

Immune checkpoint landscape of human atherosclerosis and influence of cardiometabolic factors

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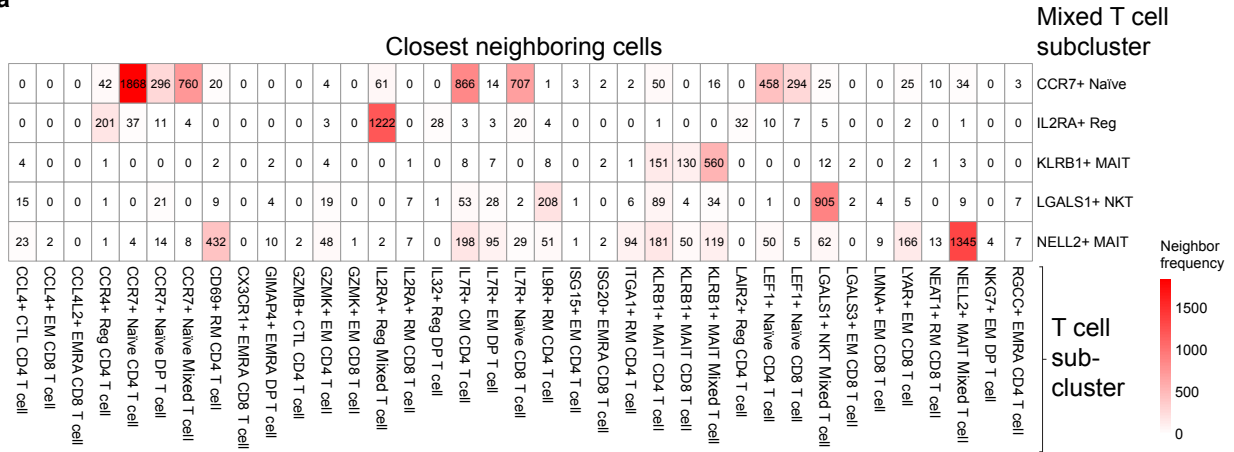
a



Supplementary Data Figure 1. Distribution and corresponding gene expression pattern of major human atherosclerotic plaque immune cell identities by single-cell RNA-sequencing (scRNA-seq). **a**, Uniform Manifold Approximation and Projections (UMAP) obtained by scRNA-seq of human atherosclerotic plaque immune cells ($n = 22$; Supplementary Table 1). Data are shown clustered by cell type. Each UMAP corresponds to one patient. CEA: carotid endarterectomy. **b**, Dot plot of immune cell marker gene expression by cluster and subcluster. Dot color represents absolute normalized counts. DP: double-positive ($CD4^+ CD8^+$); $T_{\gamma\delta}$: $\gamma\delta$ T cell; NK: natural killer; ILC: innate lymphoid cell; EM: effector memory; EMRA: terminally differentiated effector memory re-expressing CD45RA; CM: central memory; RM: resident memory; CTL: cytotoxic T lymphocyte; Reg: regulatory; MAIT: mucosal-associated invariant; NKT: natural killer T cell; Mono: monocyte; $M\phi$: macrophage; DC: dendritic cell.

