Arteriosclerosis, Thrombosis, and Vascular Biology

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Series Editor: Robert A. Hegele

Heterogeneity of T Cells in Atherosclerosis Defined by Single-Cell RNA-Sequencing and Cytometry by Time of Flight

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ABSTRACT: The infiltration and accumulation of pro- and anti-inflammatory leukocytes within the intimal layer of the arterial wall is a hallmark of developing and progressing atherosclerosis. While traditionally perceived as macrophage- and foam cell-dominated disease, it is now established that atherosclerosis is a partial autoimmune disease that involves the recognition of peptides from ApoB (apolipoprotein B), the core protein of LDL (low-density lipoprotein) cholesterol particles, by $CD4^+$ T-helper cells and autoantibodies against LDL and ApoB. Autoimmunity in the atherosclerotic plaque has long been understood as a pathogenic T-helper type-1 driven response with proinflammatory cytokine secretion. Recent developments in high-parametric cell immunophenotyping by mass cytometry, single-cell RNA-sequencing, and in tools exploring antigen-specificity have established the existence of several unforeseen layers of T-cell diversity with mixed T_H1 and T regulatory cells transcriptional programs and unpredicted fates. These findings suggest that pathogenic ApoB-reactive T cells evolve from atheroprotective and immunosuppressive $CD4^+$ T regulatory cells that lose their protective properties over time. Here, we discuss T-cell heterogeneity in atherosclerosis with a focus on plasticity, antigen-specificity, exhaustion, maturation, tissue residency, and its potential use in clinical prediction.

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

Key Words: atherosclerosis ■ autoantibodies ■ autoimmunity ■ immunophenotyping ■ peptide

T cells are regulators of adaptive immunity that are defined by surface expression of CD3 and the T-cell receptor (TCR) chains-α/β^+ or TCR-γ/δ^+. Classical functions of α/β^+ T cells include protection from intracellular pathogens, larger parasites, elimination of tumor cells, and protection from autoimmune diseases. The role of γ/δ^+ T cells, which are outnumbered by α/β^+ T cells in tissues and circulation, will not be covered in this review. T cells develop in the thymus, where bone-marrow derived progenitors gradually develop into $CD4^-CD8^-$ (double-negative [DN]) cells. At this stage, T cells divide into either α/β^+ or γ/δ^+ T cells. α/β^+ T cells transit into double-positive (DP) $CD4^+CD8^+$ cells and eventually into naive $CD4^+CD8^-$ T-helper cells or cytotoxic $CD8^+CD4^-$ T cells.[1] $CD4^+$ T cells recognize antigen-derived peptides presented on MHC (major histocompatibility complex)-II, whereas $CD8^+$ T cells recognize peptides presented on MHC-I. The maturation of T cells is accompanied by recombinase activating genes-1 and -2 dependent rearrangement of TCR genes and the generation of a random, unique TCR on each T cell. This arrangement also results in autoreactive T cells that express a high-affinity TCR recognizing endogenously expressed self-peptides. Most of these self-reactive T cells are eliminated through a process termed negative selection,

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Arterioscler Thromb Vasc Biol is available at www.ahajournals.org/journal/atvb

Nonstandard Abbreviations and Acronyms

ApoB	apolipoprotein B
CXCR5	C-X-C chemokine receptor type 5
CXCR6	C-X-C chemokine receptor type 6
CyTOF	cytometry by time of flight
DBC	deleted in breast cancer
DN	double-negative
DP	double-positive
EOMES	eomesodermin
FoxP	Forkhead box protein P
IL	interleukin
IRF	interferon regulatory factor
Lag-3	lymphocyte-activation gene 3
LDL	low-density lipoprotein
MHC	major histocompatibility complex
NFκB	nuclear factor κ-light-chain-enhancer
Pak	protein kinase
PD-1	programmed cell death protein 1
Runx	Runt-related transcription factor
scRNA-seq	single-cell RNA sequencing
TCR	T-cell receptor
TF	transcription factor
T_{FH}	follicular T helper cell
TGF-β	transforming growth factor-β
TH	T-helper type
T_{reg}	T regulatory cell

