathways linked with TCR signalling by nuclear factor of activated T cells and zeta-chain-associated protein kinase 70, T-cell differentiation, interferon, IL-2, and programmed cell death 1 signalling (see Data File S8). All of these are well-established pathways that have been extensively characterized and demonstrated to be pro-atherogenic.^{1,19–22} The association between Factor 1 and TCR signalling is exemplified by *LCK*, which encodes the lymphocyte-specific protein tyrosine kinase and is critical for TCR signalling and several other pathways linked to immune cell activation (Figure 2C; Data File S7). Importantly, *LCK* inhibition attenuates murine atherosclerosis,²³ which argues for a net pro-atherogenic role of antigen recognition. We designated this factor 'TCR signalling and immune activation'.

In contrast, Factor 4 explained only a small proportion of variance but had the second largest difference in factor values between patients with and without CAD. Factor 4 was mainly influenced by features from bulk RNA-seq. The myeloid marker genes *ITGAM* (coding for leukocyte integrin CD11b/CD18), *CTSD* (Cathepsin D), and *LTBR* (Lymphotoxin Beta Receptor) were among the genes with the highest weight on Factor 4 (Figure 3K). Deconvolution of bulk RNA-seq expression by *EnrichR* to assess its cellular constituents validated that Factor 4 predominantly originated from myeloid cells, including monocytes and dendritic cells, after mapping to the Azimuth PBMC atlas (OR of 53.8). Pathway analysis revealed strong associations with pro-atherogenic Toll-like receptor (TLR) signalling,²⁴

phagocytosis, human leukocyte antigen-II-dependent antigen-processing, and cytokine signalling (see Data File S8)—processes that are critical for dendritic cells during the take-up and processing of antigens, migration to lymph nodes, antigen-presentation, and cytokine-mediated instruction of T cells.¹ Factor 4 was also composed of genes that regulate glucose metabolism, such as aldolase-1 (*ALDOA*) and modulators of the extracellular matrix, such as ADAM metalloproteinase domain 9 (*ADAM9*) and tissue inhibitor of metalloproteinases 2 (*TIMP2*). Aldolase-1 is a glycolytic enzyme, which is known to interact with NLR family pyrin domain containing 3 (*NLRP3*) to maintain IL-1 β production in myeloid cells.²⁵ *ADAM9* and *TIMP2* have been shown to modulate extracellular matrix and plaque stability.^{26,27} Because Factor 4 covered functional components that are important for dendritic cell function, we termed this FACTOR 'Antigen-presentation by myeloid cells'. Notably, Factors 1 and 4 were not correlated (see Supplementary data online, Figure S19), suggesting that transcriptional signatures indicative of an activation of the innate myeloid and adaptive T-cell compartment can occur independently of each other.

Factors 3 and 5 predominantly reflected signalling pathways linked to cytokine signalling (see Data File S7). Factor 3—a composite of transcripts from CD4⁺ T cells, CD8⁺ T cells, and monocytes—had negative weights for IL-4, IL-13, and IL-10 signalling (see Supplementary data online, Figure S20), suggesting that Factor 3 reflects a down-regulation of mostly atheroprotective signalling cues: IL-10 is secreted from T_{H2}, T_{reg}, or T_{R1} cells and has potent immunosuppressive functions that protect from atherosclerosis.¹ In addition, atheroprotective vaccination with ApoB peptides is thought to be mediated by IL-10 expression.²⁸ The role of the T_{H2} cytokines IL-4 and -13 is less well characterized, but likely atheroprotective.¹ Factor 5 was mainly influenced by features from bulk RNA-seq data and likely originated from NK and CD8⁺ T_{EM} cells, as determined by deconvolution (OR 11.5). Factor 5 had a positive weight of transcripts important for NF- κ B activation, interferon responses, and interleukin signalling. Importantly, this factor included several transcripts implicated in cell proliferation, such as *CDK5RAP2*, *ACIN1*, and *PRKCH*. Firth's penalized logistic regression analysis demonstrated that Factors 3, 4, and 5 were significantly associated with CAD in univariable and multivariable models adjusted for age, sex, hsCRP, and N/L ratio (Figure 3L; Supplementary data online, Table S11; Data File S5). Factor 1 approached statistical significance ($P = .071$) after adjustment for age and sex (see Supplementary data online, Table S11; Figure 3L). These data indicate that distinct multicellular programmes can be detected in peripheral blood that capture different biological axes of immune cell activation.

T cells from coronary artery disease patients are characterized by enhanced secretion of pro-inflammatory cytokines

To characterize specific cytokine secretion patterns in patients with and without CAD, we used a two-step approach combining computational prediction of cytokine responsiveness with *ex vivo* functional testing (Figure 4A). Initially, we employed huCIRA (human Cytokine Response Analyzer), a framework that infers cytokine-responsive transcriptional states from bulk and scRNA-seq data using reference perturbation signatures. This

approach revealed an enrichment of IL-1 β , IL-2, IL-15, IFN- γ , TNF, and CD40L signalling in CAD samples, whereas IL-10, TSLP, and IL-32 β were relatively enriched in no-CAD samples (Figure 4B and C). CAD-associated cytokine response signatures were not restricted to a single lineage but strongly enriched in myeloid, proliferating, and activated T cells (Figure 4D). IL-1 β -associated programmes were most pronounced in monocytes and dendritic cells, whereas IFN- γ - and CD40L-associated programmes were detectable in T-cell populations (Figure 4E). For a functional validation, we stimulated CD4 $^+$ T cells with staphylococcal enterotoxin B for 6 h and quantified intracellular cytokine secretion. We observed consistently stronger responses in CD4 $^+$ T cells from patients with CAD, including CD40L, TNF, IL-4, and IFN- γ (Figure 4F–I). Consistently, expression of the activation marker CD69 was increased in CAD-derived CD4 $^+$ T cells (see [Supplementary data online, Figure S21](#)). In contrast, no significant differences in IL-10 or IL-17 production were observed between the groups (see [Supplementary data online, Figure S21](#)). Collectively, we functionally validated pronounced inflammatory cytokine secretion by CD4 $^+$ T cells from CAD patients.

