Meta-Analysis of Leukocyte Diversity in Atherosclerotic Mouse Aortas

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Abstract
The diverse leukocyte infiltrate in atherosclerotic mouse aortas was recently analyzed in 9 single cell RNA-Seq (scRNA-Seq) and 2 mass cytometry (CyTOF) studies. In a comprehensive meta-analysis, we demonstrate four macrophage subsets: resident, inflammatory, IFNIC and Trem2 foamy macrophages. We also find that monocytes, neutrophils, dendritic cells, natural killer cells, innate lymphoid cells-2 (ILC2) and CD8 T cells form prominent and separate populations. The CD4 T cells show a large population of Th17-like cells, which also contain γδ T cells. A small number of Tregs and Th1 cells is also identified. The present meta-analysis overcomes limitations of individual studies that, because of their experimental approach, over- or under-represent certain cell populations. CyTOF identifies an even larger number of clusters, suggesting that surface markers provide more discriminatory information than transcriptomes. The present analysis provides evidence to further resolve some long-standing controversies in the field. First, $Trem2^{+}$ foamy macrophages are not pro-inflammatory, but interferon-inducible cell (IFNIC) and inflammatory macrophages are. Second, about half of all foam cells are smooth muscle cell-derived, retaining smooth muscle cell transcripts rather than transdifferentiating to macrophages. Third, *Pf4*, which had been considered specific for platelets and megakaryocytes, is also prominently expressed in resident vascular macrophages. Finally, the discovery of a prominent ILC2 cluster links the scRNA-Seq work to recent flow cytometry data suggesting a strong atheroprotective role of ILC2 cells. This resolves apparent discrepancies regarding the role of Th2 cells in atherosclerosis based on studies that pre-dated the discovery of ILC2 cells.

Glossary

AAV	adeno-associated virus
Acta2	actin alpha 2
ATLO	artery tertiary lymphoid organ
Apoe	apolipoprotein E
CC	C-C chemokine
CXC	C-X-C chemokine
CX3C	C-X3-C chemokine
CD	Cluster of differentiation
CITE-seq	cellular indexing of transcriptomes and epitopes by sequencing
CTL	cytotoxic T lymphocytes
CyTOF	mass cytometry
DC	dendritic cell
GFP	green fluorescent protein
GSEA	gene set enrichment analysis
GWAS	genome-wide association study
HFD	high-fat diet
IFNIC	interferon-inducible cells
ILC	innate lymphocyte-like cells
LDL	low density lipoprotein
Ldlr	low density lipoprotein receptor
MHC	major histocompatibility complex
NK cell	natural killer cell
NKT	natural killer T cell
RAG	recombinase activating gene
scRNA-seq	single cells RNA sequencing
TCR	T cell receptor
TFH cell	T follicular helper cell
T_H cell	T helper cell
Treg	regulatory T cell
TREM2	triggering receptor expressed on myeloid cells 2
TRM	tissue resident memory
UMAP	uniform manifold approximation and projection
pDC	plasmacytoid dendritic cell
PCSK9	protein convertase subtilisin/kexin type 9
PVAT	perivascular adipose tissue
VCAM	vascular cell adhesion molecule
WD	Western diet
YFP	yellow fluorescent protein

1. Introduction

Atherosclerosis is a disease of large and mid-sized arteries with devastating consequences. The disease process is initiated by low density lipoprotein (LDL) accumulating in the subendothelial space. Rapidly, resident vascular macrophages and lymphocytes expand in number and differentiate in phenotype. Early on, neutrophils and monocytes are recruited, along with all known cell types of the adaptive immune system. Mature atherosclerotic lesions therefore contain a wide variety of immune cells.

In early studies, the immune cell infiltrate was characterized by immunohistochemistry[1]. Later, methods were developed to generate single cell suspensions from mouse aorta[2] and human atherosclerotic lesions[3]. Recently, high dimensional methods of analysis became available that generated a plethora of data allowing fine-grained and comprehensive characterization of the immune cell content of atherosclerotic lesions. Mass cytometry[4, 5] allows assessment of up to 42 cell surface and intracellular markers. Single cell RNA-sequencing (scRNA-Seq) yields expression matrices of thousands of genes per cell, depending on the single cell sequencing method and platform[6]. CITE-Seq combines scRNA-Seq with quantitative measurement of 50-200 cell surface markers[7]. The technical aspects of these high dimensional methods in vascular biology, including quality controls and validation, have recently been reviewed[6].

This review is focused on leukocyte diversity in atherosclerosis. We assembled a team of specialists in the field to provide comprehensive and, where possible, authoritative information of the immune cell phenotypes as defined by cell surface phenotype, intracellular proteins and gene expression. We will only discuss mouse data, because there are only two studies in human atherosclerosis[8, 9]. Since genetic models of atherosclerosis (*Apoe*$^{-/-}$, *Ldr*$^{-/-}$) were generated in 1992[10-12], the mouse has become the *de facto* workhorse of atherosclerosis research. Later, more sophisticated mouse models of atherosclerosis were generated[13, 14], but these are not as widely used.

One goal of this review is to assemble lists of surface markers and genes that can be used to delineate the various immune cell types. Of note, we are not concerned with the ontogeny of the immune cells in atherosclerosis. It is known that tissue resident vascular macrophages in mice[15] are not monocyte-derived, are largely seeded before birth and can proliferate locally[16]. Other macrophages in atherosclerosis are derived from infiltrating monocytes[16]. We are also not concerned with cell lineages. In immunology, the relationship between cell types and their precursors, and whether cell types can transdifferentiate into other cell types, is often quite controversial. As a case in point, there is excellent evidence for[17] and against[18] conversion of regulatory T cells to other T cell types. Thus, we remain agnostic with respect to ontogeny and lineage. Rather, we are focusing on describing the cell types and states in atherosclerotic lesions ("what is") in order to promote systematic and mechanistic investigations. Excellent reviews exist on immune cells in the heart[19, 20]. Neither heart nor veins or other blood vessels will be considered here. Another goal is to promote clarity in nomenclature, which will enable the field to move forward more rapidly. A distant goal is to translate findings in experimental atherosclerosis into prevention and therapy strategies for people with cardiovascular disease.

This review was spawned at a meeting that Andreas Zirlik organized in Graz, Austria. This meeting was focused on single cell methods in atherosclerosis research. Several of the authors were present at this meeting. Klaus Ley approached the editors of Circulation Research with the idea of putting together this broad-based review. The editor-in-chief Jane Freedman commissioned this review. Most authors who published on mouse atherosclerosis using high dimensional single cell methods accepted the invitation to contribute. In addition, we invited computational biologists to sift through the data and identify transcriptomes and surface markers across labs, approaches and platforms.

This review is focused on CD4 and CD8 αβ T cells, γδ T cells, NK and NKT cells, B cells, neutrophils, macrophages, monocytes and dendritic cells. In addition, eosinophils, mast cells and innate lymphocyte-like cells (ILCs) are found in atherosclerotic lesions. Certainly, other cell types not mentioned here will emerge as important players in atherosclerosis. In fact, single cell technologies have a unique potential to discover new cell types.

2. Introduction of Immune Cell Types

T cells are cells of the adaptive immune system that develop in the thymus, where they find niche conditions conducive to rearrangement, under the control of the recombinase activating genes (RAG)-1 and 2, of their T cell receptor (TCR) γδ or αβ subunits, expression of their unique TCRs, positive and negative selection, and maturation[21]. The γδ subunits rearrange first and, if successfully expressed, αβ rearrangement is suppressed. These γδ T cells exit the thymus with an activated phenotype that suggests antigen experience[22]. T cells that do not undergo successful γδ rearrangement then rearrange the V, (D) and J segments of their TCR α and β genes and, through various intermediates, become CD4 and CD8 T cells. CD4 T cells recognize antigenic peptides bound to major histocompatibility complex (MHC)-II and are either regulatory or helper T cells. CD8 T cells recognize peptide antigens bound to MHC-I and become cytotoxic T lymphocytes (CTLs). Some αβ T cells recognize glycolipids bound to the MHC-like molecule CD1d and become NKT cells[23]. Naïve and central memory T cells express the chemokine receptor CCR7, which drives lymph node homing[24]. The costimulatory molecule CD28 is necessary for sufficient T cell co-stimulation by antigen-presenting cells and predominantly expressed by naïve $CD4^+$ T cells[25]. Although CD28 costimulation is not necessary for a memory recall-response, its therapeutic blockade reduces pro-atherogenic responses[26]. Some T cells express the chemokine receptor CCR9, required for thymus- and mucosal tissue homing[27]. CCR5 is involved in the homing of T cells to atherosclerotic arteries[28].

CD4 T cells recognizing self antigens are mostly regulatory T cells. Regulatory $CD4^+$ T cells (T_{reg}) represent immunosuppressive T cells that are defined by the transcription factor Foxp3 and IL-2 receptor α chain (CD25) expression[29, 30]. Natural T_{regs} (nTregs) are thymus-derived, whereas peripherally induced T_{regs} (iTregs) are generated in response to antigen recognition in the presence of transforming growth factor beta (TGF-β)[31]. Tregs dampen inflammation by limiting the proliferation and function of effector CD4 and CD8 T cells.