Highlights

- T cells represent the largest population of leukocytes in the atherosclerotic plaque.
- High-parametric immunophenotyping of plaque T cells by single-cell RNA sequencing and cytometry by time of flight shows an unexpected phenotypic diversity with previously unknown immature T cells and unconventional, mixed T helper types.
- Lesional CD4+ T cells with mixed phenotypes seem to originate from initially protective T-regulatory cells that lost their immunosuppressive properties and switched into pathogenic counterparts over time.
- The phenotypic heterogeneity of plaque CD4+ T cells is driven by states of activation, exhaustion, development, and inflammation rather than by traditional types of T-helper cells.
- Appearance, transcriptomes, and proteomes of specific CD4+ T-cell populations in the atherosclerotic lesion correlate with clinical disease severity in humans.

but this process does not eliminate all autoreactive CD4+ T cells.[2] Increasing evidence argues for the presence of CD4+ T cells recognizing peptides from ApoB (apolipoprotein B)-100, an established self-antigen in atherosclerosis.[3–5] The presentation of cognate peptides by antigen-presenting cells (APCs) on surface-expressed MHC-II induces the activation and differentiation of naive CD4+ T cells into functionally and phenotypically distinct T-helper (T_H) types that express exclusive sets of transcription factors (TFs) and cytokines.[6,7] T_H type 1 (T_H1) CD4+ T cells are proatherogenic and express the TF T-bet and the lead-cytokine IFN-γ (interferon-gamma).[8] In experimental mouse models, CD4+ T cells from atherosclerosis-prone apolipoprotein-deficient ($Apoe^{-/-}$) mice home to the atherosclerotic aorta and secrete IFN-γ.[9,10] The genetic deletion of T-bet or IFN-γ reduces atherosclerosis markedly.[11,12] T_H2 cells express GATA3 (GATA-binding protein 3) and IL (interleukin)-4, -5, -10, and -13[13] and may support atheroprogression or -protection.[14,15] T_H17 cells express the lineage-defining TF RORγt (RAR-related orphan receptor) and the cytokines IL-17A, -17F, and -21. Their role in atherosclerosis is controversial.[16–19] T_H9 cells are characterized by IL-9 secretion; treatment of *Apoe*$^{-/-}$ mice with IL-9 increases atherosclerosis,[20] but the overall role of T_H9 cells in atherosclerosis remains unknown. Follicular T helper cells (T_{FH}) express the transcription factor Bcl-6, the costimulatory molecule inducible T-cell costimulator and CXCR5 (C-X-C chemokine receptor type 5).[21] T_{FH} are mostly found in germinal centers in secondary lymphoid organs where they provide help to B cells for immunoglobulin class switching. Emerging data suggest that T_{FH} contribute to the proinflammatory response in atherosclerosis.[22,23] T regulatory cells (T_{reg}), defined by expression of FoxP3 and IL-2 receptor (CD25), are immunosuppressive[24,25] and prevent from atherosclerosis by multiple mechanisms, including secretion of IL-10 and TGF-β (transforming growth factor-β).[26,27]

