Integrative single cell RNA-sequencing descrambles a substantial divergence of adaptive immune cell identities and transcriptional programs in mouse and human atherosclerosis

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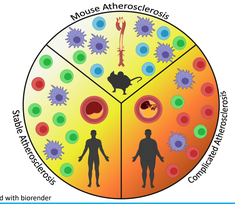
BACKGROUND



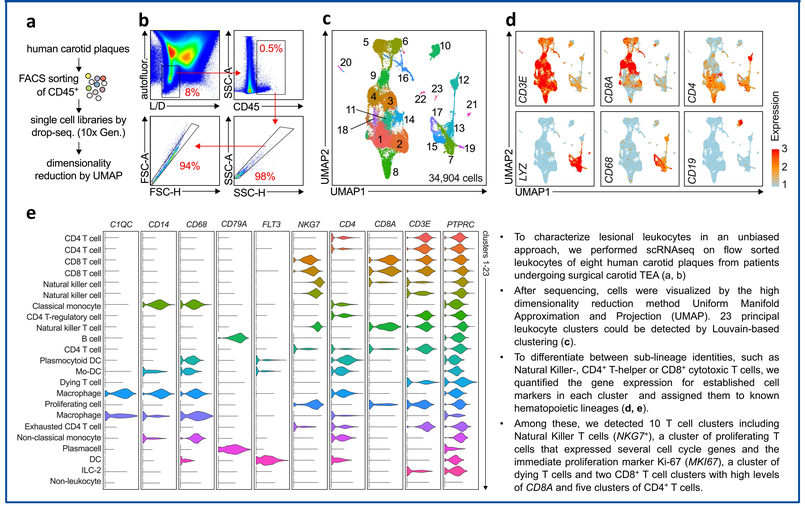
- **Atherosclerosis is a chronic inflammatory disease that is driven by the accumulation of pro- and anti-inflammatory leukocytes in the intima of affected arteries.**
- **The distinct function of immune cells in human atherosclerosis has been mostly defined by preclinical mouse studies.**
- **Contrastingly, the immune cell composition of human atherosclerotic plaques and their contribution to disease progression is only poorly understood.**
- **In this project, we optimized and applied a protocol to characterize immune cells in human carotid atherosclerotic plaques by single-cell RNA sequencing, immunofluorescence and multi-color flow cytometry**
- **As it remains uncertain whether genetic animal models allow for valuable translational approaches, we mapped our scRNAseq results to existing scRNAseq data from different mouse models, locations and diets.**
- **Further we built clinical associations between the leukocyte repertoire and cardiovascular events.**

Methods and approach

Patient with carotid atherosclerosis → **Carotid endarterectomy** → **Plaque digestion, leukocyte isolation** → **Flow Cytometry** → **Single-cell RNA sequencing & integration**

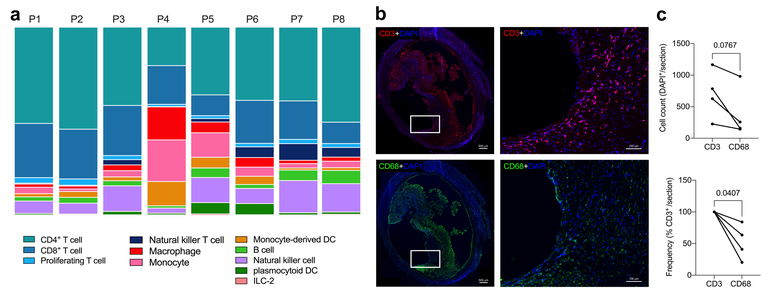
- 53 human carotid plaques were collected from patients undergoing carotid endarterectomy. The atherosclerotic plaques were collected in RPMI 1640 + 10% FCS and were processed, usually within 2 hours after surgery.
- The Plaque-tissue was dissected into multiple parts, depending on the size of the sample and was digested by an enzymatic cocktail. After digestion, we performed rescue incubation on the liberated leukocytes and stained them for FACS. All samples were analysed in flow cytometry with a pan-leukocyte panel.
- Single cell RNA-sequencing was performed to define the immune cell repertoire in human atherosclerosis on a transcriptional level in 8 selected patients. These samples were flow-sorted for CD45⁺ viable leukocytes.
- While conventional proteomic approaches, such as FACS and CyTOF, require the definition and pre-selection of anticipated cell surface or intracellular markers that may distinguish cell heterogeneity best, single-cell RNA sequencing is an unsupervised and unbiased approach.
- The resulting human scRNAseq dataset was integrated with 7 different mouse datasets in order to draw comparisons between mouse and human atherosclerosis.