Paired single-cell and T-cell receptor repertoire analysis reveals increased clonal expansion of circulating T cells in coronary artery disease

Because T-cell activation may be driven by antigen-specific responses, we aimed to analyse T-cell clonality. Therefore, we paired our scRNA-seq datasets with TCR α - and β -chain sequencing (Figure 5A). This approach enabled simultaneous assessment of transcriptional states and clonal architecture at single-cell resolution. Shared TCR clonotypes were detected across both clinical groups, consistent with shared TRA and TRB chains. Clonotypes shared exclusively among CAD patients were more common than clonotypes shared exclusively among no-CAD patients (Figure 5B). Conserved clonotypes among paired TRA–TRB sequences were not detected, indicating that increased clonal sharing in CAD is driven by recurrent single-chain usage rather than by fully conserved TCR α/β receptors, consistent with previous human repertoire studies.²⁹ To connect clonal expansion with transcriptional identity, we mapped TCR clonotypes onto the UMAP embedding of T-cell subclusters derived from scRNA-seq data (Figure 5C). Expanded and hyperexpanded clonotypes mainly appeared in effector and memory T-cell populations, such as CD4 $^+$ and CD8 $^+$ effector memory subsets. In contrast, naive T-cell clusters mostly consisted of non-expanded or singleton clonotypes (Figure 5D). When ranking T-cell subclusters by hyperexpansion level, several effector memory groups, including CCR6 $^+$ CD4 $^+$ T_{EM}, LEF1 $^+$ mixed T_{CM}, and KLRB1 $^+$ CD8 $^+$ T_{EM} subsets, were enriched for highly expanded clones in CAD, reaching up to 9.4-fold more clonal expansion in CCR6 $^+$ CD4 $^+$ T_{EM} (Figure 5E). Consistent with these findings, diversity analysis showed a significant decrease in TCR repertoire diversity in CAD: the Chao1 diversity index was lower in CAD patients for TRA chains and combined TRA/TRB clonotypes, indicating reduced clonal richness (Figure 5F). Together, these data indicate that CAD is associated with a restriction of the circulating TCR repertoire likely caused by the preferential expansion of selected T-cell clones. Importantly, these findings support a model in which circulating T cells in CAD clonally expand in response to sustained antigen exposure.

Detection of transcriptional programmes and cell states that associate with cardiovascular death and poor outcomes after acute coronary syndrome

Next, we investigated whether scRNA-seq-derived immune readouts associate with cardiovascular outcomes using a step-wise exploratory framework. Our cohort of CAD patients was designed as a case–control study, with cases defined as cardiovascular death ($n = 14$) and controls as survivors ($n = 15$) at the 2010 follow-up. During an extended observation period, we detected several deaths and crossovers by 2017 that were not initially anticipated (Figure 6A). Cases and controls were matched using nearest-neighbor matching based on hsCRP, NT-proBNP, troponin T, LDL-C, and cystatin C to account for traditional risk factors and established biomarkers known to predict cardiovascular death. We confirmed that the matching variables were balanced between the two groups; only glycated haemoglobin and age tended to be lower in cases (see [Supplementary data online, Table S12](#)).

First, we asked whether the relative abundance of immune cells across cellular resolutions R1–R3 in our scRNA-seq data allows us to discriminate survival and cardiovascular mortality at the 2010 follow-up (see [Data File S9](#)): Among all cell populations (see [Supplementary data online, Figure S22](#)), no single lineage showed a robust or consistent difference between cases and controls (see [Supplementary data online, Figure S22; Data File S10](#)). Second, a recent study indicated that MOFA-derived factors may predict ischaemic cardiomyopathy following ACS.¹⁰ Despite the small sample size and limited statistical power in this outcome analysis, we ranked MOFA-derived factors based on factor weights between both groups (Figure 6B): Factor 4 ('antigen-presentation by myeloid cells') displayed the largest absolute factor difference between the two groups (absolute difference: 0.68, $P = .042$) and showed a borderline significant association with cardiovascular death in Firth's penalized logistic regression (unadjusted: OR 2.22, 95% CI 1.01–6.10, $P = .046$; adjusted for sex and age: OR 2.24, 95% CI 0.98–6.69, $P = .058$, [Data File S10](#)). Factors 1, 3, and 5, which effectively discriminated CAD status, did not show a significant correlation with cardiovascular death. This exploratory analysis supports the hypothesis that some transcriptional programmes capture disease initiation rather than disease progression, a phenomenon also observed for LDL-C, which is less effective in risk prediction in established disease than hsCRP.²