Supplementary Data Figure 2

a

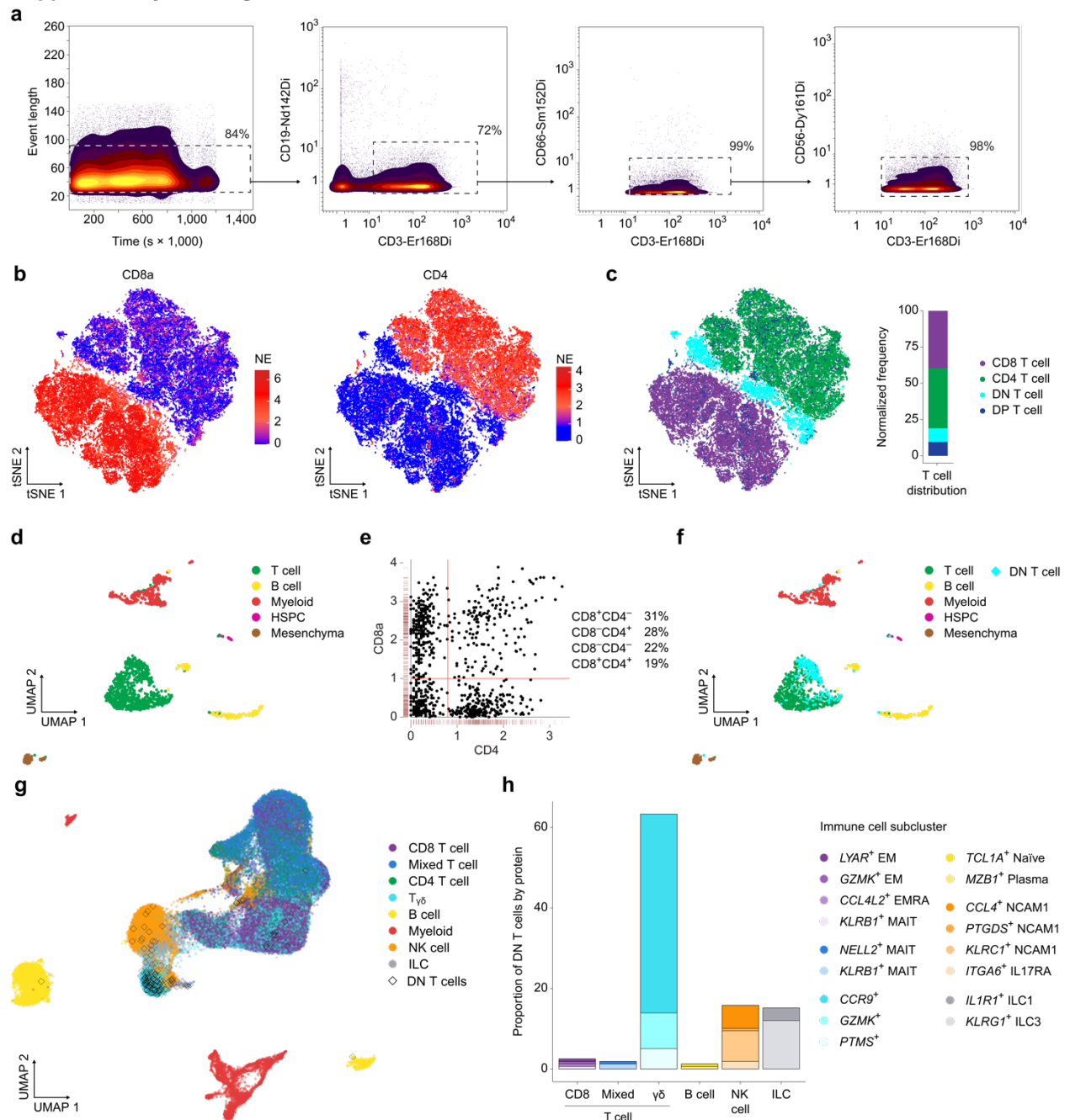


b



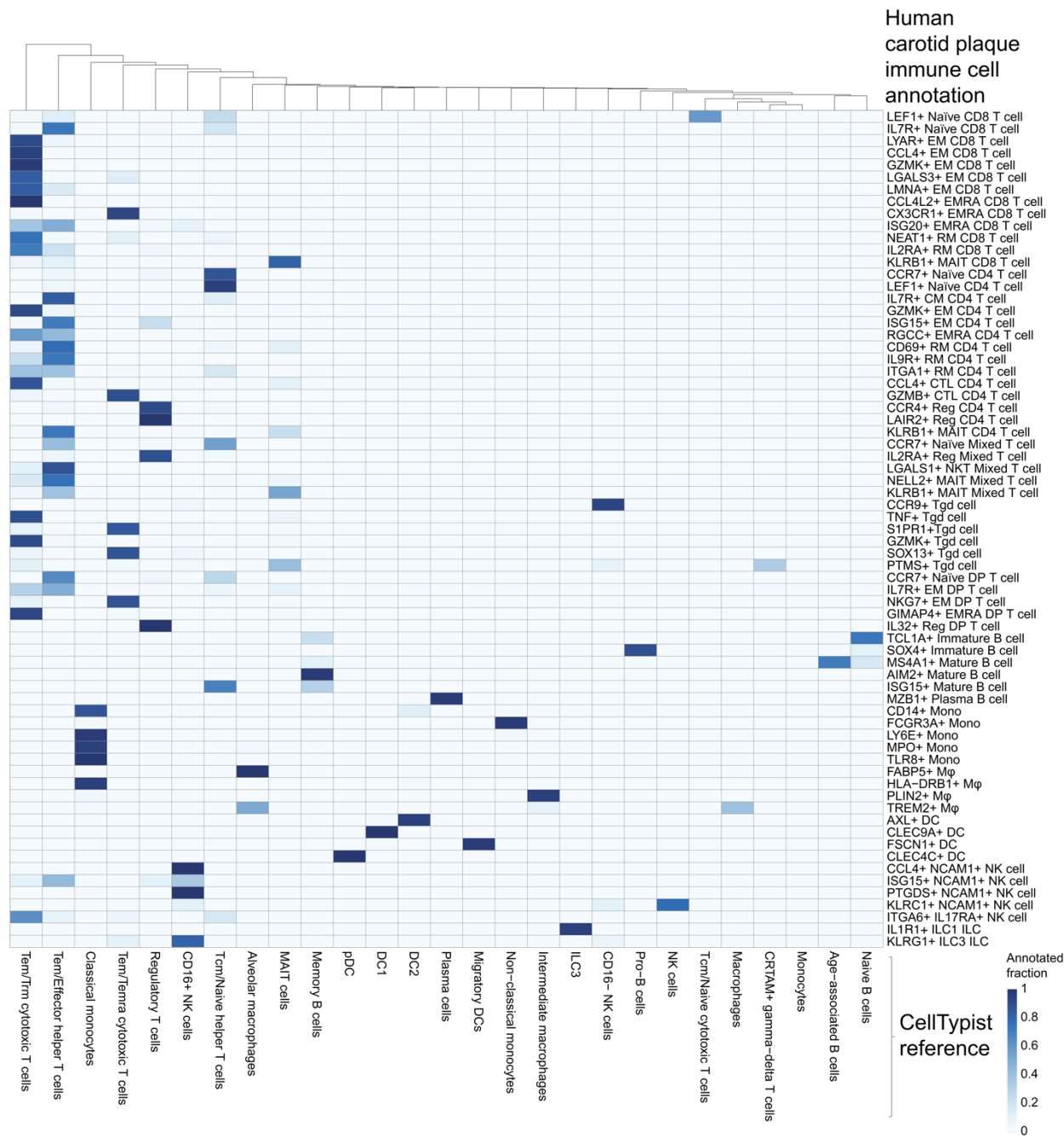
Supplementary Data Figure 2. Cluster separation assessment to determine identity of T cell subclusters that lack *CD8A*, *CD8B1*, or *CD4* and do not display the transcriptional program of $\gamma\delta$ T cells. **a**, Heatmap showing closest neighboring cell analysis of Mixed T cells against all T cell subclusters from human carotid plaque immune cell single-cell RNA-seq (scRNA-seq) dataset. Neighborhood frequencies were computed using the neighborPurity function from the bluster package (v1.11.4) on R. **b**, Heatmap showing pairwise cluster modularity analysis of all T cell subclusters from human carotid plaque immune cell scRNA-seq dataset. Pairwise modularity scores were computed using the pairwiseModularity function from the bluster package (v1.11.4) on R. DP: double-positive ($CD4^+ CD8^+$); EM: effector memory; EMRA: terminally differentiated effector memory re-expressing CD45RA; CM: central memory; RM: resident memory; CTL: cytotoxic T lymphocyte; Reg: regulatory; MAIT: mucosal-associated invariant; NKT: natural killer T cell.