Co-stimulation and antigen presentation by antigen-presenting cells induces T cell activation and differentiation into functionally and phenotypically distinct T-helper (T_H) types that contribute to atherosclerosis in a subset-dependent manner[32]. Follicular helper (TFH) cells express ICOS and the chemokine receptor CXCR5. They stay in lymphoid organs and have important helper functions for B cell maturation. In healthy vessels, CD4 T cells are found in the adventitia. In atherosclerosis, CD4 T cells are also present in the plaque. It has been suggested that $CD4^+$ T cells recognize foreign- or self-antigen in the atherosclerotic plaque in an MHC-II dependent manner. Some $CD4^+$ T cells recognize peptides from Apolipoprotein B-100, the protein backbone of LDL[33, 34], but the majority of CD4 T cells in arteries have unknown specificity.

CD4 T cells are important modulators of atherosclerosis. In experimental mouse models, $CD4^+$ T cells isolated from atherosclerosis-prone mice home to the atherosclerotic aorta and produce pro-atherogenic cytokines including IFN-γ[35, 36].T_H1 $CD4^+$ T cells express the lineage-defining transcription factor T-bet (gene name *Tbx21*) and the pro-atherogenic cytokine IFN-γ[35, 36]. The deletion of either *Tbx21* or *Ifng*, markedly reduce atherosclerosis[37, 38]. T_H2 cells express the lineage-defining transcription factor GATA3 and secrete interleukins (IL)-4, IL-5, IL-10, and IL-13[39]. Dependent on the context and model analyzed, T_H2 cells have been reported to contribute to atheroprogression or -protection[40, 41]. T_H17 cells are characterized by the transcription factor RORγt and secrete the cytokines IL-17A, IL-17F, and IL-21. The contribution

of T_H17 to atherosclerosis is controversial, as the hallmark cytokine IL-17 can hamper or enhance atherosclerosis[42-45]. This is likely related to different functions of different types of IL-17-expressing cells. Th17-Tregs have residual regulatory function (atheroprotective), whereas true Th17 cells and γδ T cells expressing IL-17 are pro-atherogenic. T_H9 T cells express the cytokine IL-9 and the transcription factor PU.1 and accelerate atherosclerosis[46].

T_{regs} prevent the progression of atherosclerosis by multiple mechanisms, including the secretion of IL-10 and TGF-β[47, 48]. At later stages of atherosclerosis, T_{regs} vanish[49] and may obtain a T_H1-like phenotype that includes expression of IFN-γ[28, 50]. While it was proposed that differentiation of T cells to distinct effector T_H cell lineages is final and irreversible, accumulating evidence suggests that T_H cells, and in particular inducible T_{regs} and T_H17 cells, are flexible in nature and can be reprogrammed to unique mixed phenotypes or can re-differentiate to other subset of T_H cells. T_H1-like T_{regs} expressing IFN-γ and Foxp3 are found in tissues and the peripheral blood of patients with type 1 diabetes, multiple sclerosis, and arthritis[51]. IL-17$^+$ T_{regs} are detected in rheumatoid arthritis, atopic asthma, and colorectal cancer, whereas GATA3$^+$Foxp3$^+$ cells are found in patients with food allergies. These findings suggest that the microenvironment promotes reprogramming of T_H cells under pathological conditions.

The first evidence for the presence of T cells expressing IFN-γ and IL-17[52] was found in human coronary artery-infiltrating T cells. These IFN-γ$^+$ IL-17$^+$ CD4$^+$ cells induced a pro-inflammatory response in vascular smooth muscle cells. In line with these data, another population of mixed-phenotype CD4$^+$ IL-17$^+$ Foxp3$^+$ cells showed higher frequencies in patients with cardiovascular disease compared to individuals with normal coronary angiography[53]. While accumulating evidence demonstrates the relevance of T cell and T_{reg} plasticity in atherosclerosis, mechanisms of generation and functions of these cells are difficult to dissect without detailed *in vivo* studies. Recent studies focusing on T cell plasticity in mice using scRNA-Seq, CyTOF, multicolor flow cytometry, and functional evaluation helped to understand the nature, phenotype and functions of T cells in atherosclerosis[5, 28, 34, 50, 54].

CD8 T cells express inflammatory cytokines, cytotoxins and death-inducing proteins including IFNγ, TNF, granzymes A and B, perforin, FasL and TRAIL. Although CD8 T cells are generally considered pro-inflammatory and cytotoxic, CD25-expressing CD8 regulatory T cells, present in humans and mice, may modulate immune responses[55]. Once they are activated by antigen-presenting cells in an MHC-I-dependent manner, CD8 T cells can potently induce target cell death via either cytotoxins, cytokines or death-inducing proteins[56]. In healthy arteries, CD8 T cells are found in the adventitia. CD8 T cell abundance increases in atherosclerosis, where they populate the plaque shoulder and the area around necrotic cores[57]. In human rupture-prone lesions, cytotoxic CD8 T cells constitute up to 50% of CD45$^+$ leukocytes[58]. CD8 T cells progressively accumulate but abruptly decline after plaque rupture[59, 60]. CD8 T cells promote atherosclerosis by increasing vascular inflammation and apoptotic cell numbers in lesions[61, 62]. CD8 T cells can also promote atherosclerosis by controlling monopoiesis and circulating monocyte levels[57, 63]. Some CD8 T cells recognize epitopes in ApoB, but most CD8 T cells in arteries have unknown specificities. Recently, a study comparing single cell transcriptiomes of immune cells isolated from plaques (carotid endarterectomy) and blood described abundant numbers of CD8 T cells with higher expression of activation markers in plaques. Exhausted CD8 T cells were also observed in this same study[8]. Expression of CD103 in lesion CD8 T cells with higher expression of CD69 compared to blood CD8 T cells are consistent with resident memory CD8 T cells[8]. CD103$^+$ CD69$^+$ tissue resident memory (TRM) CD8 T cells that are highly cytotoxic have a unique transcriptional profile, different from other CD8 T cells subsets[64]. Different transcriptional regulators are responsible for the development and maintenance of TRM CD8 T cells in different tissues[65]. TRM CD8 T cells show clonal expansion in plaques[8].

NKT cells are a minor T cell subset. They are considered a bridge between innate and adaptive immunity. NKT cells do not have immunological memory[66]. Besides innate receptor-dependent activation, NKT cells are activated by CD1d-bound glycolipid antigens[66]. Upon activation, they rapidly release large amounts of cytokines and cytotoxins[67], which can aggravate atherosclerosis[68-70]. Among different subsets, NKT cell research has been focused on invariant NKT (iNKT) cells, also known as type I NKT cells expressing an invariant TCR α chain (Vα14-Jα18 in mouse and Vα24-Jα18 in human). Phenotypically, NKT cells can be identified by using combinations of the NK cell marker, killer cell lectin-like receptor subfamily B member 1, also known as KLRB1, NK1.1 or CD161 and one of the T cell markers, CD3. Human and mouse work using lineage tracking or double immunostaining have identified the presence of NKT cells in atherosclerotic lesions[69, 71, 72]. Recent single-cell immunophenotyping methods have identified NKT cells in human and mouse atherosclerotic lesions[8].

γδ T cells were first reported in human atherosclerotic lesions in 1993[73], yet their exact role in atherosclerosis remains unclear. γδ T cells are a minor subset (1-10% of T cells)[74]. They express TCRs composed of γ- and δ-chains that are activated independent of MHC. Upon activation through innate immune receptors such as NKG2D or DNAM-1, γδ T cells can produce large amounts of inflammatory cytokines, chemokines and cytotoxins[75]. Grossly, they can be divided into $CD27^+$ γδ T cells and $CD27^+$ γδ T cells that secrete IFN-γ and IL-17, respectively[76]. Another subset of γδ T cells expressing the Vγ9Vδ2 TCR are reported to be cytotoxic[77]. Current research on γδ T cells in atherosclerosis reports inconsistent results; one study reported that hyperlipidemia increased γδ T cells population, yet had no effect on atherosclerosis[78]. Another study suggested that γδ T cells are atherogenic by promoting atherosclerosis via an IL-17-dependent mechanisms[79]. Recently, CyTOF-based mapping of lesion immune cells identified γδ T cells in plaque immune cells prepared from WD-fed *Apoe*$^{-/-}$ mice[80].

NK cells are cytotoxic cells that kill cells that lack expression of MHC-I. NK cells have no direct effect on atherosclerosis or lesion phenotype[81]. However, NK cells may contribute to atherosclerotic lesion formation when activayed, such as under conditions of chronic viral infection[81, 82].

B cells are adaptive immune cells, but unlike T cells, do not require the thymus for development. The B cell receptor (BCR) has V, (D) and J segments rearranged. This forms the "germline-encoded" set of BCRs, often with low affinity to antigen. Immature B cells display IgM and IgD with the same sequence as their BCR. In the presence of follicular helper T cells (TFH), B cells can switch their isotype to various IgG isotypes, IgE or IgA. In germinal centers, their BCR undergoes many cycles of affinity maturation. This is under the control of the AID enzyme and results in high affinity antibodies. B cells express CD19 and CD20.