Third, we evaluated whether cell type-specific pathways, which emphasize intracellular rather than multicellular transcriptional regulation, associate with outcomes (Figure 6C). At the pathway level, we detected several immune programmes with a differential enrichment in death cases involving antigen presentation, innate immune activation, and adaptive lymphocyte responses (Figure 6C; [Supplementary data online, Table S13](#)). Two pathways ['antigen processing and presentation' (GO:0019884) in plasma blasts and 'adaptive immune responses' (GO:0002250) in proliferating cells] were more strongly enriched in cells from dead patients than other markers in logistic regression modelling (Figure 6D). In contrast, hsCRP, plasma IL-6, and N/L ratio were equally distributed across groups (Figure 6E). In ROC analysis, the GO:0002250 enrichment score in proliferating cells substantially outperformed

established clinical markers, including hsCRP ($P = .0008$, 95% CI -0.6814 , -0.1797) and IL-6 ($P = .004$, 95% CI -0.5357 , -0.1032), for 10-year cardiovascular mortality prediction, and retained the highest discriminatory performance in the extended follow-up (AUC 0.86; [Figure 6F](#); [Supplementary data online, Tables S14 and S15](#)). Notably, the abundance of proliferating and IFN $^+$ cells correlated only weakly with hsCRP, but not with IL-6 (see [Supplementary data online, Figure S23](#)), suggesting that inflammatory biomarkers are not reliable surrogates for cellular proliferation. These findings indicate that cell-resolved immune programmes capture dimensions of immune activation that are not adequately reflected by systemic inflammation.

Fourth, we compared DEGs in proliferating cells from CAD patients who had deceased by 2010 with event-free CAD controls. We detected an enrichment of interferon-stimulated and cytotoxic lymphocyte genes, including *IFIT2*, *IFIT3*, *TNFRSF13B*, *IFIT1*, *GADD45G*, *EOMES*, *XCL1*, *XCL2*, *OASL*, and *CD38* ([Figure 6G](#)). Pathway analysis linked this signature to programmes of adaptive immune responses, lymphocyte-mediated immunity, interferon signalling, chromatin organization, epigenetic regulation, and cellular senescence (see [Supplementary data online, Figure S24](#)), indicating a coordinated immune response in these cells. In an exploratory Kaplan–Meier analysis, both the abundance of proliferating cells (log-rank $\chi^2(1) = 7.72$, $P = .035$) and Factor 4 (log-rank $\chi^2(1) = 11.57$, $P = .0003$) discriminated survival trajectories across the extended follow-up to 2017 in the full cohort ([Figure 6H](#)).

Because of the consistent association of proliferating cells (at the level of abundance and transcriptional regulation) with CAD disease status and outcomes, we next assessed proliferating immune cell dynamics in an independent validation cohort of patients presenting with ACS¹⁰ ([Figure 6I](#)). Mapping of scRNA-seq datasets in this cohort to our annotations allowed us to specifically detect the rare population of proliferating cells, which increased dynamically after ACS during the initial 5–8 days ([Figure 6J](#)). Patients with a low abundance of proliferating cells (below the median) had a favourable outcome, defined by a constant left ventricular ejection fraction (LVEF). In contrast, all patients with high abundance (above the median) experienced a decline of LVEF (bad outcome, [Figure 6K](#)). Although these findings are consistent with the cross-sectional observations in LURIC, they should be interpreted with caution given the different clinical study designs.

Taken together, these data establish that transcriptional programmes of adaptive immunity, interferon signalling, and proliferation identify immune states associated with adverse cardiovascular outcomes and could therefore capture a finer granularity of risk dimensions undetectable by traditional stratification tools.

Local and peripheral cellular proliferation is a feature of coronary atherosclerosis

The transcriptional signature of proliferation may interfere with cell-specific signatures, thereby masking distinct cellular components within this cluster. Therefore, we re-clustered the population of proliferating cells and found that it consisted of NK cells, activated CD4 $^+$ and CD8 $^+$ T cells, and a smaller fraction of plasma blasts ([Figure 7A](#)). In a differential abundance analysis by MiloR, most of their cellular subpopulations could be detected in neighbourhoods that were enriched in CAD patients, suggesting that all constituents of proliferating cells increased in the context of

CAD ([Figure 7B](#)). This led to the hypothesis that cellular proliferation in blood may be driven by the same contextual factors as in atherosclerotic plaques itself. Interestingly, immune cells from the blood of CAD patients had much greater similarity to lesional immune cells than immune cells from the blood of control patients, as assessed by cross-compartment alignment with Spearman correlations between patient-level pseudobulk profiles of proliferating cells from blood and plaque ([Figure 7C](#)). We wondered whether cell populations up-regulated in CAD patients, including proliferating and NK cells, would resemble lesional cells in human atherosclerotic plaques. Reference mapping to our human carotid plaque single-cell atlas⁵ indeed identified cell states transcriptionally overlapping with proliferating and NK cells ([Figure 7D](#)). Finally, we validated *in situ* proliferation in human carotid plaques by mass cytometry and spatial imaging ([Figure 7E](#)). Mass cytometry detected Ki67 $^+$ fractions in CD4 $^+$ T cells (33.9%), CD8 $^+$ T cells (21%), and CD56 $^+$ NK cells (24.8%) ([Figure 7F and G](#)). In addition, spatial CODEX imaging demonstrated that proliferating CD4 $^+$, CD8 $^+$, and NK cells were present in distinct regions of the human atherosclerotic plaque, including shoulder and cap-associated areas, rather than being confined to discrete lymphoid aggregates ([Figure 7H](#)). Together, these data show that cellular proliferation is a common feature of patients with CAD, both in circulating immune cells and locally in atherosclerotic plaques.

Discussion

This proof-of-concept study provides evidence for changes in the peripheral immune cell landscape, its transcriptional programmes, and associations with long-term cardiovascular mortality in patients with CAD. We show that particularly cellular proliferation appears to be a prominent feature of the adaptive immunity in human atherosclerosis, detectable both systemically and locally within the plaque. This feature is independent of myeloid-driven systemic inflammation involving IL-6 and hsCRP and, therefore, may reflect a class of immune signatures associated with disease progression ([Structured Graphical Abstract](#)).