The regulation of T_{regs} in mouse and human atherosclerosis and the relationship to pathogenic T_H-types has been controversial: fractions of FoxP3+ T_{regs} decrease in murine atherosclerotic aortas and blood but increase in the spleen of the same animals.[28] A proportion of T_{regs} in the aorta and in lymph nodes of mice seems to obtain a T_H1-like phenotype and expresses IFN-γ.[29] While numbers of T_{regs} decrease during acute coronary syndromes in humans,[30–32] frequencies of blood T_{regs} are higher in patients with angiographically documented atherosclerosis compared with healthy individuals.[5] Other studies have failed to establish that frequencies of blood T_{regs} predict future myocardial infarction.[33] Traditional TF staining in mouse atherosclerotic aortas by intracellular flow cytometry (fluorescence-activated cell sorting) and a canonical gating strategy has suggested a mix of FoxP3+ (T_{reg}), RORγT+ (T_H17), and T-bet+ (T_H1) CD4+ T cells,[5,29,34–36] while the quantification of T_H-associated surface-expressed chemokine-receptor patterns in humans suggests a predominant T_H1 and T_H2 phenotype.[8,37] These partially conflicting findings have sparked several fundamental questions: are patterns of cytokines, chemokine

 Arterioscler Thromb Vasc Biol. 2021;41:549–563. DOI: 10.1161/ATVBAHA.120.312137

receptors, and transcription factors sufficient to define functionally relevant phenotypes of lesional and circulating T-helper cells? Do transcriptional programs in T_H more precisely define T_H cell diversity than single key TFs or cytokines? How are T_H-cells regulated in different stages of disease and may these predict clinical complications?

RECENT DEVELOPMENTS IN HIGH-PARAMETER IMMUNOPHENOTYPING

Fluorescence-activated cell sorting is considered the gold standard for immunophenotyping of aortic leukocytes since 2006.[38] Clinical routine fluorescence-activated cell sorting-workflows allow the simultaneous quantification of up to 18 parameters (marker expression, size, and granularity) on single cells by fluorescently tagged antibodies, albeit up to 40 markers are technically possible. Several reports have used either pan-leukocyte antibody panels to cover major principal hematopoietic lineages, that is, myeloid, T-, and B cells, or lineage-specific sets of antibodies to obtain phenotypic insight in particular lesional leukocyte lineages.[38–40] These approaches, however, cover cellular heterogeneity only incompletely. Two newer technologies have demonstrated the ability to overcome this limitation. Cytometry by Time of Flight (CyTOF) uses rare metals conjugated to antibodies, which expands the number of simultaneously detectable extra- and intracellular protein targets up to 40 to 50. To date, CyTOF has been used to study leukocytes in atherosclerotic plaques in 3 studies.[37,39,41] Single-cell RNA sequencing (scRNA-seq), on the other hand, provides transcriptomes of single cells with an average of 1300 to 8000 genes per cell, which enables the detection of transcriptionally defined cell populations. The first application of scRNA-seq in atherosclerosis used a microfluidics-based platform for 96-well sorting that allowed to study only limited numbers of cells.[29] Today, technologies encapsulating up to 10000 individual cells into oil droplets are commercially available (drop-sequencing). These have successfully been applied to atherosclerotic plaques from mice and humans.[39,40,42–52] A newer strategy enables the combination of single cell transcriptomes and cell surface expression of proteins detected by RNA-barcoded antibodies (cellular indexing of transcriptomes and epitopes by sequencing).[37,53] In scRNA-seq based approaches, genes encoding for *Cd3d* or *Cd3e* can be used to identify T cells. In contrast to *Cd8*, *Cd4*-transcripts are only weakly expressed. The segregation of T-cell lineages by scRNA-seq is challenging as the sensitivity to detect genes with low expression differs across technical pipelines: for example, the sensitivity of Smart-Seq2 is 2.5× higher compared with droplet-based sequencing by 10× Genomics 3' v.2.[54] Other technical challenges include gene dropouts caused by low RNA-expression, inefficient mRNA capture, and the stochastic, pulsatile nature of gene expression on a single-cell level.[55] Accordingly, cells positive for TF-coding and other genes at low expression are often sparse in scRNA-seq datasets. Even in the meta-analysis of several mouse datasets, expression of transcripts coding for T-bet and FoxP3 was rare and did not allow the identification of distinct T_H1 or T_{reg}-clusters based on TFs alone.[47]