The immune cell repertoire of human carotid plaques is highly diverse



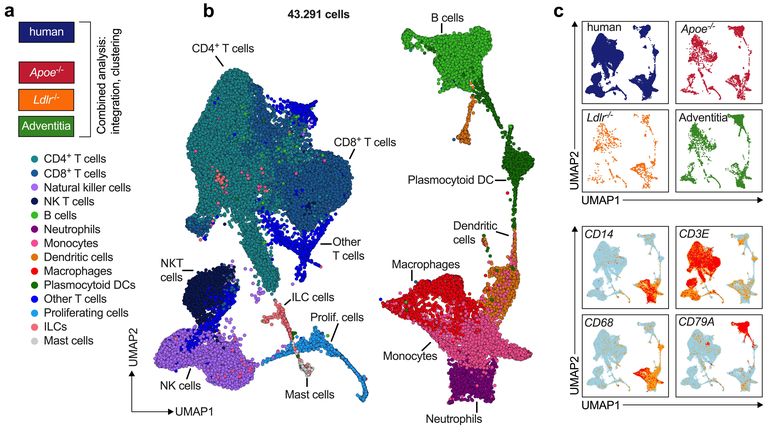
- To characterize lesional leukocytes in an unbiased approach, we performed scRNAseq on flow sorted leukocytes of eight human carotid plaques from patients undergoing surgical carotid TEA (a, b)
- After sequencing, cells were visualized by the high dimensionality reduction method Uniform Manifold Approximation and Projection (UMAP). 23 principal leukocyte clusters could be detected by Louvain-based clustering (**c**).
- To differentiate between sub-lineage identities, such as Natural Killer-, CD4⁺ T-helper or CD8⁺ cytotoxic T cells, we quantified the gene expression for established cell markers in each cluster and assigned them to known hematopoietic lineages (**d**, **e**).
- Among these, we detected 10 T cell clusters including Natural Killer T cells (*NKG7*⁺), a cluster of proliferating T cells that expressed several cell cycle genes and the immediate proliferation marker Ki-67 (*MKI67*), a cluster of dying T cells and two CD8⁺ T cell clusters with high levels of *CD8A* and five clusters of CD4⁺ T cells.

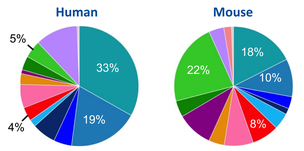
Human atherosclerotic plaque leukocytes are dominated by T cells



- Surprisingly, T cells represented the predominant cell type (62.3±10.2%) in scRNAseq, with CD4⁺ T cells accounting for 41.3 ± 7.0% and CD8⁺ T cells for 21.0 ± 3.8%, while myeloid cells only represented 25.6 ± 11.1% of all leukocytes (**a**).
- We found similar results in Immunofluorescence of human plaques, which were stained for CD3 as a T cell marker and CD68 as a macrophage/myeloid marker (**b**). In this approach, T cells outnumbered macrophages (**c**). Collectively, these data show the dominance of T cells among human atherosclerotic plaque leukocytes.

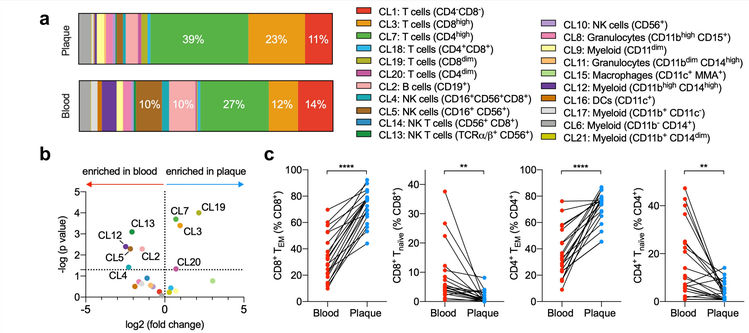
Leukocyte frequencies in mouse models do not resemble the immune cell landscape in human atherosclerosis