The acknowledgment of inflammation as a causal factor in developing and progressing atherosclerosis illustrates a paradigm shift in cardiovascular medicine: While established care pathways focus on LDL-C and other modifiable risk factors, it is now clear that chronic low-grade inflammation, driven by pro-inflammatory pathways such as those mediated by IL-1 β and IL-6, promotes atherosclerosis.¹⁷ Inflammatory biomarkers have drawn attention for their potential to enhance long-term cardiovascular risk prediction, which is best illustrated by hsCRP.³⁰ We replicate this inflammatory response by detecting several instances of myeloid-driven inflammation, including IL-1 and IL-6 signalling, by bulk RNA-seq. Similarly, anti-inflammatory therapies have been shown to reduce cardiovascular events in patients with otherwise well-managed risk factors.¹⁷ However, the indiscriminate use of anti-inflammatory therapies without proper risk stratification is ineffective; the CANTOS trial demonstrated that the cardioprotective effects of canakinumab were predictable from the decrease in hsCRP levels under therapy.³¹ Therefore, effective inflammation management must include biomarkers to identify patients at risk and monitor therapeutic efficacy.³² On the other hand, the utility of hsCRP as a marker of low-grade inflammation is limited due to its low specificity. Our observation that

specific adaptive immune programmes, cell types, and cytokines are regulated independently of hsCRP argues for the existence of pro-inflammatory responses that are not reflected by biomarkers of the IL-1 β –IL-6–hsCRP axis. Consequently, hsCRP may not be a reliable marker of cardiovascular inflammation.

The central finding of our study is the discovery of multicellular programmes in several adaptive immune cell lineages, including CD4⁺ and CD8⁺ T cells that are associated with TCR signalling, cell activation, cell differentiation, and cytokine secretion. These programmes are best presented by Factor 1 ('TCR signalling and immune activation') that originates from central-memory and effector-memory T cells and increases in patients with CAD—an effect that remains significant even after adjusting for markers of myeloid cell inflammation, such as hsCRP and the N/L ratio. We observed the same patterns of T-cell engagement in two independent experiments: First, we detected a higher functional capacity of pro-inflammatory cytokine secretion in T cells from patients with CAD involving TNF α , IFN- γ , and CD40L. Second, we detected oligoclonal enrichment of T cells based on parallel TCR α and TCR β DNA sequencing. These findings are well in accord with increased T-cell activation, proliferation, and pathogenic differentiation. The existence of a proliferating cell population, which has largely remained undetected by most targeted immunophenotyping approaches in the past, represents a phenotypically distinct population comprising T cells, NK cells, and plasmablasts. Cells in this cluster are highly activated, rich in interferon signalling, and therefore likely represent an advanced stage of cell activation. In addition to these more T-cell-centred findings, we observed another class of regulation that is best captured by Factor 4: signalling cascades captured by this factor are linked to TLR signalling, phagocytosis, MHC-II presentation, and interactions with adaptive immune cells. Although this factor likely originates from myeloid cells, it should not be confused with IL-1 β and IL-6 signalling, as we observed independence from hsCRP. Together, these findings suggest that major detectable regulatory axes in this dataset are confined to mechanisms of adaptive immunity and interaction with antigen-presenting cells in human blood. While the participation of adaptive immune cells in atherosclerosis has been demonstrated by numerous observations in mice, this report is among the first studies to characterize multicellular regulatory programmes in human blood associated with CAD. Our exploratory finding that some of these signature factors and cell types are associated with cardiovascular death in our case-control scenario and link to cardiovascular outcomes in the extended follow-up period and the validation cohort suggests that adaptive immunity may be an important, yet underrated, causal factor in cardiovascular disease progression.

The interplay of immune cells in atherosclerosis involves cells in the plaque, lymphoid organs, and peripheral blood. Different subsets of cell types often exhibit opposing outcomes, such as pro- and anti-inflammatory roles^{1,33}: for example, T_{reg} cells are generally protective, while conventional T cells can be pathogenic, even though they are activated by the same antigens through similar mechanistic pathways, partially by binding to the exact same TCR sequences.³⁴ Additionally, T cells are highly adaptable, switching from protective to pathogenic phenotypes in atherosclerotic plaques and lymphoid organs.³⁵ Although our data do not allow to infer the definitive function of cellular clusters and regulated pathways, we observed cell-type specific regulation at the level of differentiation states and memory status, as exemplified by

naive and memory T cells or naive/intermediate and memory B cells/plasmablasts. Their abundance is regulated in opposing directions in patients with and without CAD. Our data suggest a stronger memory phenotype, particularly in CD4⁺, CD8⁺ T cells, and B cells, in the blood of CAD patients (and, conversely, an increase in naive subsets in people without CAD). Notably, we found no associations with T_H-effector types, known to be distinctly regulated in single antigen-specific T cells within tissue,³³ even when applying T_H-module scores optimized for scRNA-seq data sets. Instead, we identified global patterns of regulation encompassing TCR signalling pathways, a clonal enrichment of TCR clones in TCR sequencing, memory formation, cellular activation across pro- and anti-inflammatory subsets, and cellular proliferation, which are hallmarks of antigen-specificity, regardless of the actual antigen. Because proliferating cells contained a mixed population of T cells, NK cells, and plasmablasts, this cluster may contain antigen-experienced cells that proliferate as result of antigen-recognition and co-stimulation. Activation and enhanced cellular proliferation are observed in antigen-specific lymphocytes, which proliferate and undergo clonal expansion in response to antigen recognition.¹ Although antigen-independent proliferation has been reported,³⁶ the discovery of atherosclerosis-associated autoantigens³⁵ primarily supports the idea of an autoimmune response against self-antigens in CAD patients. We propose that this response may be traceable by transcriptional signatures of proliferation. While our findings are in accord with previous reports on T-cell phenotypes in human and murine atherosclerotic plaques, which proposed a strong auto-reactive component of adaptive immunity,⁶ we cannot exclude the existence of anti-non-self T cells or an unspecific clonal enrichment of TCRs as potential confounders. The increase in type 2 interferon signatures at the level of proliferating cells and bulk RNA-seq, as well as the involvement of NK cells, is not uncommon in infections with intracellular pathogens. The OPTICO-ACS study has demonstrated a correlation of T-cell enrichment (abundance locally divided by abundance in peripheral blood) in coronary blood at the site of atherosclerotic plaque erosion.⁷ This may indicate a specific role of T cells in acute coronary syndrome, although it remains unknown whether the shift in T-cell ratios is a cause or a complication of plaque inflammation.