Many IgM-producing B cells are B1 cells, further divided into B1a and B1b based on the expression of CD5. B2 cells are much more abundant than B1 cells in spleen, bone marrow and circulation and make IgG, IgE and IgA. B1 cells are more abundant in serosal cavities and adipose tissue including perivascular adipose tissue (PVAT) where they produce IgM locally[83]. Both B1 and B2 cells express MHC-II and can present antigenic peptides to CD4 T cells. One specialized type of B1 cells are Innate Response Activator (IRA) B cells, which are strongly pro-inflammatory by expressing *Csf2* (GM-CSF)[84]. Their role in atherosclerosis is unknown.

In healthy arteries, B cells are found in the adventitia[2] and perivascular adipose tissue (PVAT)[83]. B cells in the artery wall and adventitia of normal arteries probably do not secrete antibodies, but can traffic to other sites and become antibody-secreting plasma cells. Antibody production in the artery appears localized to PVAT[83] and artery tertiary lymphoid organs (ATLOs) adjacent to advanced plaques[85]. IgM and IgG to LDL and other antigens are present in

atherosclerotic vessels and the blood of subjects with atherosclerosis[86-89]. However, the contribution of circulating IgM and IgG versus local production in PVAT and ATLOs is unclear.

Integrated analyses of GWAS and whole blood expression data suggest a causal role for B cells in coronary heart disease[90]. Moreover, a substantial amount of experimental data implicate B cells as important immune cells in the development of murine atherosclerosis[91-96]. B cells regulate atherosclerosis lesion development in a subset-dependent manner[97-99] with B2 cells promoting atherosclerosis through production of inflammatory cytokines and germinal center-derived IgG[91, 95, 99-102] and B1 cells attenuating atherosclerosis through production of anti-inflammatory cytokines and natural IgM antibodies (NAbs)[87, 103-106].

Neutrophils are short-lived cells of the innate immune system. They are the main defenders against bacterial and fungal infections. A few neutrophils are present in normal arteries. Under conditions of atherosclerosis, some neutrophils are found on the endothelial surface and others in the plaque. Although their numbers are small, neutrophils have important roles in atherosclerosis and thrombosis[107]. Neutrophils, as all granulocytes, are highly granular cells with a distinct lobed nuclear morphology. They are typically produced in the bone marrow in a process called granulopoiesis. Under conditions of metabolic stress such has hypercholesterolemia, however, neutrophil production also occurs in extramedullary tissues including the spleen, thus contributing to heightened circulating neutrophil counts[108]. Neutrophils can be identified in mouse blood as well as in single cell suspensions of tissues including aortic tissue as $CD45^+CD11b^+Ly6G^+CD115^-$[109]. With Ly6G being a rather specific marker of mature neutrophils, antibodies to Ly6G can be used to identify neutrophils in sections of atherosclerotic lesions. Expression of Cre recombinase under control of Ly6G has provided a tool to specifically manipulate neutrophils, e.g. for neutrophil tracking or cell-specific gene deletion[107, 110].

While aortas from *Apoe*$^{-/-}$ mice on chow diet contain only a limited number of neutrophils, their proportion increases to 7-10% after four weeks of high fat diet feeding[5, 111]. In cross sections of atherosclerotic lesions, neutrophils account for up to 5% of lesional cells[107, 111]. However, given the short live span neutrophils typically exhibit, low counts at any given time may simply be a reflection of their vast turn over in the tissue. Under conditions of neutrophilia as caused by infection, stress or sleep fragmentation, neutrophil counts in digested aortas and lesion cross sections can drastically increase[112-114].

Monocytes are also innate immune cells, which typically express CD115 (Csf1r) and CD11b. In mice, they come in two main flavors, classical and non-classical. Classical monocytes express Ly-6C and the chemokine receptor CCR2[115]. Non-classical monocytes highly express the transcription factor Nr4a1[116] and the chemokine receptor CX3CR1; in contrast, they have little or no expression of Ly6C and CCR2[117]. In healthy arteries, monocytes are rare. However, in atherosclerosis-prone regions (such as the lesser curvature of the ascending aortic arch), classical monocytes can be recruited through VCAM1-dependent interactions on vascular endothelium[118, 119]. Monocytes rapidly infiltrate atherosclerotic lesions. Some differentiate into macrophages, others to monocyte-derived dendritic cells, and some stay monocytes. Some monocytes express high levels of MHC-II and can present antigenic peptides to CD4 T cells. Non-classical monocytes patrol the endothelial surface and promote endothelial integrity[120]. Patrolling is highly intensified under conditions of atherosclerosis[121, 122]. Nonclassical monocytes patrol in response to oxidized LDL and ingest greater amounts of oxidized LDL than classical monocytes, processes that are dependent upon the scavenger receptor CD36[122].

Dendritic cells (DCs) are found in secondary lymphoid organs, where they present antigens to naive T cells and start a specific immune response. DCs can be divided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Among the conventional DCs, lymphoid organ resident $CD8\alpha^+$ and non-lymphoid organ $CD103^+$ type 1 cDC1 expressing Xcr1 and Clec9a (DNGR1)

can be discriminated from cDC2[123]. Plasmacytoid DCs produce type I interferons (IFNα and β)[124]. Due to difficulties in specific targeting, the function of cDCs in atherosclerosis remains controversial. pDCs promote atherosclerosis[125, 126].

Macrophages are the most common and probably the most important cell type in atherosclerosis. They express F4/80 and CD64, which is the activating Fcγ receptor-III. In healthy arteries, macrophages are in the adventitia, where they contribute to the physiology and diameter of the vessel wall via cross-talk with smooth muscle cells[127]. These vascular macrophages are largely derived from embryonal precursor cells and self-renew in situ[15]. A small subset of macrophages is located below the endothelium in certain areas of the arterial circulation such as the aortic arch. Atherosclerosis is characterized by an accumulation of a very large number of macrophages, derived from infiltrating monocytes and proliferating vascular macrophages[16]. Macrophages function to clear apoptotic cells (efferocytosis), to phagocytose debris, to produce inflammatory and inflammation-resolving cytokines and lipid meditators, and to present antigens[128, 129]. In addition to resident vascular macrophages[15], Trem2$^+$ macrophages have been identified in atherosclerosis[130] and in other diseases[131-136].

3. Meta-Analysis of scRNA-Seq Data

This review and meta-analysis is based on scRNA-Seq and CyTOF data from mouse aortas (figure 1). scRNA-Seq data from 9 data sets (table 1) were analyzed using the latest bioinformatics integration metho,d Harmony[137] . Harmony allows to simultaneously account for multiple experimental and biological batches across data from different labs. After quality control, a total of 15,288 cells were projected into a shared embedding in which cells were assigned and corrected for dataset-specificity using fuzzy clustering. Then, all cells were visualized using the non-linear high-dimensionality deduction method, UMAP (Uniform Manifold Approximation and Projection)[138]. This Louvain-based clustering method was then used with the Seurat method[139] that yielded 17 clusters (figure 2). One cluster had an overwhelming proliferation signature. When regressing out cell cycle genes, this cell cluster seemed to contain macrophages, T and B cells. One cluster contained *Acta2*$^+$ cells that were not leukocytes, but smooth muscle cell-derived foam cells. The remaining 15 clusters distributed into 4 macrophage clusters, 2 monocyte/DC clusters, one DC cluster, one neutrophil cluster, one NK cell cluster, 2 B cell clusters, 3 T cell clusters and one innate lymphoid cell (ILC) cluster.

Overall, macrophages are the most abundant cell type in atherosclerotic mouse aortas. The Louvain clustering algorithm implemented in Harmony clearly identified four subsets. We also identified two monocyte/DC subsets and one cluster of Xcr1$^+$ cDC1 cells *(Xcr1, Irf8*). Xcr1 is a chemokine receptor characteristic of cDC1. One of the clusters of the monocyte/DC clusters has a gene signature suggesting it may contain classical monocytes, the other a mixture of monocyte-derived DCs, and yet unidentified cells. The B cell compartment shows 2 clusters. The B1-like cluster is enriched in B1 cell genes and the B2-like cluster shares gene expression with germinal center and marginal zone B cells. The three T cell clusters include the expected CD8 T cells, CD4$^+$ Th17-like cells and an unexpected subset of CD4$^+$CD8$^+$ T cells. The fourth cluster, although located right next to the T cells, mainly contains ILC2 cells based on marker genes like *Klrg1, Il1rl1* and *Areg* and the absence of *Cd3* expression. It is striking that Th1 cells, shown to be abundant in atherosclerotic lesions in mice[140] and humans[141] by immunostaining, do not appear to form an identifiable cluster. However, Tbx21$^+$ T cells are found in the T cell and NK cell clusters (supplemental figure 1). Similarly, Tregs are not easily identified in scRNA-Seq experiments. This is likely due to technical problems, because low-expressed transcription factors like FoxP3 are often missed by scRNA-Seq. Also, FoxP3 expression can be pulsatile. Although they do not form a separate cluster, we find a few FoxP3$^+$ CD4 T cells on the edge of

the Th17-like cluster (supplemental figure 1). Th1 Tregs[50, 142], a type of T cells with features of Tregs and Th1 cells, were not found in in this analysis.