Of note, all high-parametric tools require the mechanical disruption of tissue-resident cells by enzymatic digestion, which may lead to an underestimation of fragile cellular subsets, such as macrophages that populate up to 50% of the atherosclerotic plaque area based on imaging- or histology-based detection.[51,56] Other technical requirements and limitations of CyTOF and scRNA-seq have been reviewed recently.[57] An ongoing challenge remains to combine and integrate different datasets into one cellular atlas of atherosclerotic lesions with a unified nomenclature—an attempt recently undertaken in the first meta-analysis of mouse plaque-resident leukocytes.[47] Ultimately, novel immunophenotyping workflows may be combined with reagents to detect antigen-specific T_H cells recognizing particular antigenic peptide laden MHC multimers labeled with fluorochromes.[58] Recently, we have demonstrated the existence of human and mouse ApoB-specific T_H cells on a single-cell level by MHC-II tetramers[3,5] and linked these to single-cell transcriptomes.[5] These technical developments helped to describe several novel layers of T-cell diversity in atherosclerosis with respect to spatial, temporal, phenotypic, antigen-specific, and transcriptional resolution that fundamentally differs from traditional discrimination and nomenclature of $CD4^+$ T cells.

QUANTITIES AND LOCATIONS OF LESIONAL T CELLS

The presence of T cells in atherosclerotic plaques and the surrounding adventitial tissue has been first observed >30 years ago. In immunohistochemistry, $CD4^+$ and $CD8^+$ T cells accumulate in the shoulder region, the fibrous cap, and the intima of human atherosclerotic plaques as well as in adventitial tissue and account for up to ≈54% of all leukocytes in the shoulder region and ≈14% in the necrotic core.[59,60] Contrastingly, (dying) macrophages dominate the necrotic core of plaques while B cells can only be found in relevant numbers in adventitial tissue.[39,61] Recent immunophenotyping has validated the frequency of T cells in human atherosclerotic tissue, where T cells accounted for up to 65% of all leukocytes in CyTOF[37] and ≈52% in scRNA-seq of human atherosclerotic plaques.[45] In mice, relative frequencies of T cells seem to be lower: in 2 studies, myeloid cells, T cells, B cells, and other smaller leukocyte subsets accounted each for ≈25% of all leukocytes in atherosclerotic aortas of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice,[39,46] while another study found ≈6% of all leukocytes to be $CD3^+TCR\text{-}\beta^+$ T cells by CyTOF of atherosclerotic mouse

aortas.[41] Notably, the approximate frequencies of parental leukocyte lineages seem to be consistent between conventional flow cytometry, CyTOF, and scRNA-seq in direct comparison.[37,39] Besides potential differences related to the species, experimental protocols, and tested tissues (whole aorta versus plaque), the loss of particular cell types during tissue digestion may explain these inconsistent findings.[39,46,49] T cells are already detectable in adventitial tissue before the initiation of atherosclerosis: scRNA-seq of adventitial tissue demonstrated the existence of several T-cell subsets already in wild-type animals,[52] including 2 CD4$^+$ subsets (clusters 12, 13 in the original dataset) and one CD8$^+$ population (cluster 2 in the original dataset).[52] CD8$^+$, but not CD4$^+$, T cells were also found in the healthy arterial vessel of *Apoe*$^{-/-}$ mice,[39] whereas the highest numbers of CD4$^+$ and CD8$^+$ T cells were detected in atherosclerotic aortas. Notably, frequencies of T cells do not substantially differ between *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice.[39,46] In aggravated hypercholesterolemia, T-cell fractions in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice decrease, while total numbers increase,[39,46] suggesting that the relative contribution of myeloid cells is increasing in advanced atherosclerosis. It is noteworthy that adventitial CD4$^+$ T cells increase up to 10-fold in the proximity of atherosclerotic plaques.[62,63] These may be partially located in artery tertiary lymphoid organs, structures that harbor significant numbers of CD4$^+$ T cells, B cells, and other lymphocytes in aged mice.[61] It is therefore tempting to speculate how the spatial organization of T cells impacts on lesion development: while CD4$^+$ T cells in atherosclerotic lesions could instruct and interact with other tissue-resident cells, specialized T_{FH} cells in the adventitia could interact with highly concentrated B cells in artery tertiary lymphoid organs to induce immunoglobulin switching. Such side-specific cellular functions will have to be determined in future work, which could take advantage of the recent development of spatial single-cell transcriptomics that provide simultaneous gene expression and spatial information.[64]