Supplementary Data Figure 3



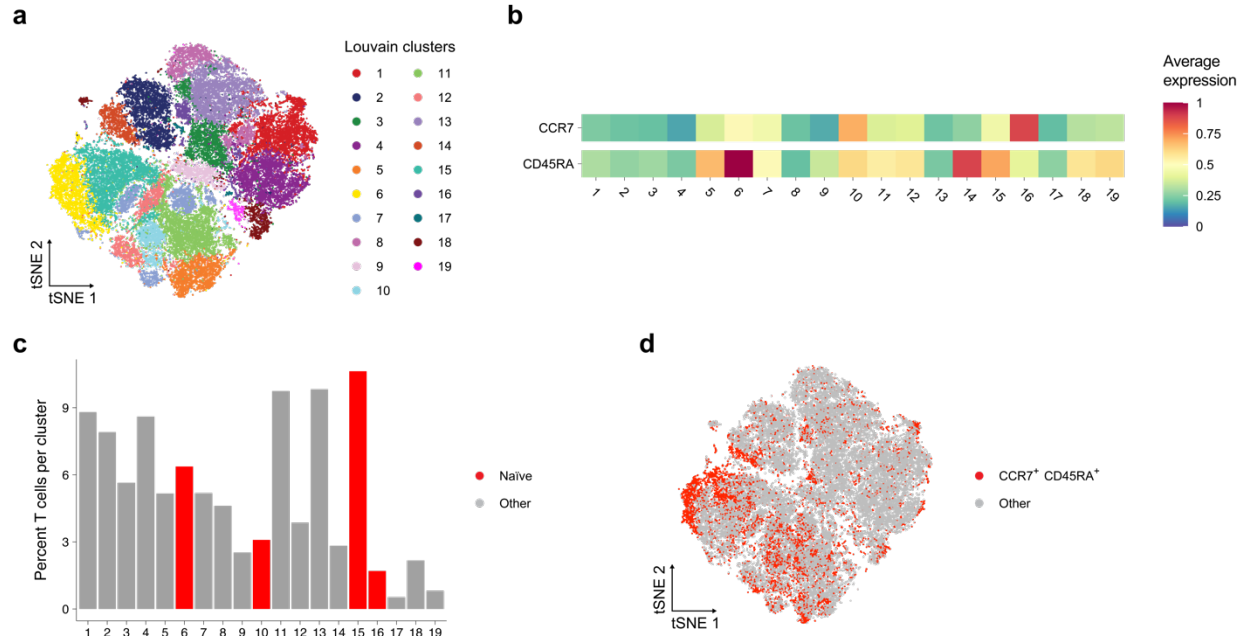
Supplementary Data Figure 3. Assessment of CD8 and CD4 transcript and protein expression for T cell identity deconvolution. **a**, Representative cytometry by time-of-flight (CyTOF) plots of human carotid plaque immune cells showing gating strategy to obtain T cells for downstream analyses. **b**, t-distributed Stochastic Neighbor Embedding (tSNE) plots depicting CD8a (left) and CD4 (right) protein expression by CyTOF ($n = 28$ plaques). NE: Normalized expression. **c**, tSNE plot showing T cell annotation based on Louvain clustering and surface protein marker expression (left) and stacked bar plot depicting frequency of each subgroup (right). DN: double-negative ($CD8^- CD4^-$); DP: double-positive ($CD8^+ CD4^+$); $T_{\gamma\delta}$: $\gamma\delta$ T cell. **d**, Uniform Manifold Approximation and Projection (UMAP) obtained by cellular indexing of transcriptomes and epitopes followed by sequencing (CITE-seq) of human carotid plaque immune cells ($n = 1$). Data are shown clustered by cell type. **e**, Scatter plot depicting normalized CD8 and CD4 protein expression by CITE-seq. Each dot represents a T cell. Maroon rugs show data distribution along axes; cutoff lines are drawn in red at $y = 1$ and $x = 0.8$. The proportions of cells in each quadrant are shown on the right. **f**, UMAP shown in (d), highlighting DN T cells as determined by protein expression in (c). **g**, UMAP of whole human atherosclerotic plaque immune cell scRNA-seq dataset ($n = 22$) clustered by cell type (see Fig. 1b), highlighting DN T cells as determined by protein expression in (c) largely on top of the $\gamma\delta$ T ($T_{\gamma\delta}$) cell cluster. **h**, Stacked bar plot showing proportion of DN T cells as determined by protein expression and the corresponding cluster and subcluster identities assigned by scRNA-seq analyses of whole integrated human carotid plaque immune cell dataset.