The cellular composition of mouse aortaic leukocytes varied widely among the 9 data sets (figure 3). This is likely due to differences in the experimental protocols used (table 1) and to biological differences between the different atherosclerosis models. Some datasets show a bias towards or directly focus on macrophages[143, 144], while others are more biased towards lymphoid cells, especially Winkels et al.[5]. A very interesting finding is that the foam cells, identified by Bodipy staining in Kim[143], are exclusively Trem2 macrophages[145]. Thus, based on this meta-analysis, we propose to call this cell type foamy Trem2 macrophages, a classic cell type in atherosclerosis[128]. About half of the foam cells as defined by Bodipy staining are non-leukocytes, probably smooth muscle cells, as expected[146, 147].

CD8 T cells are found in all data sets except in the sorted foam cells[143] and in the regression study that focuses on flow cytometry purified CX3CR1^{+} CD11b^{+} myeloid cells[144]. Th17-like T cells are probably a mixture of Th17 and γδ T cells. The ILC2 cluster was only found in the Cochain[130] and Winkels[5] datasets. Interestingly, these cell types are already present in the healthy aorta, prior to onset of atherosclerosis, whereas the CD8 T cells are rare in healthy aortas[130]. CD4^{+}CD8^{+} T cells are present in healthy and atherosclerotic aortas.

Among the B cells, B2-like cells are found in healthy aortas and are less abundant at 11 or 20 weeks of high fat diet[130]. B1-like cells are only found in the Winkels dataset[5], which contains data from *Apoe*$^{-/-}$ mice. When comparing the different models of atherosclerosis, it seems that the *Apoe*$^{-/-}$ model shows preferential accumulation of B2 cells and the appearance of a small cluster of B1 cells.

As expected[15], the healthy aorta contains only resident macrophages. Also expected, macrophage frequencies increase with the duration of high fat diet in *Ldlr*$^{-/-}$ mice[130] and with western diet in *Apoe*$^{-/-}$ mice[5]. Interestingly, macrophage diversity increases with atherosclerosis progression. Four clear subsets are seen in all datasets: Trem2+ foamy macrophages, resident macrophages, inflammatory macrophages and IFNIC macrophages.

The gene expression driving the various cell types is presented as a dot plot (figure 4). Inflammatory macrophages highly express the inflammatory chemokines *Ccl4, Cxcl2, Ccl3* and *Ccl2*. They also express *Cd14* at a higher level than the other macrophage subsets. Many express *Il1b* and *Tnf* and a few express *Cxcl1*. The Trem2 foamy macrophages have a gene expression profile that partially overlaps with the inflammatory macrophages. They highly express *Lgals3*, the gene encoding galectin-3, a known biomarker for atherosclerosis[148], the tetraspanin *Cd9* and *Ctsd*, the gene encoding cathepsin D. Some express the fatty acid binding protein *Fabp5*. Resident macrophages are very different from the other subsets. They express the chemokine *Ccl8*, the chemokine-like molecule CXCL4 (*Pf4*), which was previously thought to be platelet-specific[149], coagulation factor XIII (*F13a1*) and *Wfdc17*. Some express *Lyve1*, a marker associated with tissue resident perivascular macrophages in the aorta[15, 127, 150]. The IFNIC macrophages have a type I interferon signature, prominently expressing *Isg15, Irf7, Ifit 1* and *Ifit 3*. They express the chemokine *Ccl12*, which encodes an important ligand for CCR2.

A few neutrophils are found in all data sets except the sorted foam cells, even in healthy aorta. This is somewhat surprising and unlikely to be due to blood contamination, as Cochain[130] used intravascular CD45 staining to exclude blood contamination. Thus, this finding suggests that a few neutrophils may be present in the mouse aorta even under control conditions. Neutrophils share many genes with monocytes and macrophages, but characteristically express *Cd7, S100a9* and *S100a8*.

The B cell clusters express typical B cell genes like CD79a and b, Ccr7 and Mzb1. The expression profile between B1-like and B2-like cells shows only minor differences

Two monocyte/DC subsets are well represented in all datasets with atherosclerosis. As mentioned above, few monocytes are present in healthy aortas, and monocytes are not found among foam cells.

The cluster denominated classical mono, DCs contains cells expressing genes found in classical monocytes, i.e. *Lyz1*, and the chemokine receptor *Ccr2*, but also *Fn1*, encoding fibronectin, *Retnla* encoding Relm-α, and the chemokine *Ccl6*. The other monocyte cluster (mixed mono, DCs) expresses aspartic peptidase *Napsa1* and *Cd209a*, encoding DC-SIGN, which may suggests the presence of monocyte-derived DCs. This cluster also shows expression of interferon genes *Ifi30* and *Ifitm1*. The Xcr1$^+$ DCs highly express the transcription factor *Irf8*, *Cst3* encoding cystatin 3, the N-acylethanolamine acid amidase *Naaa*, the N-acylgalactosaminidase *Naga* and the phospholipase *Plbd1*.

NK cells express *Nkgh7, Klre1, Klra7, Klrk1* and granzyme B (*Gzmb*). Interestingly, they also express the chemokine *Xcl1*, the only known ligand for dendritic cell receptor Xcr1, and the chemokine *Ccl5*, also known as RANTES.

ILC2 cells are negative for CD3-encoding transcripts (*Cd3d*, *Cd3g*) and express genes associated with ILC2 (*Rora*, *Gata3*, *Areg*, *Il1rl1* encoding ST2). The Th17-like T cells express CD3-encoding genes, but are negative for *Cd4*, *Cd8a* or *Cd8b1* transcripts. These cells are enriched for *Il17a* expression. This cluster likely contains γδT cells also expressing lymphotoxin B (*Ltb*), *Il7r* and *Icos*.

Meta-Analysis of CyTOF Data

Two of the studies of mouse aortic leukocytes[5, 80] used CyTOF to characterize the immune cell landscape of *Apoe*$^{-/-}$ mouse aortas. Each study used 35 markers, 33 of which were conjugated to monoclonal antibodies recognizing cell surface and intracellular molecules. Although the number of markers (33) is much smaller than in scRNA-Seq (~2,000), these markers are very informative, because they were picked based on 30 years of experience with flow cytometry and 15 years of experience with flow cytometry of mouse aortas[2].

Cole[80] used 19 myeloid markers, 5 T cell markers, 2 B cell markers and 8 others. They identified 4 macrophage subsets (table 2). All macrophages expressed CD11b, CD64 and CD68[151]. High levels of CD64 distinguish macrophages from other mononuclear phagocytes including monocytes Markers that differed among subsets were CD11c$^+$CD44$^+$ for Mac 1, CD11c^{low} CD44$^+$ CCR2+ CD206^{med} CD169$^-$ for Mac 2, CD11c$^-$ CD44$^-$ CD206$^+$ CD169$^+$ CD209b$^-$ for Mac 3 and CD11c$^-$ CD44$^-$ CD206$^+$ CD169$^+$ CD209b$^+$ for Mac 4.

Three of these four subsets were also identified in Winkels et al[5], who used 7 myeloid, 7 T cell, 7 B cell and 10 other markers. Again, all macrophages expressed CD11b and CD64; CD68 was not in the panel of Winkels et al. CD68 in Cole et al. was expressed by all clusters of monocytes, macrophages and dendritic cells, including pDCs[80]. Winkels' Mac cluster 14 was CD11c^{med} and CD43$^+$ and likely corresponds to Mac 1 in Cole[80]. Winkels' Mac cluster 3 was CD11c$^-$ and expressed CD103 and FcεR1. It might correspond to Cole's Mac 3 or Mac 4, which are CD11c$^-$ CD206$^+$ CD169$^+$ CD209b$^\pm$. Winkels' Mac cluster 9 was also CD11c- but expressed CD160 and CD138. Cole et. Al.[80] identified a shift in macrophage populations induced by Western diet, with a reduction in resident-like macrophages (CD11c$^-$ CD206$^+$ CD169$^+$) and an increase in the CD11c$^+$ CD44$^+$ population.

Both Cole[80] and Winkels[5] found the two known subsets of mouse monocytes, classical and non-classical. Both subsets express CD11b and CD68. Ly6C$^+$ classical monocytes expressed CD68, CCR2, CD44, CD43 but not MHC-II or CD11c (table 2). Ly6C$^-$ nonclassical monocytes expressed CD68, CD43, CD44 and some MHC-II and CD11c. It is interesting that the CyTOF studies both identify classical and non-classical monocytes in atherosclerotic mouse aortas, but the scRNA-Seq data (figures 2 and 4) do not yield such clear signatures. This suggests that the monocytes may rapidly change their transcriptome upon entering the arterial wall.

Both Winkels and Cole found cDC1 cells, called cluster 16 in Winkels et al.[5]. They expressed CD68, CD11c, MHC-II, CD103 and XCR1, but little CD11b. cDC2 (cluster 22 in Winkels) also expressed CD68, CD11c and MHC-II, but not CD103. Instead, they expressed CD11b, CD41 and CD172a. Winkels found a third DC subset (cluster 10) that differed from cluster 22 by lower expression of CD11b and absence of CD41, while Cole identified pDCs defined by expression of B220 and SiglecH.