It remains uncertain whether biomarkers exist that fully capture the immune-associated risk in cardiovascular disease. Titres of antibodies³⁷ directed against known auto-antigens and the enumeration of antigen-specific immune cells³⁸ may serve as parameters to quantify immune activation. However, the entirety of cardiovascular (auto-)antigens and tools that allow for high-throughput screening still need to be defined, despite first promising attempts to detect ApoB-specific autoimmunity.^{35,38–40} Immunophenotyping, aimed at creating a comprehensive snapshot of the immune cell landscape that incorporates states of cellular activation and differentiation, may overcome these limitations by identifying broader characteristics of immune regulation.^{41,42} Traditional techniques, often using flow cytometry, introduce a relevant bias due to experimental design constraints, such as pre-selected parameters used for phenotyping. In contrast, transcriptome-wide methodologies generate comprehensive maps of immune cell states and phenotypes without preconceived assumptions and provide detailed insights into transcriptional regulation. Down the same line, spatial transcriptomics have identified

transcriptional signatures in human atherosclerotic plaques associated with plaque rupture.⁴³ The integration of various axes of immune regulation (including numerical vs multicellular programmes vs monocellular pathways and systemic cytokines) poses significant challenges for interpretability. For example, the relationship between numeric and transcriptional regulation remains unclear. Effector T cells typically proliferate in response to appropriate signals through the T-cell receptor, leading to a strong correlation between numeric and transcriptional changes, as observed in proliferating cells. In contrast, T_{regs} may not proliferate to a significant extent but can exert their effects through increased cytokine expression or alternative mechanisms. Another example is the positive association of pathways related to plasmablast activation in CAD, despite the absence of notable numeric regulation of this cell type. Our findings indicate that the correlation between numeric and transcriptomic changes is weak and does not permit definitive conclusions.

While our study is the first to link long-term outcomes to single-cell transcriptomes in patients with stable chronic coronary syndromes, scRNA-seq has previously been conducted in the context of ACS.^{10,11} These earlier studies raise the question whether the transcriptional programmes identified earlier fundamentally differ from those seen in other clinical settings. Pekayvaz *et al.*¹⁰ applied MOFA to patients after ACS and found multicellular immune signatures, originating from monocytes, NK and T cells that were associated with short-term treatment outcomes assessed by LVEF. We used data sets from patients with and without CAD as a validation cohort. While the enrichment of our MOFA-defined factors could not be benchmarked in this study due to inconsistent multiomic readouts used as input to MOFA, the regulation of most principal leukocyte subsets could be replicated. A notable difference remained the involvement of NK cells in both clinical scenarios. The study by Pekayvaz *et al.* indicates that one MOFA factor termed 'Integrative ACS repair' was comprised by NK cells with decreased cytotoxic activity and correlated with improved myocardial repair, suggesting that a low cytotoxicity of NK cells may be protective and aid myocardial repair after ACS. In contrast, our study shows that NK cells with an anticipated higher cytotoxicity are overrepresented in CAD. Accordingly, the higher number in CAD patients could not be validated in patients at high risk of myocardial infarction. Proliferating cells, however, were associated with adverse outcomes in both studies, suggesting that either cellular proliferation may precede myocardial infarction and cardiac events, or that it reflects an acute, immunity-driven state of acute responsiveness.

Overall, our findings encourage the systematic investigation of transcriptional biomarkers, including their validation in larger, prospective cohorts. Ideally, these validation studies would utilize standardized immunophenotyping protocols to enable high-throughput testing in routine clinical settings. Additionally, it should be examined whether complex multicellular programmes or pathways can be reduced to relevant core motifs suitable for large-scale testing, such as quantification of MKI67⁺ proliferating T and NK cells by flow cytometry or cytokine secretion patterns measured by proximity extension assays (e.g. by Olink). It will also be essential to identify distinct transcriptional markers that are specific for certain clinical outcomes, such as for myocardial healing or vascular inflammation.⁴⁴ Transcriptional or cellular

biomarkers should have the ability to detect a reversal of pathogenic immune phenotypes by immunomodulatory therapies. A study by Zhao *et al.*⁴⁵ recently demonstrated that low-dose IL-2 in the LILACS trial led to a dose-dependent clonal expansion of T_{reg} cells and an induction of T_{reg} transcriptional pathways, such as these defined by mammalian target of rapamycin (mTOR) signalling, hypoxia, oxidative metabolism, and IL-2-STAT5 signalling, trackable by scRNA-seq, indicating that immunomodulation holds the potential to partially reverse immune deviation. Our study supports further exploration of transcriptomic tools for risk stratification and monitoring of immune activation through transcriptional tools that guide personalized therapy decisions in addition to the existing biomarkers IL-6 and hsCRP.