Supplementary Data Figure 4



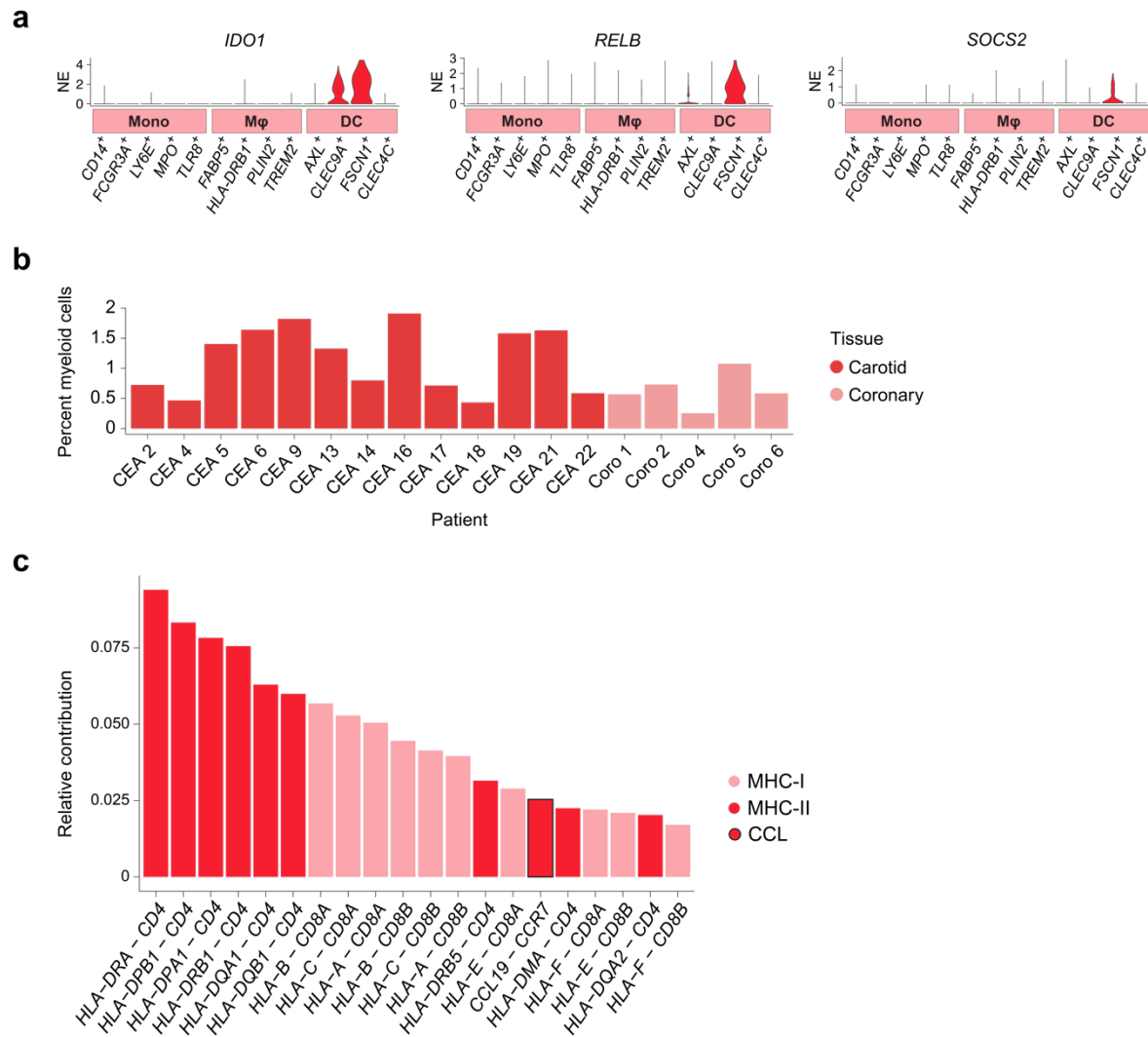
Supplementary Data Figure 4. Reference annotation of human carotid plaque immune cell single-cell RNA sequencing dataset. a, Heatmap depicting annotated fraction of carotid plaque immune cell subclusters against CellTypist reference. DP: double-positive ($CD8^+ CD4^+$); Tgd: $\gamma\delta$ T cell; Mye: myeloid cell; NK: natural killer; ILC: innate lymphoid cell; EM/em: effector memory; EMRA/emra: terminally differentiated effector memory re-expressing CD45RA; CM/cm: central memory; RM/rm: resident memory; CTL: cytotoxic T lymphocyte; Reg: regulatory; MAIT: mucosal-associated invariant; NKT: natural killer T cell; Mono: monocyte; M ϕ : macrophage; (p)DC: (plasmacytoid) dendritic cell.