CyTOF identified two granulocyte populations, neutrophils ($CD11b^+Ly6C^+Ly6G^+$) and eosinophils ($CD11b^+$ $SiglecF^+$ $F4/80^+$). B lymphocytes ($CD19^+$) were clustered into 4 clusters in Winkels et al.[5] based on the expression of B220, CD117, IgM and CD43, and 2 clusters ($B220^+$ and $B220^-$) in Cole et al.[80].

The two studies showed 5 common T lymphocyte populations including $CD4^+$ $Ly6C^-$, $CD4^+$ $Ly6C^+$, $CD8^+$ $Ly6C^-$, $CD8^+$ $Ly6C^+$ and $TCR\beta^-$ $TCR\gamma\delta^+$ cells[5, 80]. Additionally, the Winkels panel identified $CD4^{low}$ $CD5^{low}$ $CD8^{high}$ $FR4^-$ and $TCR\beta^{low}$ IgM^{low} $CD11c^{low}$ T cell clusters[5].

NK cells that express NKp64 and NK1.1 were identified in both studies[5, 80] and innate lymphoid cells defined as $CD3\varepsilon^-$ $TCR\beta^-$ $CD90.2^+$ $IL7R\alpha^+$ were detected in Cole et al.'s study[80].

Overall, the CyTOF studies, albeit more limited in scope when compared to the scRNASeq date, offer a solid steer on an extensive panel of antibodies that are useful to disambiguate the leukocyte populations within the atherosclerotic aorta (e.g. macrophages from dendritic cells), leading the way towards more effective and multi-analyte lesion phenotyping. Further integration of CyTOF with scRNASeq-led markers will further refine our capture of other subsets that emerge from the scRNASeq, including Trem2 foamy macrophages.

Macrophage Subsets in Atherosclerosis

As revealed by our meta-analysis, four subsets of macrophages, namely resident-like macrophages, foamy Trem2 macrophages, inflammatory and IFNIC macrophages are distinguishable in atherosclerosis.

Resident-like macrophages expressed genes previously associated with resident aortic macrophages, such as *Lyve1* and *Mrc1*[15, 127]. These marker genes have been described in yolk sac-derived and embryonic precursor-derived tissue resident macrophages in the adventitia of the aorta and other organs. GSEA analysis of the transcriptome of resident aortic macrophages, obtained by Affimetryx[15], and our integrated scRNA-seq data set indeed showed that vascular macrophages of healthy mouse arteries were most similar to the resident-like macrophages found in healthy and atherosclerotic mouse aortas[130]. Resident-like macrophages are not found in the normal arterial intima, but it is possible that these cells may also be located in the atherosclerotic plaque.

Within the population of resident-like macrophages, Cochain et al. identified a subset of macrophages that showed higher expression of Ccr2 (but lower expression of Lyve1)[130], suggesting that atherosclerotic aortas may contain monocyte-derived macrophages that adopt a phenotype similar to bone fide resident aortic macrophages, which self-renew independently of CCR2-dependent monocyte recruitment[15].

Among the most abundant genes expressed in this cell cluster (figure 4), resident-like macrophages also showed enrichment in Pf4. Pf4 was originally thought to be specific for platelets and megakaryocytes. Pf4 expression was proposed to reflect platelet/macrophage conjugates. However, several studies in reporter mice driven by Pf4-Cre have demonstrated widespread macrophage expression of this gene, notably in peritoneal macrophages[152] and (peri)vascular macrophages[153]. Thus, Pf4 can no longer be considered platelet-specific. If Pf4-Cre mice are used to drive a gene modification, the intended gene modification will occur in megakaryocytes, platelets and resident macrophages of the peritoneum and artery wall.

The Trem2 macrophage population predominantly found in atherosclerotic aortas was identified by Cochain et al[130]. This macrophage population displays high expression of *Mmp12, Mmp14, Itgax* (CD11c) and markers of lipid loading (*Abcg1, Trem2, Fabp4*), lysosomal cathepsins (*Ctsd*, *Ctsl*) *Cd9*, and *Spp1* (osteopontin). Establishing a flow cytometry-based method of lipid staining of macrophages, Kim et al. detected lipid-laden foam cells in the intima of atherosclerotic aortas[143]. Cross-comparison of data from Kim and Cochain et al. indicated that foamy macrophages and Trem2^{hi} macrophages are identical[145]. Gene ontology or KEGG pathway analysis assigned foamy Trem2 macrophages putative functions in lipid metabolism, cholesterol efflux and lysosome function[130, 143]. Foamy Trem2 macrophages had low inflammatory gene expression, in line with prior findings showing that foam cells are characterized by low expression of inflammatory-response genes[154]. This resolves an important controversy: atherosclerotic arteries show a pro-inflammatory milieu[155, 156], yet foam cells are not pro-inflammatory[154]. With the improved resolution of macrophage subsets provided by scRNA-Seq and CyTOF, it is now clear that the Trem2 macrophages[130] are the foam cells[143], but do not supply the inflammatory cytokines and chemokines so characteristic of atherosclerotic arteries.

Mononuclear phagocytes sharing transcriptomic features with aortic foamy Trem2 macrophages have been observed across various disease contexts in mice. In particular, this signature is associated with disease-associated microglia in neurodegeneration[131, 132], and demyelinating disease[133], non-alcoholic hepatosteatosis- and fibrosis-associated macrophages in the liver[134, 135], and lipid-associated macrophages in obese adipose tissue[136]. How these cells acquire this specific transcriptomic signature remains to be fully determined. Recent evidence implicates TREM2 itself and downstream control of cholesterol metabolism in phagocytes[131-133, 136], and a TREM2-ApoE pathway[157]. Insights from other diseased tissues suggest that macrophage ontogeny may have little impact on acquisition of the foamy Trem2 signature. Both yolk sac-derived microglia[131] and monocyte-derived macrophages in adipose tissue[136] can acquire the foamy Trem2 macrophage state. Importantly, results so far indicate that ApoE deficiency does not affect acquisition of the foamy Trem2 macrophage transcriptional signature in atherosclerosis (Cochain et al.[130] and the meta-analysis here).

Inflammatory macrophages[130]**,** primarily evidenced in atherosclerotic aortas by scRNA-seq, show enrichment in inflammatory transcripts, including the chemokines *Cxcl1*, *Cxcl2, Ccl2*, *Ccl3*, *Ccl4,* and the inflammatory cytokines *Il1b*, and *Tnf*. Inflammatory macrophages, also designated as chemokine^{high} macrophages[144] or non-foamy macrophages[143], show a strong proinflammatory gene profile. They are not foam cells and express CCR2, suggesting they are likely derived from blood monocytes. How Trem2 macrophages and inflammatory-macrophages are interrelated is currently unclear. An algorithm to predict differentiation pathways[158] from scRNA-seq data suggested that differentiation pathways into these macrophage subsets may be distinct and independent from each other[156]

These three main aortic macrophage populations have been observed across studies and in three distinct models of atherosclerosis in mice: *Ldlr*$^{-/-}$ mice, *Apoe*$^{-/-}$ mice[5, 130, 143], and in progressing and regression atherosclerotic lesions of C57Bl/6 mice treated with PCSK9-AAV[144].

Interferon-inducible macrophages (IFNIC) form a small cluster with a signature characteristic of a type 1 interferon response. IFNIC are enriched for numerous interferon-inducible genes including *Ifit3, Irf7 and Isg15.* IFNIC were found in the two studies with large numbers of macrophages[143, 144], but not by Cochain et al.[130] or Winkels et al.[5] Identification of the IFNIC cluster was likely favored by the large number of macrophages sequenced in[80, 84]. After the meta-analysis (figure 3), small numbers of IFNIC macrophages became visible in the Winkels[5] and Cochain[130] studies. Thus, the present meta-analysis resolves a third issue in the field, whether there are 3 or 4 macrophage subsets in atherosclerotic arteries. The answer is there are 4, but the IFNIC subset is smaller than the others.

IFNIC macrophages are reminiscent of macrophages described in the ischemic heart by King et al.[159]. IFNIC may originate from remote type I IFN-mediated priming of monocytes and their progenitors in the bone marrow after consecutive tissue injury[160]. However, macrophages with a similar gene expression signature have been found at steady state in the heart[161]. Thus, it is not clear whether IFNIC macrophages serve a homeostatic role or whether they are strictly disease-induced. Given the pro-atherogenic role of type I IFN signaling[162], this cell cluster could be relevant to disease progression.