How circulating T cells correspond to T cells in the plaque has not been investigated in-depth, but a recent report applying cellular indexing of transcriptomes and epitopes by sequencing indicated that CD4$^+$ and CD8$^+$ effector-memory T cells[37] and T cells with a clonal restriction of the TCR[37,65] are overrepresented in atherosclerotic plaques in comparison with circulating T cells. These findings raise the question whether activated T cells primarily home to the plaque as recently suggested[9,66,67] or are generated in situ by local proliferation as a result of their interaction with local APCs.[9] Interestingly, 2 scRNA-seq reports have detected clusters of proliferating leukocytes that include T cells,[39,47] arguing for in situ proliferation. Consistently, earlier evidence has suggested that DNA-synthesis, which typically occurs in proliferating cells, colocalizes with T cells.[68] It is therefore likely that both mechanisms—migration and in situ proliferation—contribute to the pool of lesional T cells.

DISTINCTION OF T-HELPER CELL PHENOTYPES IN THE PLAQUE

Traditional immunophenotyping by fluorescence-activated cell sorting is per se limited by the usage of predefined sets of antibodies: in most studies, staining for CD62L and CD44, which discriminates between naive, effector-memory T cells, and central-memory T cells in the mouse, has been combined with intracellular expression of T_H-defining TFs or cytokines for canonical gating and identification strategies.[2,3,5,28,69] Contrastingly, in most available scRNA-seq studies, T_H-cell phenotypes were inferred from dimensionality reduction algorithms to detect clusters of cells with similar transcriptome without the bias of preselecting markers. Several T-cell populations that partially overlap have been identified by these screening approaches: 4 in *Apoe*$^{-/-}$[39] and in *Ldlr*$^{-/-}$ mice,[46] 3 populations in adventitial cell preparations from *Apoe*$^{-/-}$ and wild-type mice,[52] and 5 clusters in an integrative analysis of published datasets.[47] The latter meta-analysis expands over the original studies by identifying one discrete population of T_{regs}, which did not form a separate cluster in the original datasets. Some datasets contained a cluster with a proliferation and T-cell gene signature, albeit it is not clear whether this cluster contains other cell types as well.[39] The most consistent unique T-cell phenotypes across these studies include: a multilineage-committed (T_{reg}-T_H1-T_H17-T_H2) *Cxcr6*-expressing T cell (1), a naive T cell (2), a thymocyte-like T cell (3), and a *Cd8*$^+$ cytotoxic T cell (4). Phenotypes, frequencies, and proposed functions of these populations will be discussed in detail (Figure 1) in the following.

CYTOTOXIC CD8$^+$ T CELLS

One cluster of CD8$^+$ (*Cd8b*$^+$) cytotoxic T cells has been detected by scRNA-seq in atherosclerotic and healthy aortas but not in the adventitia,[39,46,52] suggesting CD8$^+$ T cells are already present in the healthy intima or media of arteries. Genes differentially expressed by CD8$^+$ T cells include *Cd8a*, *Cd8b*, *Nkg7* (coding for natural killer cell granule protein 7), *Ms4a4b*, the CD20 homologue in T cells, *Ccl5* (coding for C-C motif chemokine-ligand 5), *Eomes* (coding for the TF eomesodermin), and *Gzmk* (coding for Granzyme K). In scRNA-seq, 31% to 41% of lesional T cells were identified as cytotoxic T cells depending on tested genotypes and diets.[39,46] The overall frequency of CD8$^+$ T cells was confirmed by 2 CyTOF-studies of mouse aortas (≈30% of T cells).[39,41] In humans, ≈30% of all leukocytes (and ≈50% of T cells) in plaques are CD8$^+$ T cells.[37] In histology, the accumulation of CD8$^+$ T cells seems to be particularly