Supplementary Data Figure 5



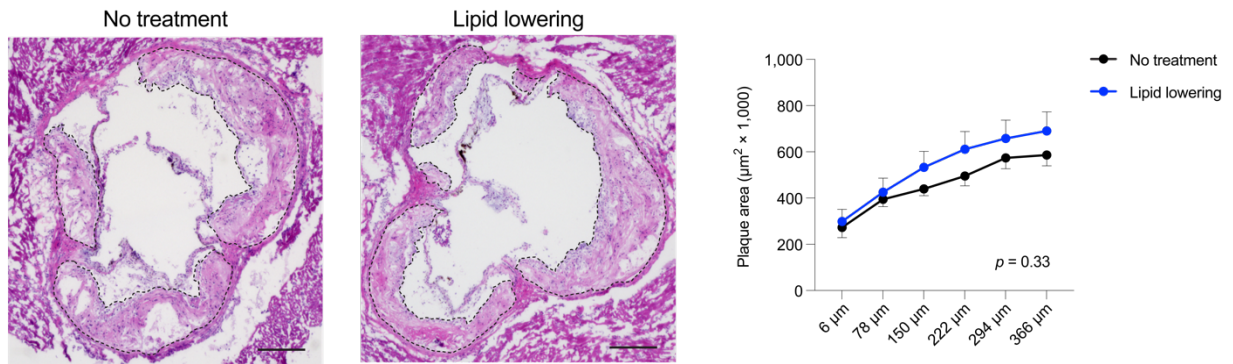
Supplementary Data Figure 5. Carotid plaque naïve T cell assessment by cytometry by time-of-flight (CyTOF). **a**, t-distributed Stochastic Neighbor Embedding (tSNE) showing carotid plaque T cell clustering by CyTOF ($n = 28$). **b**, Heatmap depicting normalized surface protein expression of naïve T cell markers CCR7 or CD45RA by cluster. NE: normalized expression. **c**, Bar plot showing T cell cluster proportions. Naïve T cell clusters, as determined by CCR7 and CD45RA expression in (c), are shown in red. **d**, tSNE showing individual cells co-expressing CCR7 and CD45RA in red.

Supplementary Data Figure 6



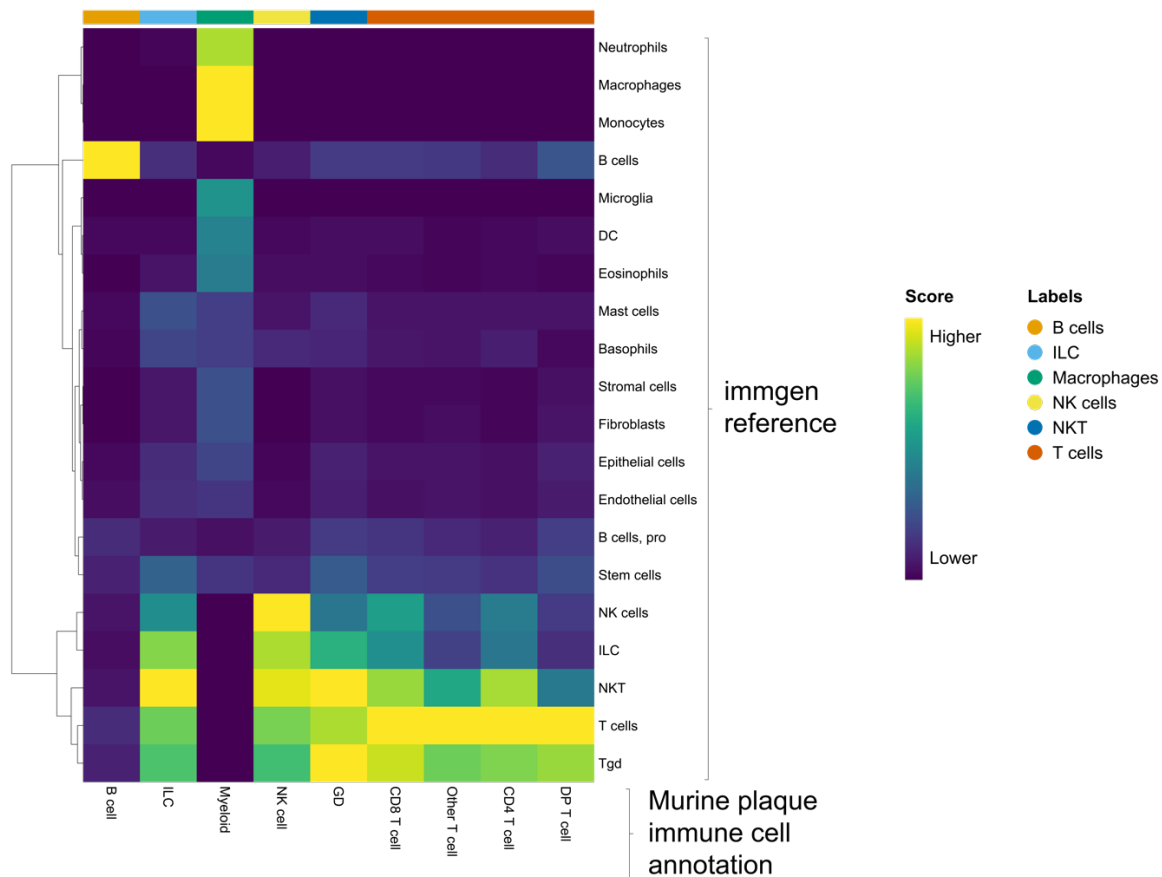
Supplementary Data Figure 6. Characterization of $CCR7^+$ $FSCN1^+$ dendritic cells in human atherosclerotic tissue. **a**, Violin plots showing normalized transcript abundance and distribution in human plaque myeloid cell subclusters engaging in IC interactions. NE: normalized expression. **b**, Bar plot depicting the proportion of $CCR7^+$ $FSCN1^+$ dendritic cells as a percentage of myeloid cells per patient in the carotid or coronary datasets analyzed by single-cell RNA-sequencing. CEA: carotid endarterectomy. **c**, Bar plot depicting the relative contribution of the strongest ligand-receptor interactions to overall cell-cell communication engaged by $CCR7^+$ $FSCN1^+$ dendritic cells in human atherosclerotic plaques, as predicted by our model. MHC: major histocompatibility cluster. CCL: chemokine ligand.