Relationship between Macrophage Subsets Identified by scRNA-Seq and CyTOF

How the 4 macrophage subsets identified by scRNA-Seq correspond to the 4 subsets identified by CyTOF is not completely clear. Future work with CITE-Seq where the cell surface phenotype and the transcriptome is available for the same cells[8] may provide a definitive answer. However, at this point, the evidence suggests that the resident-like, foamy Trem2 and inflammatory macrophage populations may correspond to the main macrophage populations identified by mass cytometry. The largest macrophage cluster displayed a $CD11b^+$ $CD64^+$ $CD206^+$ $CD169^+$ cell surface phenotype, and could further be discriminated by expression of the C-type lectin receptor CD209b (SIGNR1) into $CD209b^-$ and $CD209b^+$ cell clusters[80]. A large majority of these $CD206^+$ macrophages also expressed other resident macrophage markers such as Lyve1 and Tim4 (Monaco et al., unpublished observation). Thus, $CD11b^+$ $CD64^+$ $CD206^+$ $CD169^+$ macrophages, encompassing Mac 3 and Mac4 in Cole et al.[80] likely correspond to the resident-like macrophages identified by scRNA-seq.

Another cluster of vascular macrophages expressed CD11c, CD44, CD11b and CD64 on the cell surface but lacked resident markers such as CD206 and CD169[80]. MHCII expression appeared to subdivide this subset further into two clusters, however, it is yet to be determined whether these two are functionally or ontogenetically distinct. Given high expression of CD11c in foamy intimal macrophages[143], this cell cluster may correspond to foamy Trem2 macrophages. Mass cytometry also identified a small macrophage subset expressing monocyte chemokine receptor CCR2 and low/intermediate levels of CD206[80], which may map to inflammatory macrophages.

Macrophage and Smooth Muscle Foam Cells

It has been postulated that vascular smooth muscle cells can transdifferentiate to macrophage-like cells in murine and human atherosclerotic lesions[146, 147, 163]. These cells acquire some macrophage markers like CD68. However, scRNA-Seq of fate-mapped SMCs demonstrated that smooth muscle cells transform into unique fibroblast-like cells, termed 'fibromyocytes', rather than into macrophages. Their portfolio of macrophage genes is limited[9]. Such 'fibromyocytes' are not recovered by cell isolation strategies focusing on leukocytes or cells derived from $Cx3cr1^+$ precursors[5, 130, 144]. However, in a study focused on foam cells[143], almost half of the foam cells are smooth muscle cell-derived, as indicated by expression of smooth muscle α actin (*Acta2)* (figure 3). Thus, the present meta-analysis corroborates that smooth muscle cells significantly contribute to foam cells, but do not become macrophages.

Comparing Macrophage Subsets Defined by scRNA-Seq, bulk RNA-Seq and Gene Chips

It is also not clear how the four macrophage subsets identified by scRNA-Seq correspond to the four macrophage subsets GFP^+, YFP^+, GFP^+YFP^+ and unlabeled in Cx3cr1-GFP^+/CD11c-YFP *Apoe*$^{-/-}$ mice[164]. McArdle et al. re-analyzed the macrophage data from the Winkels[5] study. Using genomic gating, they found 4 macrophage subsets (rather than the 3 originally reported in Winkels). One of the macrophage subsets identified by scRNA-Seq expressed *Cx3cr1*, one expressed *Itgax,* one expressed both and one expressed neither. Thus, it is possible that the GFP^+ macrophages correspond to the *Cx3cr1*$^+$, the YFP^+ to the *Itgax*$^+$, the GFP^+YFP^+ to the *Cx3cr1*$^+$*Itgax*$^+$ and the unlabeled to the *Cx3cr1*$^-$*Itgax*$^-$. To address this question, we formally

mapped the four subsets from the McArdle study to the 4 subsets from the Cochain and Winkels studies[5, 130]. We compared the genes that were significantly up- or downregulated in one macrophage subset versus the three others in McArdle et al. with the genes that were significantly up- or downregulated in one macrophage subset versus the three others in the present meta-analysis (figure 5). Cx3cr1-GFP macrophages were most similar to resident macrophages as suggested by 36 genes upregulated in common (figure 5) and 10 genes downregulated in common. CD11c-YFP macrophages were most similar to Trem2 foamy macrophages suggested by 21 genes upregulated in common (figure 5) and 24 genes downregulated in common. GFP+YFP+ cells did not map clearly. They showed 11 and 12 genes downregulated in common with inflammatory and Trem2 foamy macrophages, respectively. Thus, based on the intravital 2-photon microscopy analysis in [164] the Trem2 foamy macrophages may be motile and the resident macrophages not.

Monocytes in Atherosclerosis

Monocyte recruitment intensifies in the setting of vascular inflammation. Parabiosis studies have shown that recruited monocytes persist within the tissue or become tissue macrophages in early lesions[16, 165, 166]. This is supported by observing the integrated data from *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice under normo- and hypercholesterolemic conditions. Monocytes have been characterized in the bone marrow and blood using RNA sequencing of sorted monocyte subsets. These studies revealed the enrichment of several notable genes including *Lgals3*, *Mmp8*, Ccr2*, Ly6c2*, and *Cebpd* in classical Ly6C^{+} monocytes. Nonclassical Ly6C^{int}/Ly6C^{-} monocytes expressed elevated levels of *Nr4a1*, *Cebpb*, *Mef2a*, *Pparg*, *Cd209a*, and *Itgal* transcripts[167]. The monocyte population in the atherosclerotic aorta does follow a classical Ly6c^{+} (Ly6c2a^{+}Ccr2^{+}) or non-classical Ly6c^{-} (Ccr2^{-}Nr4a1^{hi}Cx3cr1^{hi}) transcriptomic profile as defined by Thomas and colleagues[168] and Mildner and colleagues[167]. Thus, aortic monocytes possess characteristics of both classical and non-classical monocytes. It remains to be determined how soon and how completely they differentiate into macrophages or monocyte-derived DCs. Monocyte differentiation programs may initiate soon upon entry to the atherosclerotic lesion or adventitia. However, in a model of skin inflammation, monocytes have been shown to retain transcriptomes similar to blood monocytes during their migration to draining lymph nodes[169]. Indeed, the meta-analysis conducted on scRNA-Seq data from atherosclerotic aortas shows gene signatures compatible with classical and non-classical monocytes.

The present meta-analysis shows that the vast majority of monocytes were detected in aortas of WD or HFD-fed mice. Furthermore, when comparing cell populations from CD-fed *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice, the small number of monocytes contributed from “healthy” mice are from the *Apoe*$^{-/-}$ contributed cells. This is likely due to the 2-fold elevated circulating plasma cholesterol in *Apoe*$^{-/-}$ mice that occurs under basal conditions, which is known to induce spontaneous lesion formation in the absence of diet-induced hypercholesterolemia[170]. This is in keeping with previous functional studies reporting that the recruitment of classical monocytes to atherosclerotic lesions begins to increase approximately 2 weeks after the initiation of diet-induced hypercholesterolemia[171] and that monocyte recruitment to the ascending aorta is elevated in 10 week-old *Apoe*$^{-/-}$ mice[119]. It remains to be fully understood why there is a delay between the recruitment of monocytes to nascent lesions (immediately) and the detection of macrophage foam cells (detectable within a few days of initiating hypercholesterolemia). Although recruitment of monocyte persists in advanced lesions[119, 165, 166], parabiosis experiments showed that recruited monocytes contribute minimally to lesion macrophages[16].

Hypercholesterolemic mice also display monocytosis in the bone marrow[166]. Hypercholesterolemia in mice expands monopoiesis, resulting in increased numbers of Ly6C^{+} monocytes in blood[172]. High levels of hypercholesterolemia in mice induce the formation of foamy monocytes[173]. The cytoplasmic lipid droplets result in a high side scatter when analyzed by flow cytometry. Monocyte lipid uptake is associated with upregulated expression of CD11c,

chemokine receptors, and activation of α4 integrin (CD49d), which mediates adhesion to vascular cell adhesion molecule-1[122, 174]. Studies designed to detect monocyte fate-differentiation during atherosclerotic lesion development will require the utilization of peripheral monocyte-inducible systems (Ccr2 or Ms4a3) with periodic retrieval to map differentiation programs from these cells. These approaches will answer long-standing questions regarding kinetics of fate-specification and heterogeneity of differentiation programs of monocytes entering inflamed lesions.

B Cells in Atherosclerosis

Although some B cells are found, especially in the *Apoe*$^{-/-}$ data set[5] (figure 3) but also in the healthy aorta[130] (figure 3), single cell analyses of atherosclerotic plaques show that B cells are not a predominant cell type in atherosclerotic aortas[5, 8, 83]. Resting B cells do not produce antibodies, but can differentiate to antibody-producing plasma cells in supportive niches of the spleen and bone marrow. Hence, it is not surprising that antibody production in the artery wall itself is minimal[83]. However, antibodies can be produced in aortic tertiary lymphoid organs (ATLO)[175, 176] located in the adventitia adjacent to atherosclerotic plaques and in perivascular adipose tissue (PVAT) of aged mice. In 1 year-old mice, B cells are abundant in ATLOs[5, 83] and may be more differentiated. Earlier studies[2, 97] suggesting a greater abundance of B cells in atherosclerotic vessels likely included the adventitial and PVAT compartments. Flow cytometry[83], CyTOF and RNAseq[5] confirm the presence of both B1 and B2-like cells in atherosclerotic vessels. Although the exact location is not known, many of them may reside in the adventitia and PVAT. These findings underscore the need to further study adventitia and PVAT as vascular compartments important in immune regulation of atherosclerosis. Moreover, future characterization of the unique BCR sequences associated with different B cell subsets and functions will provide important insights into the specific antigens that are recognized and enhance our understanding of B cell immunity in the context of atherogenesis.