Limitations

This study was designed as a hypothesis-generating, proof-of-concept study to demonstrate that transcriptome-resolved immune programmes in peripheral blood associate with CAD status and adverse outcomes, but several limitations apply by design. First, primary outcome analyses were performed in a small, nested case-control subset of LURIC (*n* = 44). Although adjudicated long-term follow-up is a major strength, the modest sample size limits power and precision. To improve robustness, we integrated and validated CAD-associated compositional and transcriptional features in two independent cohorts (a total of 136 patients) and reproduced key findings, including the expansion of proliferating immune cells and the contraction of naive T-cell compartments. However, a comparable 20-year mortality validation was not available due to limited follow-up in the external cohorts. In the ACS cohort from Munich, proliferating immune cells increased after an acute event and were associated with worse short-term recovery, providing important biological consistency. Still, larger prospective studies with long-term outcomes are needed to define thresholds and incremental predictive value. Second, PBMCs were cryopreserved for long periods. Although cryopreservation in liquid nitrogen is the accepted gold standard, it can, in principle, reduce recovery and introduce stress-related transcriptional effects or selectively deplete fragile subsets. We applied stringent viability criteria and benchmarking, detected no systematic loss of major lineages, and obtained even better similarity indexes to fresh cells than the current gold-standard dataset from Hao *et al.*¹⁵ but we cannot exclude effects on rare or activation-sensitive states. Third, despite matching and adjustment, residual confounding may remain. CAD groups differed in age and medication exposure, and therapies may influence immune states. Variance partition analyses suggested that medications and covariates explained little transcriptional variance relative to CAD status, and key associations remained robust after adjustment for major clinical and inflammatory variables in the validation cohorts. However, unmeasured confounders and incomplete characterization of treatments may remain. Finally, the observational, largely cross-sectional design limits causal inference. Cytokine-response programmes likely reflect responsiveness to circulating cues rather than cell-intrinsic cytokine production; TCR data indicate antigen experience without pinpointing specificity; and mapping circulating states to plaque programmes does not establish cell trafficking or tissue origin. Resolving these questions will require longitudinal sampling and more functional and mechanistic characterization. Overall, these

constraints are typical of contemporary human single-cell cardiovascular studies and do not undermine the central finding that peripheral immune transcriptomes capture reproducible, biologically grounded multicellular programmes linked to CAD and cardiovascular risk. Importantly, the convergence of transcriptional, clonal, functional, and tissue-level evidence supports the biological relevance of the identified immune programmes despite our exploratory design and weak statistical power due to small sample sizes.

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Supplementary data

Supplementary data are available at [European Heart Journal](#) online.

Declarations

Disclosure of Interest

All authors declare no disclosure of interest for this contribution.

Data availability

All data used in this study will be made available in the Gene Expression Omnibus (GEO) for researchers interested in reproducing the results or replicating the procedures. The single-cell RNA sequencing data are available at the Gene Expression Omnibus under accession number GSE289422, and the bulk RNA-seq data can be accessed via the Sequence Read Archive (SRA) under accession number PRJNA1244274.

Ethical Approval

The study was performed according to the declaration of Helsinki and approved by the local Institutional review boards [ethics committee of the Landesärztekammer Rheinland-Pfalz (approval number 837.255.97(1394))].

Pre-registered Clinical Trial Number

The pre-registered clinical trial number is 0001-4016.