Supplementary Data Figure 7



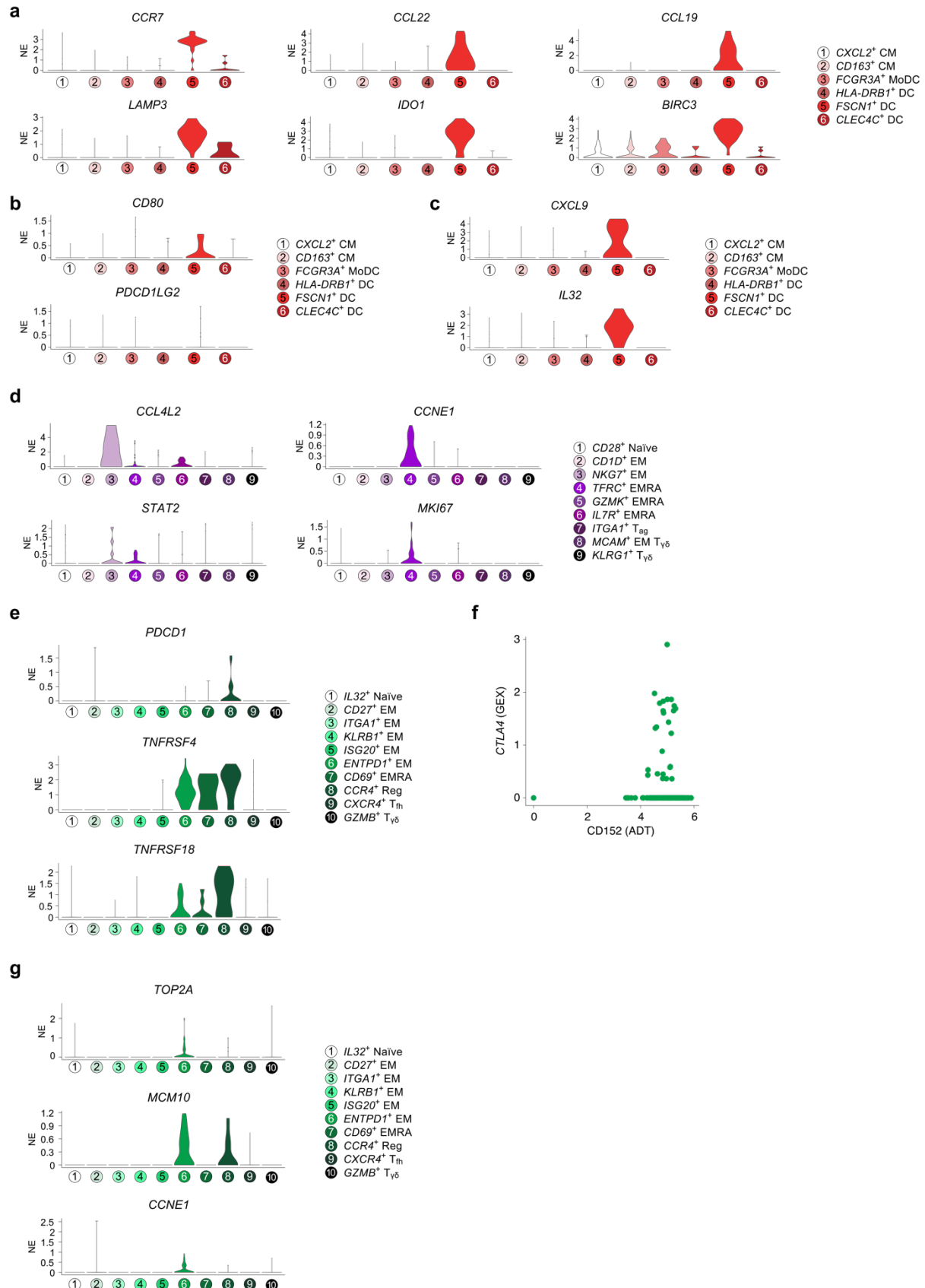
Supplementary Data Figure 7. Murine atherosclerotic plaque characterization. a, Representative images of hematoxylin and eosin immunohistochemical staining of aortic roots from atherosclerotic mice, stratified by treatment (left) and quantification of plaque area (right). Dotted lines circumscribe atherosclerotic plaques. Data are presented as mean values $\pm$ SEM. p value was determined by two-way ANOVA ($n = 9$ per group). Scale bars are 200 μm .