Neutrophils in Atherosclerosis

While neutrophils are consistently found in atherosclerotic lesions and aortic tissue when using antibody-based detection methods, some studies employing scRNAseq of both human and mouse atherosclerotic tissue have failed to present a neutrophil cluster[5, 8]. This discrepancy is likely explained by the very low mRNA content of neutrophils when compared to other leukocyte subsets. In addition, neutrophils are rich in easily releasable ribonucleases that rapidly and potently degrade endogenous RNA[177]. Regardless of such intrinsic obstacles, neutrophils have been detected in healthy and atherosclerotic arteries by scRNAseq[130], and in this meta-analysis. In atherosclerosis, these neutrophils have recently been proposed to segregate into *Siglecf*^{hi} and *Siglecf*^{low} neutrophil subsets, similar to the neutrophil subpopulations infiltrating the heart after myocardial infarction[178]. In this meta-analysis we report *Siglecf*^{hi} neutrophils in atherosclerotic but not healthy aortas, a new discovery.

T cells in Atherosclerosis

Four studies employed single cell RNA-sequencing of flow-sorted CD45^{+} leukocytes in murine and human atherosclerosis[5, 8, 130, 179]. In these studies, T cells were identified as cells expressing the mRNA coding for CD3d or CD3e (*Cd3d*, *Cd3e*). Coding genes for the T cell receptor (TCR) as frequently used in flow cytometry are less reliably found in scRNAseq data sets. In these studies, T cells were present at all stages of atherosclerosis development. Depending on models, diets and time points, *Cd3e*$^{+}$ cells accounted for ~46 to 65% of leukocytes enzymatically released from mouse aortas. The presence of *CD3d/e*$^{+}$ cells in different layers of atherosclerotic arteries has been inferred from a genetic deconvolution strategy of microdissected tissues[180] with highest absolute cell numbers in atherosclerotic lesions and the highest fraction among all leukocytes in the adventitial layer of established atherosclerosis. In

aged mice, the adventitia may contain arterial tertiary lymphoid organs (ATLOs)[5, 180, 181]. Direct scRNAseq of adventitial leukocytes confirmed the presence of T cells in WT and *Apoe*$^{-/-}$ mice in the adventitia of aortas from 12-week-old mice on a chow diet[179]. Notably, T cells were also found by scRNAseq in healthy arteries from *Apoe*$^{-/-}$ mice[5]. Fractions of *Cd3d*/*e*$^{+}$ cells among all lesional leukocytes seem to be relatively higher in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice fed with a chow diet (61 and 54%, respectively), while the fraction of monocytes and macrophages increases during WD and HFD feeding in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice. This suggests that in the setting of aggravated atherosclerosis, the contribution of myeloid cells increases relatively to T cells. In humans, CD4^{+} and CD8^{+} T cells account for ~65% of all leukocytes in carotid endarteriectomy specimens as measured by CyTOF[8]. These results are at odds with the predominance of macrophages and the low percentage of T cells (5 to 20%) in immunohistochemistry of atherosclerotic plaques[1, 182], suggesting that enzymatic isolation, which is required before flow cytometry and scRNAseq, may overestimate relative T cell content due to a loss of fragile myeloid cells including macrophages during tissue digestion. Genetic deconvolution of human carotid plaque gene expression data sets[183] suggests that T cells may account for about 1/8 of all leukocytes, while macrophages dominate in atherosclerotic plaques.

The role of T cells in atherosclerosis was recently reviewed[184]. As introduced above, phenotypes of T cells are traditionally attributed to lineages that are defined by the expression of specific transcription factors and cytokines[185]. In CD4 T cells, the expression of adhesion and chemokine receptors correlates with T_H-lineage assignment and may be used as surrogates of T_H phenotypes[24]. One technical limitation of scRNAseq, in particular of commercial drop-sequencing approaches, is the only incomplete coverage of transcription factor expression at a single cell level. Transcription factors are not highly expressed. Thus, scRNA-seq may reveal a transcription factor-coding mRNA or not, depending whether transcripts were present at the time of sampling[186]. In addition, expression of T_H-specific cytokines at the mRNA and protein levels may only be detectable after cell stimulation, e.g. by PMA and ionomycin, for several hours. The latter considerations make scRNAseq a less reliable tool for detecting expression of T_H-defining transcription factors or cytokines that would identify T_H-lineage commitment. Dimensionality reduction tools may overcome this limitation by clustering non-transcription factor and non-cytokine coding T_H-specific genes.

So far, T cell heterogeneity in atherosclerosis has only been characterized in single cell suspensions of aortic leukocytes. This provides sufficient discrimination between major principal leukocyte lineages, e.g. T- vs myeloid cells, but underestimates heterogeneity and differentiating genes within principal lineages. For example, one macrophage cluster in the study of Winkels et al.[5] was re-clustered and revealed three distinct subsets[164]. Only one study focusing on T_{reg} plasticity has employed scRNAseq of flow sorted CD4^{+} T cells[50]. In the available studies, several distinct T cell clusters were identified: Winkels et al. detected 5 T cell populations in *Apoe*$^{-/-}$ mice[5], Cochain et al 4 in *Ldlr*$^{-/-}$ mice[130], and Gu et al. 3 populations in adventitial cell preparations from *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice[179].

In the atherosclerotic plaque, one of these clusters across studies represents **CD8**$^{+}$ (*Cd8b1*$^{+}$) cytotoxic T cells[5, 130]. No CD8-coding genes were differentially expressed in adventitial cells[179]. Differentially expressed genes in the CD8 T cell cluster included *Nkg7* (Natural Killer Cell Granule Protein 7), *Ms4a4b*, the CD20 homologue in T cells, *Ccl5*, and *Gzmk* (Granzyme K). Notably, this cluster was also present in healthy arteries of 8-week-old *Apoe*$^{-/-}$ mice, indicating the existence of tissue-resident CD8^{+} T cells. Inconsistent marker gene expression that argues for both, *Cd4*$^{+}$ and *Cd8*$^{+}$ T cells, has been detected in the atherosclerotic plaque in two studies suggesting the existence of clusters containing both, **CD4**$^{+}$ and **CD8**$^{+}$ **T cells** (termed 'mixed'[5, 130]).

The most discrete cluster among non-CD8^{+} T cells identified in two independent studies expresses the chemokine receptor *Cxcr6* and the transcription factor *Rora*$^{+}$ (ROR-α) and accounts for ~9 % of cells (*Ldlr*$^{-/-}$) and ~15 % (*Apoe*$^{-/-}$) of non-CD8 T cells, respectively. CXCR6

is a chemokine receptor that is expressed by $CD4^+$ T cells, Natural Killer T (NKT) cells and γδ T cells. Global deficiency of CXCR6 reduced atherosclerosis and $CD4^+$ T cell accumulation[187], particularly of the pro-inflammatory IL-17A-producing $CD4^+$ T cell subset[188], consistent with the concomitant expression of *Rora* in this subset. In this cluster of **Th17-like cells**, *Cd4* was not detected, arguing that γδ T cells and not Th17 cells dominate.

Our meta-analysis in addition identified another cell cluster displaying an ILC2 gene signature[5]. This cluster was mostly negative for *Cd3* and *Cd4*, but showed enrichment in *Areg* encoding amphiregulin[189] and *Il1rl1* (encoding the IL-33 receptior ST2), consistent with type 2 innate lymphoid cells (ILC2s) that require RORA and GATA3 for their development[190, 191]. This cell cluster may also contain a few bona fide **Th2** cells. Further analysis of larger cell numbers combined with CITE-seq detection of surface markers and 5'-seq of T cell receptor encoding transcripts will likely help resolve the full spectrum of T cell heterogeneity in atherosclerotic vessels.

CyTOF and scRNA-Seq data inherently do not contain spatial information. Thus, it remains unknown in which regions of the atherosclerotic plaque T cells accumulate. Immunohistochemistry has indicated that T cells are mostly found in the fibrous cap regions of the plaque[1]. Spatial transcriptomics could precisely address this question by combining single cell gene expression and locations. No such studies are available in mouse or human atherosclerosis.

Association Between Clinical Outcome and Plaque T cell Phenotypes

A recent study conducted by Fernandez and colleagues describes the T cell landscape of carotid artery endarteriectomy specimens from patients with symptomatic (recent stroke or transient ischemic attack) or asymptomatic disease[8]. Single-cell proteomic and transcriptomic analyses revealed a distinct distribution of leukocytes between blood and atherosclerotic plaques with a population of $CD4^+$ and $CD8^+$ cells being the most abundant in the plaques. CyTOF analysis identified 13 clusters of aortic $CD4^+$ T cells that include central memory, effector memory, terminally differentiated effector memory, and T_{regs}. One of the most striking phenotypic features of plaque-derived T cells is elevated expression of the activation marker CD69, the chemokine receptor CCR5, and PD-1, a negative regulator of T cell activation and a marker of T cell exhaustion, within $CD4^+$ T cells. These data further support the idea that continuous and repeated activation of T cells in the aorta results in dysfunctional T cells that correlate with inflammation. Based on additional $CD4^+$ subsets discovered by CyTOF and scRNA-Seq, it will be important to elucidate transcriptional regulation of T_H cell subsets and to identify molecules that drive phenotypes and functions of Th cells in atherosclerosis. In a genetic deconvolution strategy, a gene signature of a scRNAseq-derived T cell cluster from mouse plaques negatively correlated with the clinical outcome of carotid stenosis, corroborating the concept that T cell phenotypes may predict plaque outcomes[192].