References

1. Wolf D, Ley K. Immunity and inflammation in atherosclerosis. *Circ Res* 2019; **124**:315–27. <https://doi.org/10.1161/CIRCRESAHA.118.313591>
2. Ridker PM, Bhatt DL, Pradhan AD, Glynn RJ, MacFadyen JG, Nissen SE, et al. Inflammation and cholesterol as predictors of cardiovascular events among patients receiving statin therapy: a collaborative analysis of three randomised trials. *Lancet* 2023; **401**:1293–301. [https://doi.org/10.1016/S0140-6736\(23\)00215-5](https://doi.org/10.1016/S0140-6736(23)00215-5)
3. Nidorf SM, Fiolet ATL, Mosterd A, Eikelboom JW, Schut A, Opstal TSJ, et al. Colchicine in patients with chronic coronary disease. *N Engl J Med* 2020; **383**:1838–47. <https://doi.org/10.1056/NEJMoa2021372>
4. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med* 2017; **377**:1119–31. <https://doi.org/10.1056/NEJMoa1707914>
5. Horstmann H, Michel NA, Sheng X, Hansen S, Lindau A, Pfeil K, et al. Cross-species single-cell RNA sequencing reveals divergent phenotypes and activation states of adaptive immunity in human carotid and experimental murine atherosclerosis. *Cardiovasc Res* 2024; **120**:1713–26. <https://doi.org/10.1093/cvr/cvae154>
6. Marchini T, Mitre LS, Wolf D. Inflammatory cell recruitment in cardiovascular disease. *Front Cell Dev Biol* 2021; **9**:635527. <https://doi.org/10.3389/fcell.2021.635527>
7. Leistner DM, Krankel N, Meteva D, Abdelwahed YS, Seppelt C, Stahl BE, et al. Differential immunological signature at the culprit site distinguishes acute coronary syndrome with intact from acute coronary syndrome with ruptured fibrous cap: results from the prospective translational OPTICO-ACS study. *Eur Heart J* 2020; **41**:3549–60. <https://doi.org/10.1093/eurheartj/ehaa703>
8. Hamers AAJ, Dinh HQ, Thomas GD, Marcovecchio P, Blatchley A, Nakao CS, et al. Human monocyte heterogeneity as revealed by high-dimensional mass cytometry. *Arterioscler Thromb Vasc Biol* 2019; **39**:25–36. <https://doi.org/10.1161/ATVBAHA.118.311022>
9. Adamstein NH, MacFadyen JG, Rose LM, Glynn RJ, Dey AK, Libby P, et al. The neutrophil-lymphocyte ratio and incident atherosclerotic events: analyses from five contemporary randomized trials. *Eur Heart J* 2021; **42**:896–903. <https://doi.org/10.1093/eurheartj/ehaa1034>
10. Pekayvaz K, Losert C, Knottenberg V, Gold C, van Blokland IV, Oelen R, et al. Multiomic analyses uncover immunological signatures in acute and chronic coronary syndromes. *Nat Med* 2024; **30**:1696–710. <https://doi.org/10.1038/s41591-024-02953-4>
11. van Blokland IV, Oelen R, Groot HE, Benjamins JW, Pekayvaz K, Losert C, et al. Single-cell dissection of the immune response after acute myocardial infarction. *Circ Genom Precis Med* 2024; **17**:e004374. <https://doi.org/10.1161/CIRCGEN.123.004374>
12. Winkelmann BR, Marz W, Boehm BO, Zotz R, Hager J, Hellstern P, et al. Rationale and design of the LURIC study—a resource for functional genomics, pharmacogenomics and long-term prognosis of cardiovascular disease. *Pharmacogenomics* 2001; **2**:S1–73. <https://doi.org/10.1517/14622416.2.1.S1>
13. Aragam KG, Jiang T, Goel A, Kanoni S, Wolford BN, Atri DS, et al. Discovery and systematic characterization of risk variants and genes for coronary artery disease in over a million participants. *Nat Genet* 2022; **54**:1803–15. <https://doi.org/10.1038/s41588-022-01233-6>
14. Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM 3rd, et al. Comprehensive integration of single-cell data. *Cell* 2019; **177**:1888–902 e1821. <https://doi.org/10.1016/j.cell.2019.05.031>
15. Hao Y, Hao S, Andersen-Nissen E, Mauck WM 3rd, Zheng S, Butler A, et al. Integrated analysis of multimodal single-cell data. *Cell* 2021; **184**:3573–87 e3529. <https://doi.org/10.1016/j.cell.2021.04.048>
16. Rebuffet L, Melsen JE, Escaliere B, Basurto-Lozada D, Bhandoola A, Björkstam NK, et al. High-dimensional single-cell analysis of human natural killer cell heterogeneity. *Nat Immunol* 2024; **25**:1474–88. <https://doi.org/10.1038/s41590-024-01883-0>
17. Soehnlein O, Libby P. Targeting inflammation in atherosclerosis - from experimental insights to the clinic. *Nat Rev Drug Discov* 2021; **20**:589–610. <https://doi.org/10.1038/s41573-021-00198-1>