Supplementary Data Figure 8



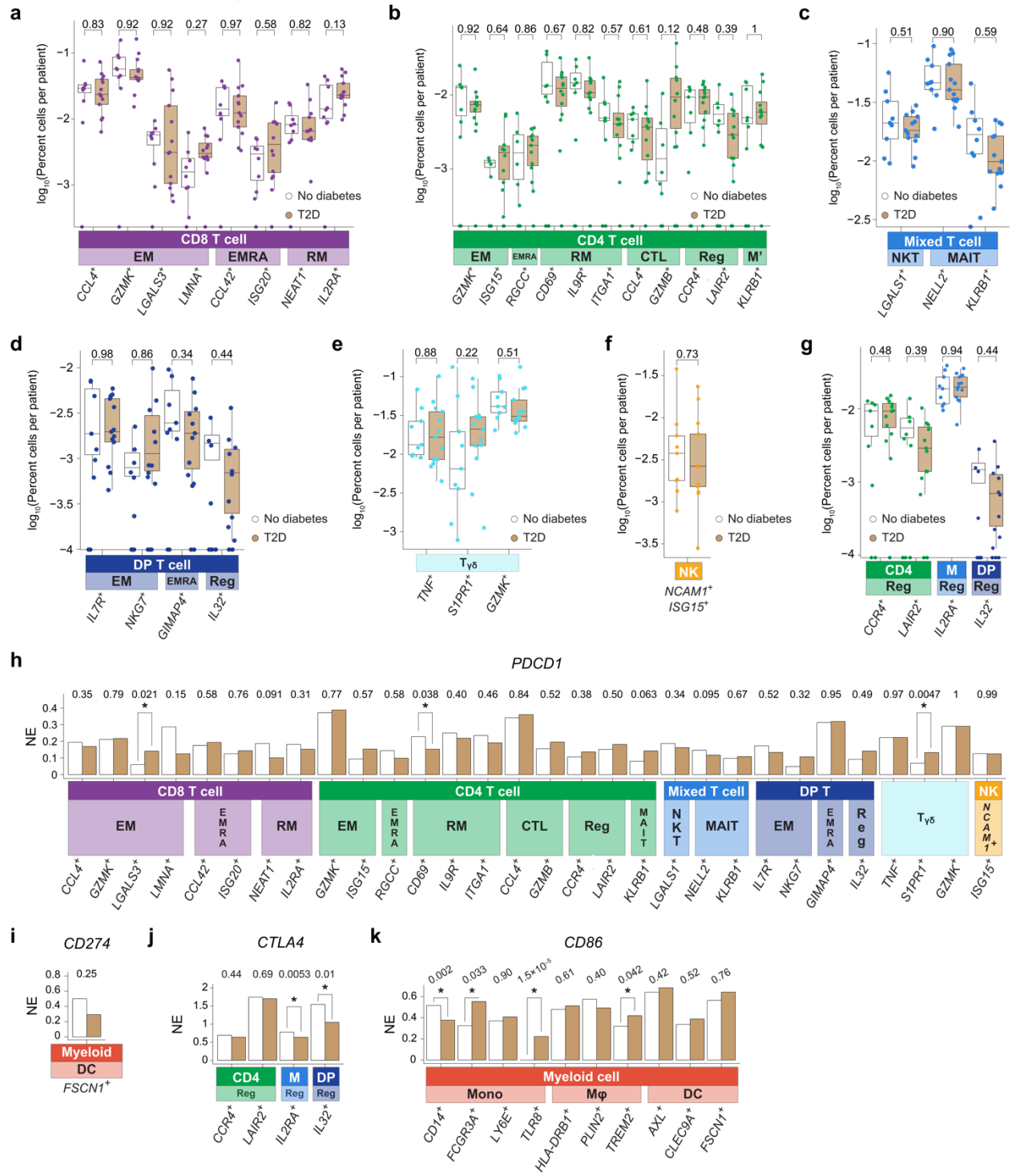
Supplementary Data Figure 8. Reference annotation of murine aortic arch immune cell single-cell RNA sequencing dataset. a, Heatmap generated using the `plotScoreHeatmap()` function from the `SingleR` package (v1.10.0) in R (v4.0.3) depicting identity scores of major clusters against the Immunological Genome Project reference. DP: double-positive ($CD8^+ CD4^+$); Tgd/GD: $\gamma\delta$ T cell; Mye: myeloid cell; NK: natural killer; ILC: innate lymphoid cell; NKT: natural killer T cell; DC: dendritic cell.

Supplementary Data Figure 9



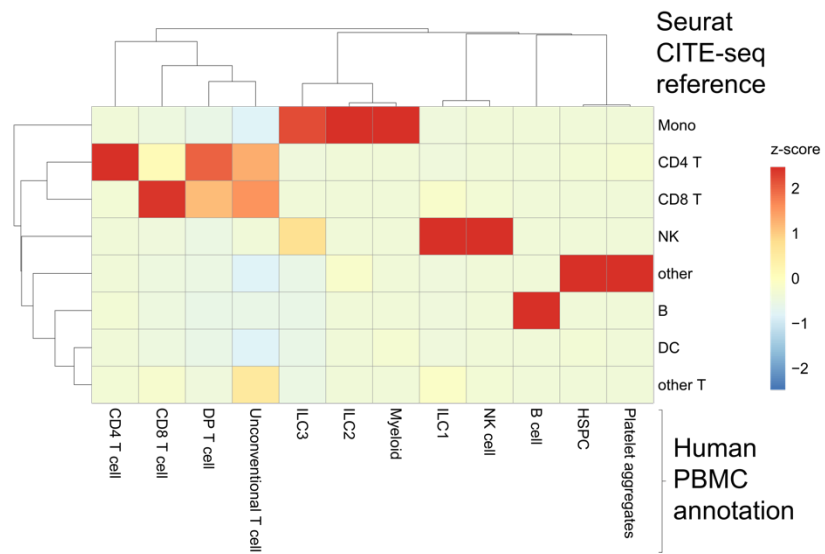
Supplementary Data Figure 9. Gene expression signatures of human peripheral blood mononuclear cell subclusters by cellular indexing of transcriptomes and epitopes followed by sequencing. **a–c**, Violin plots illustrating normalized transcript abundance and distribution of, respectively, mature regulatory dendritic cell markers as defined in Magen et al.⁶⁹, genes encoding secondary CTLA4 and PD-1 ligands and dendritic cell activation and chemotaxis markers across myeloid cell subclusters. **d**, Violin plots illustrating normalized transcript abundance and distribution of, respectively, inflammation and proliferation markers across CD8⁺ T cell subclusters. **e** and **g**, Violin plots illustrating normalized transcript abundance and distribution of immunoregulatory and proliferation markers, respectively, across CD4⁺ T cell subclusters. **f**, Scatter plot depicting normalized *CTLA4* transcript expression and CD152 (CTLA4) protein expression in CD4⁺ T cells. Each dot represents a cell. GEX: gene expression; ADT: antibody-derived tag. NE: normalized expression. CM: classical monocyte; MoDC: monocyte/dendritic cell; DC: dendritic cell; EM: effector memory; EMRA: terminally differentiated effector memory re-expressing CD45RA; T_{ag}: aging T cell; Reg: regulatory; T_{fh}: follicular helper T cell.