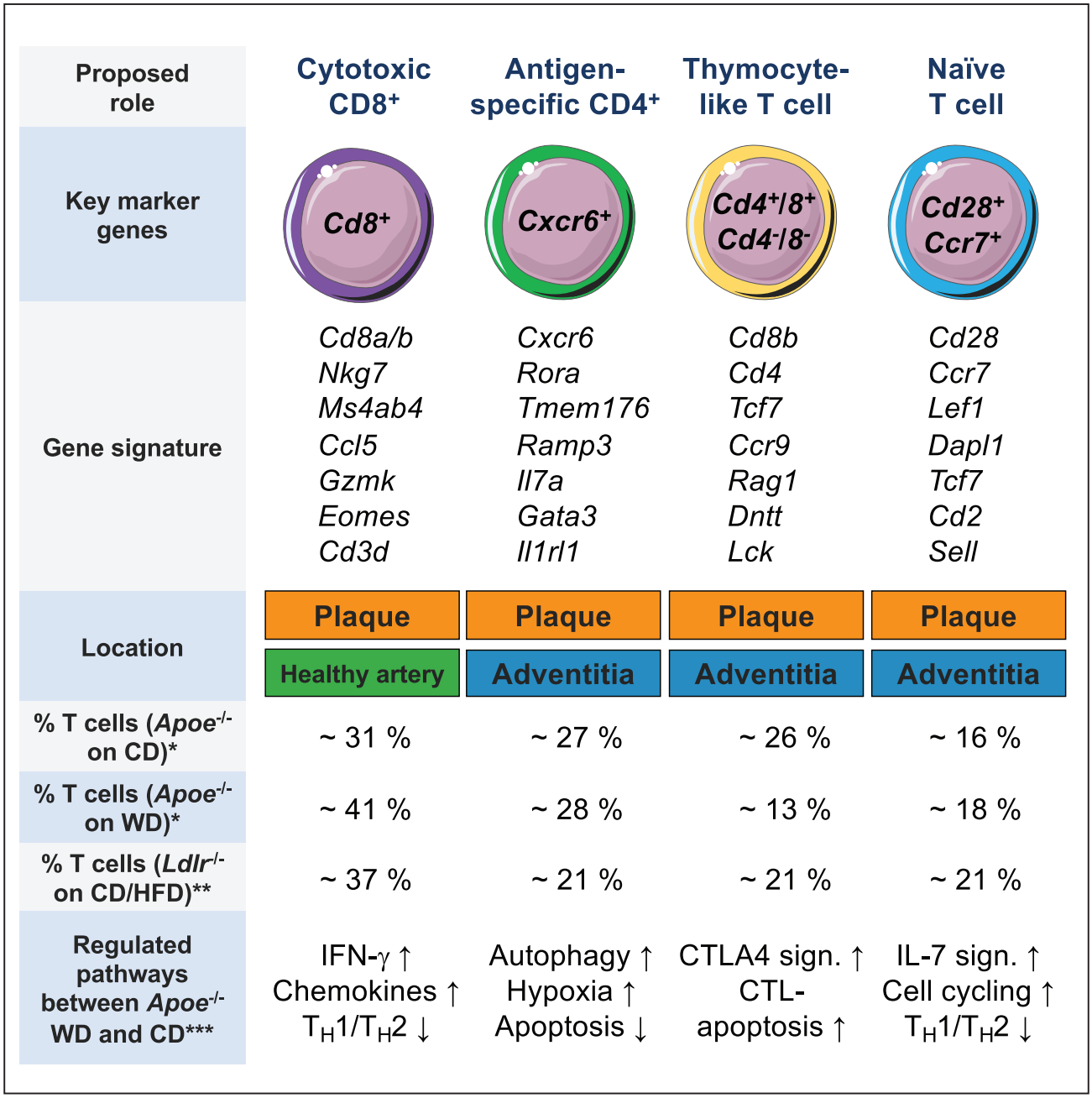

Figure 1. Distinct vascular T-cell phenotypes in the healthy and atherosclerotic mouse aortas.
Overview of the 4 major T-cell phenotypes identified by single-cell RNA-sequencing with key gene expression, frequencies, and regulated pathways during atherosclerosis progression. Gene signatures were retrieved by differentially expressed genes among all leukocytes across several studies. Frequencies of clusters were retrieved by an analysis of the original data set (*),[39] or estimations based on the original publication (**).[46] CD indicates chow diet–consuming mice; CTL, cytotoxic T-cell–mediated apoptosis; HFD, high-fat diet–consuming mice; IFN-γ, interferon-gamma; and WD, Western diet–consuming mice. ***Regulated pathways between CD and WD-fed mice were retrieved from study by Winkels et al.[39] Schematics adapted from "Smart Servier Medical Art."

dense in the fibrous cap region,[70] where they outnumber CD4+ T cells.[70,71] The frequency of some CD8+ T-cell subsets found only in CyTOF correlates to TCR-clonality in plaques,[37] suggesting their antigen-specific expansion. However, cognate antigens for CD8+ T cells remain to be determined, although HSP60 (heat shock protein 60)[72] and ApoB[73,74] are among the prime candidates. Generally, functions of CD8+ T cells include the removal of virus-infected and cancer cells.[75] The function of CD8+ T cells in atherosclerosis is ambivalent and stage dependent: antibody-mediated depletion of CD8+ T cells prevented atherosclerosis[76–78] by attenuating proinflammatory cytokine levels and myelopoiesis.[78] In established atherosclerosis, the depletion of CD8+ T cells was proatherogenic and reduced plaque stability, which was accompanied by elevated macrophage and T