18. Argelaguet R, Velten B, Arnol D, Dietrich S, Zenz T, Marioni JC, *et al.* Multi-omics factor analysis - a framework for unsupervised integration of multi-omics data sets. *Mol Syst Biol* 2018;**14**:e8124. <https://doi.org/10.15252/msb.20178124>
19. Fernandez DM, Rahman AH, Fernandez NF, Chudnovskiy A, Amir ED, Amadori L, *et al.* Single-cell immune landscape of human atherosclerotic plaques. *Nat Med* 2019;**25**:1576–88. <https://doi.org/10.1038/s41591-019-0590-4>
20. Goossens P, Gijbels MJ, Zernecke A, Eijgelaar W, Vergouwe MN, van der Made I, *et al.* Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions. *Cell Metab* 2010;**12**:142–53. <https://doi.org/10.1016/j.cmet.2010.06.008>
21. Barcia Duran JG, Das D, Gildea M, Amadori L, Gourvest M, Kaur R, *et al.* Immune checkpoint landscape of human atherosclerosis and influence of cardiometabolic factors. *Nat Cardiovasc Res* 2024;**3**:1482–502. <https://doi.org/10.1038/s44161-024-00563-4>
22. Du M, Yang L, Liu B, Yang L, Mao X, Liang M, *et al.* Inhibition of NFAT suppresses foam cell formation and the development of diet-induced atherosclerosis. *FASEB J* 2021;**35**:e21951. <https://doi.org/10.1096/fj.202100947R>
23. Liu J, Guo Z, Zhang Y, Wu T, Ma Y, Lai W, *et al.* LCK inhibitor attenuates atherosclerosis in ApoE(-/-) mice via regulating T cell differentiation and reverse cholesterol transport. *J Mol Cell Cardiol* 2020;**139**:87–97. <https://doi.org/10.1016/j.yjmcc.2020.01.003>
24. Jin M, Fang J, Wang JJ, Shao X, Xu SW, Liu PQ, *et al.* Regulation of toll-like receptor (TLR) signaling pathways in atherosclerosis: from mechanisms to targeted therapeutics. *Acta Pharmacol Sin* 2023;**44**:2358–75. <https://doi.org/10.1038/s41401-023-01123-5>
25. Bai D, Du J, Bu X, Cao W, Sun T, Zhao J, *et al.* ALDOA maintains NLRP3 inflammasome activation by controlling AMPK activation. *Autophagy* 2022;**18**:1673–93. <https://doi.org/10.1080/15548627.2021.1997051>
26. Brauningner H, Kruger S, Bacmeister L, Nystrom A, Eyerich K, Westermann D, *et al.* Matrix metalloproteinases in coronary artery disease and myocardial infarction. *Basic Res Cardiol* 2023;**118**:18. <https://doi.org/10.1007/s00395-023-00987-2>
27. Kremastiotis G, Handa I, Jackson C, George S, Johnson J. Disparate effects of MMP and TIMP modulation on coronary atherosclerosis and associated myocardial fibrosis. *Sci Rep* 2021;**11**:23081. <https://doi.org/10.1038/s41598-021-02508-4>
28. Kimura T, Tse K, McArdle S, Gerhardt T, Miller J, Mikulski Z, *et al.* Atheroprotective vaccination with MHC-II-restricted ApoB peptides induces peritoneal IL-10-producing CD4 T cells. *Am J Physiol Heart Circ Physiol* 2017;**312**:H781–90. <https://doi.org/10.1152/ajpheart.00798.2016>
29. Depuydt MAC, Schaftenaar FH, Prange KHM, Boltjes A, Hemme E, Delfos L, *et al.* Single-cell T cell receptor sequencing of paired human atherosclerotic plaques and blood reveals autoimmune-like features of expanded effector T cells. *Nat Cardiovasc Res* 2023;**2**:112–25. <https://doi.org/10.1038/s44161-022-00208-4>
30. Ridker PM, Moorthy MV, Cook NR, Rifai N, Lee IM, Buring JE. Inflammation, cholesterol, lipoprotein(a), and 30-year cardiovascular outcomes in women. *N Engl J Med* 2024;**391**:2087–97. <https://doi.org/10.1056/NEJMoA2405182>
31. Ridker PM, MacFadyen JG, Everett BM, Libby P, Thuren T, Glynn RJ, *et al.* Relationship of C-reactive protein reduction to cardiovascular event reduction following treatment with canakinumab: a secondary analysis from the CANTOS randomised controlled trial. *Lancet* 2018;**391**:319–28. [https://doi.org/10.1016/S0140-6736\(17\)32814-3](https://doi.org/10.1016/S0140-6736(17)32814-3)
32. Bugger H, Zirlik A. Anti-inflammatory strategies in atherosclerosis. *Hamostaseologie* 2021;**41**:433–42. <https://doi.org/10.1055/a-1661-0020>
33. Saigusa R, Winkels H, Ley K. T cell subsets and functions in atherosclerosis. *Nat Rev Cardiol* 2020;**17**:387–401. <https://doi.org/10.1038/s41569-020-0352-5>
34. Ko A, Watanabe M, Nguyen T, Shi A, Achour A, Zhang B, *et al.* TCR repertoires of thymic conventional and regulatory T cells: identification and characterization of both unique and shared TCR sequences. *J Immunol* 2020;**204**:858–67. <https://doi.org/10.4049/jimmunol.1901006>
35. Wolf D, Gerhardt T, Winkels H, Michel NA, Pramod AB, Ghosheh Y, *et al.* Pathogenic autoimmunity in atherosclerosis evolves from initially protective apolipoprotein B100-reactive CD4(+) T-regulatory cells. *Circulation* 2020;**142**:1279–93. <https://doi.org/10.1161/CIRCULATIONAHA.119.042863>
36. Ponchel F, Morgan AW, Bingham SJ, Quinn M, Buch M, Verburg RJ, *et al.* Dysregulated lymphocyte proliferation and differentiation in patients with rheumatoid arthritis. *Blood* 2002;**100**:4550–6. <https://doi.org/10.1182/blood-2002-03-0671>
37. Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ. B cells and humoral immunity in atherosclerosis. *Circ Res* 2014;**114**:1743–56. <https://doi.org/10.1161/CIRCRESAHA.113.301145>
38. Marchini T, Hansen S, Wolf D. ApoB-specific CD4(+) T cells in mouse and human atherosclerosis. *Cells* 2021;**10**:446. <https://doi.org/10.3390/cells10020446>
39. Roy P, Sidney J, Lindestam Arlehamn CS, Phillips E, Mallal S, Suthahar A, *et al.* Immunodominant MHC-II (major histocompatibility complex II) restricted epitopes in human apolipoprotein B. *Circ Res* 2022;**131**:258–76. <https://doi.org/10.1161/CIRCRESAHA.122.321116>
40. Marchini T, Malchow S, Caceres L, El Rabi AAH, Hansen S, Mwinnyella T, *et al.* Circulating autoantibodies recognizing immunodominant epitopes from human apolipoprotein B associate with cardiometabolic risk factors, but not with atherosclerotic disease. *Front Cardiovasc Med* 2022;**9**:826729. <https://doi.org/10.3389/fcvm.2022.826729>
41. Gollmer J, Zirlik A. Identifying leukocyte phenotypes by scRNA-seq to predict cardiovascular risk. *Nat Rev Cardiol* 2023;**20**:439–40. <https://doi.org/10.1038/s41569-023-00891-1>
42. Huang YV, Waliany S, Lee D, Galdos FX, Witteles RM, Neal JW, *et al.* The role of single-cell profiling and deep immunophenotyping in understanding immune therapy cardiotoxicity. *JACC CardioOncol* 2022;**4**:629–34. <https://doi.org/10.1016/j.jacc.2022.08.012>
43. Sun J, Singh P, Shami A, Kluza E, Pan M, Djordjevic D, *et al.* Spatial transcriptional mapping reveals site-specific pathways underlying human atherosclerotic plaque rupture. *J Am Coll Cardiol* 2023;**81**:2213–27. <https://doi.org/10.1016/j.jacc.2023.04.008>
44. Pekayvaz K, Heinig M, Stark K. Predictive cardio-omics: translating single-cell multiomics into tools for personalized medicine. *Nat Rev Cardiol* 2025;**22**:305–6. <https://doi.org/10.1038/s41569-025-01132-3>
45. Zhao TX, Sriranjani RS, Tuong ZK, Lu Y, Sage AP, Nus M, *et al.* Regulatory T-cell response to low-dose interleukin-2 in ischemic heart disease. *NEJM Evid* 2022;**1**:EVIDoA2100009. <https://doi.org/10.1056/EVIDoA2100009>