Supplementary Data Figure 10



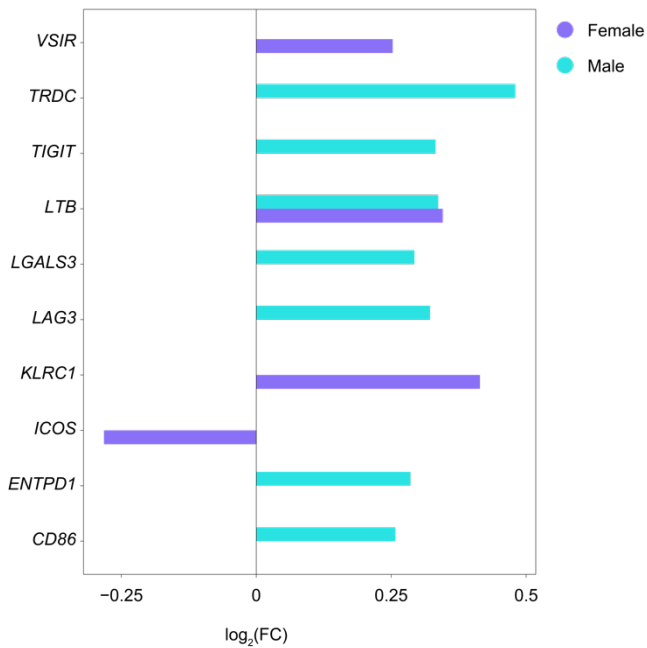
Supplementary Data Figure 10. Proportions and gene expression profile of human atherosclerotic plaque immune cell clusters and clinically relevant subclusters by single-cell RNA-sequencing of type 2 diabetic (T2D) vs. non-diabetic patients. **a–g**, Proportions of: *PDCDI*-expressing (a), CD8⁺; (b) CD4⁺; (c), Mixed (CD8⁺ or CD4⁺); (d), double-positive (DP; CD8⁺ CD4⁺) T cell; (e) T_{γδ}: γδ T cell; and (f) natural killer (NK) cell subclusters engaging in the strongest ($p < 0.05$) predicted interactions with PD-L1 (encoded by *CD274*); or (g) *CTLA4*-expressing CD4⁺, Mixed, and DP T cell subclusters engaging in the strongest ($p < 0.05$) predicted interactions with CD86. Each dot represents one patient. Boxes represent interquartile ranges; center lines depict medians. Whiskers below and above boxes represent extent of lower and upper quartiles, respectively ($n = 9$ non-diabetic and 13 T2D). **h–k**, Bar plots illustrating normalized transcript abundance of (h), *PDCDI* (encoding PD-1); (i), *CD274* (encoding PD-L1); (j), *CTLA4*; and (k), *CD86* in the indicated immune cell clusters and subclusters. All plots are split by diabetes status. p values were determined by unpaired, two-tailed Student's t tests. (*) $p < 0.05$. p values for interactions were calculated by the CellChat package (v1.1.3) in R (v4.0.3). AU: arbitrary units. EM: effector memory; EMRA: terminally differentiated effector memory re-expressing CD45RA; RM: resident memory; CTL: cytotoxic T lymphocyte; Reg: regulatory; M'/MAIT: mucosal-associated invariant; NKT: natural killer T cell; Mono: monocyte; Mφ: macrophage; DC: dendritic cell.

Supplementary Data Figure 11



Supplementary Data Figure 11. Reference annotation of human peripheral blood mononuclear cell single-cell RNA sequencing dataset. a, Heatmap depicting annotated fraction of major clusters against Seurat's CITE-seq reference. DP: double-positive ($CD8^+ CD4^+$); Mye: myeloid cell; NK: natural killer; ILC: innate lymphoid cell; Mono: monocyte; DC: dendritic cell; HSPC: hematopoietic stem and progenitor cell.

Supplementary Data Figure 12



Supplementary Data Figure 12. Differential immune checkpoint (IC) gene expression in human peripheral blood mononuclear cells following lipid-lowering treatment, stratified by sex. a, Bar plot showing significantly (FDR < 0.05) differentially expressed IC genes. FC: fold change.