Limitations of this study

scRNA-Seq preferentially yields highly expressed genes, because genes with low expression or periodic expression have many dropouts and thus are less likely to be significantly different between cell types. This is particularly obvious for transcription factors. One way around this issue is to define gene signatures of abundantly expressed genes that correlate with the low-expressed genes of interest.

mRNA levels for cell surface markers are poorly correlated with protein expression on the cell surface. This is because cell surface proteins must undergo glycosylation, cleavage of the signal peptide, vesicular trafficking and sometimes enzymatic modifications to appear at the cell surface. Experience teaches that it is useful to measure the cell surface phenotype in addition to single cell transcriptomes. In fact, 35 CyTOF markers provide a more fine-grained

picture of leukocyte heterogeneity in atherosclerosis than single cell transcriptomes with ~2,000 genes[5]. As mentioned above, cell surface phenotype can now be linked to transcriptomes by using oligonucleotide-tagged antibodies in CITE-Seq[7]. There is no CITE-Seq dataset available for mouse aortas.

csRNA-Seq and CyTOF retain no information on cell position within the vessel wall or relative to other immune cells. Spatial transcriptomics methods[193, 194] are on the horizon, but we currently still lack information on spatial gene expression, including the exact localization of immune cells in the plaque versus the adventitia.

scRNA-Seq is a discovery tool. As such, it is great at identifying new cell types. For example, scRNA-Seq provided clarity with respect to the macrophage types in atherosclerotic mouse aortas. Once the phenotype of the cells of interest is known, with the cell surface phenotype defined by CITE-Seq, flow cytometry-based cell sorting can be used to sort homogeneous cell populations, extract RNA and perform bulk RNA-Seq. This yields much better, deeper transcriptomes than scRNA-Seq[164].

Conclusions

The modern single cell methods scRNA-Seq and CyTOF consistently defined four macrophage subsets, two monocyte subsets, 3-5 T cell subsets, 2 B cell subsets, one NK cell subset, neutrophils, eosinophils and dendritic cells in atherosclerotic mouse aortas. This approach, albeit in its infancy, has promise in terms of disambiguation of leukocyte cell populations and states within lesions giving us the tools for multi-dimensional atherosclerotic lesion phenotyping. The advances are, however, not limited to technical development.

This meta-analysis resolved several apparent controversies. First, foam cells in atherosclerotic aortas are macrophage-derived and smooth muscle cell-derived. Although these smooth muscle foam cells acquire some markers including CD68, they do not become macrophages. Second, macrophage-derived foamy Trem2 cells do not express a pro-inflammatory gene signature. This reconciles a study by Glass et al.[154] with many studies showing pro-inflammatory functions of atherosclerotic macrophages. This is resolved by the discovery of two inflammatory macrophage subsets, called IFNIC and inflammatory macrophages. Third, resident-like macrophages are found in the atherosclerotic aorta and express Pf4. Pf4 is not platelet-specific, because Pf4 is consistently expressed in these resident vascular macrophages. Fourth, the discovery of an ILC2 cluster links the scRNA-Seq work to recent work by Mallat's group[195], showing that ILC2 cells are strongly atheroprotective. This could resolve the controversy about the role of Th2 cells in atherosclerosis: Some of these Th2 cells in earlier studies may have been ILC2 cells instead. Finally, the observation of SiglecF^{+} neutrophils in atherosclerosis is striking. Such neutrophils had previously only been observed in cancer[196].

This meta-analysis also generated new hypotheses that can be tested in future work. The Treg switch hypothesis has received much support from the recent single cell studies, but it remains a hypothesis that needs to be tested. In particular, the switch mechanism is not known, neither is the switch direction. There is evidence for a switch to Th1[28, 50], Th2[5], Th17[34] and TFH[54]. Other hypotheses flowing from this work include the NK cell hypothesis. NK cells probably do not accelerate or curb atherosclerosis[81], but are more like bystanders. This cannot be gleaned from the NK cell transcriptomes consistently found in the scRNA-Seq studies and awaits further experimental testing.

The single cell interrogation techniques have ushered in a new wealth of information that will eventually lead to new depths of understanding. Such information from experimental model systems is needed to translate the insights from the immunology of atherosclerosis into therapies that can benefit patients.

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Figure legends

Figure 1. Overview. This analysis is based on published studies where atherosclerosis was induced in mouse models using genetic knockouts *Ldlr*$^{-/-}$ [130, 143], *Apoe*$^{-/-}$ [5], diet (chow, western diet, high fat diet, refs) or AAV-PCSK9 induced lipoprotein changes[144], resulting in atherosclerotic aortas. In some studies, lineage tracking (Cx3cr1-Cre)[144] or chemical labeling (Bodipy)[143] labeled specific cell types. All studies used enzymatic digestion with the attendant problems of cell death and loss of surface markers[6]. Single cells were phenotyped by RNA-Seq[130, 143, 144], CyTOF[80] or both[5]. Dimensionality reduction and clustering identified cell types and gene signatures. Matching CyTOF with scRNA-Seq data is challenging. Based on gene signatures, genetic labeling was used to visualize, image and sort some cell types to gain functional insights and deep transcriptomes[164].

Figure 2. Integration of scRNA-Seq data from 9 atherosclerosis studies on mouse aortas. scRNA-Seq data was retrieved from NCBI GEO for Harmony integration and visualized using UMAP[137]. 17 clusters were identified by Louvain clustering. One cluster (lilac) was dominated by non-leukocyte genes including Acta2 and one cluster (dark yellow) was dominated by proliferation genes including Top2a and Tuba1b, leaving 15 bone fide leukocyte clusters: 2 B cell clusters (red and pink), one CD8 T cell cluster (light green), 4 macrophage clusters (inflammatory, blue, IFNIC, light purple, resident, purple and Trem2, light blue), 2 CD4 T cell clusters (Th17, brown and Th2, teal), $CD4^+CD8^+$ cells (turquoise), 2 monocyte/DC clusters (orange), $Xcr1^+$ DCs (hot pink), neutrophils (light pink), NK cells (dark green). Total n= 15,288 cells

Figure 3. Different abundance of aortic leukocyte subsets in 9 scRNA-Seq mouse atherosclerosis studies. The UMAP from figure 2 was separated into the 9 different studies. Studies identified by first author[5, 130, 143, 144] and abbreviated conditions (see table 1 for full conditions). Cell subsets as in figure 2.

Figure 4. Top gene signatures for 15 aortic leukocyte subsets. The top 10 up-regulated genes in each subset compared to all other subsets are shown by Dot plot. Expression level indicated by saturation of blue (dark blue is highest expression, log2 scale where 0 is global average). Dot diameter represents percentage of cells in cluster expressing corresponding genes (largest circle is 100%).

Figure 5. Relationship between Cx3cr1-GFP+ and CD11c-YFP+ macrophages[107] and the resident, IFNIC, inflammatory and Trem2 foamy macrophages from the scRNA-Seq studies. Significantly differentially expressed (DE) genes were determined for Cx3cr1-GFP+, CD11c-YFP+, GFP+YFP+ and unlabeled macrophages against the other 3 subsets. The same was done for resident, IFNIC, inflammatory and Trem2 foamy macrophages. The DE gene lists were intersected. Only the genes that were upregulated in common are shown.

Supplemental Figure 1. Mapping of Th1, Th2 and ILC2, Th17 and Treg cells. The lineage-characteristic transcription factors Tbx21 for Th1, Gata3 for Th2 and ILC2, Rorc for Th17 and other IL-17-expressing cells and FoxP3 for Tregs were mapped onto the UMP shown in figure 2. Gene expression shown in grey (0) to blue (3), log2 scale.

Tables

Table 1. Experimental details of the studies on which this meta-analysis is based. Data on mouse strains, diet, treatments, digestion, scRNA-Seq and CyTOF. Empty fields indicate that this step was not done or is not applicable.

Table 2. Identification of immune cells from atherosclerotic mouse aortas by CyTOF. Data compiled from Winkels et al. and Cole et al.

Supplemental Table 1. Differentially expressed genes for each of the clusters in figure 2 against all other clusters. p_val: significance level, unadjusted. avg_logFC: average log fold change for each gene in the cluster compared to the average in all other clusters. pct.1: fraction of cells in the cluster that express each gene. pct.2: fraction of the cells in all other clusters that express each gene. p_val_adj: significance level, adjusted for multiple comparisons. Cluster: cell type identified, labeled as in figure 2. Gene: gene symbol.

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