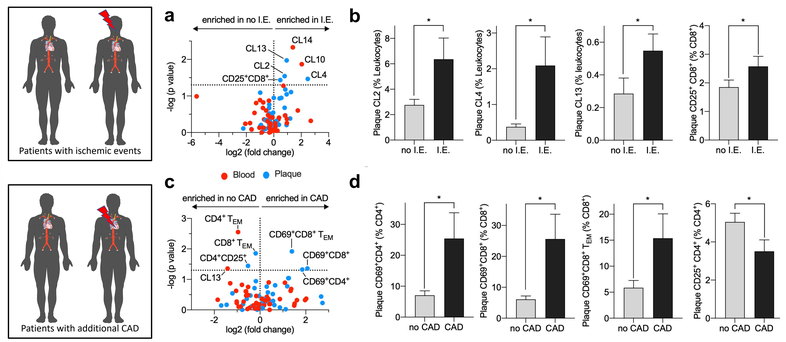
- As we aimed to compare mouse and human atherosclerosis simultaneously in the same analysis pipeline, we integrated our human scRNAseq dataset with seven mouse data sets, that were previously generated by us and others, including atherosclerotic aortas from *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ and adventitial preparations from *Apoe*$^{-/-}$ and wildtype (**a**)
- The resulting 43.291 transcriptomes from two species, three genetic backgrounds, and different locations uniformly distributed over the UMAP projection without apparent batch effects (**b**, **c**).
- In this cross-species approach, human and mouse genes served for cell type identification alike resulting in the identification of 14 distinct metaclusters.
- In a direct comparison, we detected a considerable divergence of the immune cell composition in mice and humans. For instance, neutrophils (0.8 vs 8.7%), B cells (5 vs 22%) and macrophages (4 vs 8%) were under-represented in human compared with mice plaques, respectively.
- Contrastingly, cells of the T cell lineage (52% vs 28%) dominated human plaques. Some T cell clusters were only detectable in in human plaques, while others were exclusive to mice.
- Taken together, we present the first cross-species atlas of mouse and human atherosclerosis, indicating that the frequencies in moues models do not resemble the immune cell landscape in human atherosclerosis adequately.

Protein surface marker-defined immune cell landscape of human carotid plaques



- In order to validate the immune cell composition in human plaques, we tested a total of 43 carotid plaques from surgical endarterectomy and characterized these with a multi-colour panel with 13 cell surface markers capable of identifying principal hematopoietic lineages. We applied the same gating strategy to peripheral arterial blood samples from a subgroup of patients to establish the relative over- or underrepresentation of lesional leukocytes in the plaque (**a**).
- By this strategy we could verify that T cells are the most dominant fraction among atherosclerotic plaque leukocytes (64.8%) , while myeloids and macrophages represented only 14% in this protein based approach. Interestingly, T cells were relatively more abundant in the plaque than in the blood of the same patient (**b**) and showed an effective memory phenotype, indicating previous antigen recognition (**b**, **c**).

Leukocytes in atherosclerotic plaques associate with cardiovascular events



- In a last step we associated clinical signs of complicated carotid atherosclerosis (stroke or a transient-ischemic attack) within 6 months before surgery with immune cell frequencies. We found that NK T cells (CL4,13) and B cells (CL2) were more frequent in patients with an ischemic event (I.E.) compared to asymptomatic patients (no ischemic event, no I.E.). In addition the population of activated CD25⁺CD8⁺ T cells was slightly increased in patients with ischemic events (**a**, **b**). Interestingly, frequencies of NK cell containing clusters CL10 and -14 were higher in the blood of patients with an ischemic event (**a**).
- Next, we tested the association between the incidence of CAD with leukocyte frequencies in blood and carotid plaques in the same cohort of patients. In patients with CAD, more CD8⁺, CD8⁺ T_{EM} and CD4⁺ T cells expressed the activation marker CD69. Conversely, the population of non-activated CD8⁺ T cells (CD69^{neg}) and of atheroprotective CD25⁺CD4⁺ T-regulatory cells was larger in carotid plaques from patients without CAD.
- These results indicate that the activation status of T cells in carotid plaques associates with an increased likelihood of generalized and complicated atherosclerosis and may serve as future riskmarkers .

CONCLUSION

- **By combining unsupervised cluster detection algorithms and integration of mouse scRNAseq data sets, we uncovered several leukocyte clusters with unique cell surface marker expression, suggesting an unexpected high diversity of mouse and human plaque leukocytes.**
- **Here, we identify several cellular identities that are unique to human disease, overrepresented in plaques, and associate with plaque vulnerability and systemic atherosclerosis in humans. Our data indicates, that the frequencies in mouse models do not resemble human atherosclerosis adequately.**
- **Distinct leukocyte populations in atherosclerotic plaques may represent future cellular targets for cardiovascular immunotherapy or atheroprotective vaccination and could serve as riskmarkers for generalized and complicated atherosclerosis.**