cell reponses.[77,79] Vaccination studies with HSP60 and ApoB peptides evidenced antiatherogenic effects by CD8+ T cells,[72–74] although no effect on atherosclerosis was observed in a humanized HLA mouse model.[80] Vaccination-induced atheroprotection was discussed to be a consequence of lower dendritic cell numbers in aortas,[74] enhanced macrophage death, and a blockade of T_H17 cell formation.[81] Atheroprotective properties of CD8+ T cells have been linked to CD8+ T_{regs},[82] a known subpopulation with immunosuppressive propensities.[83] It remains unclear how this functional dichotomy of CD8+ T cells corresponds to distinct subsets found in recent CyTOF studies.[39,41] It is well possible, that some CD8+ T cells with distinct features occur uniquely in atherosclerotic lesions similar to recently discovered subsets of noncytotoxic CD8+ T cells in the tumor microenvironment.[84]

NAIVE T CELLS

A *Cd28*+*Ccr7*+ cluster is detectable in the atherosclerotic aorta and the adventitia across several scRNA-seq data sets.[39,46,47,52] The consensus genes for this cluster include *Lef1*, *Dapl1*, *Tcf7*, *Sell*, and *Cd2* (Figure 1). The chemokine receptor CCR7 (gene *Ccr7*) is expressed by naive and central memory cells and instrumental for lymph node homing.[85] The costimulatory molecule CD28 (gene *Cd28*) is required for effective T-cell co-stimulation by APCs and predominantly expressed by naive CD4+ T cells.[86] Although CD28 costimulation is not necessary for a memory recall-response, its therapeutic blockade reduces proatherogenic T_H1 responses.[87] *Cd28*+*Ccr7*+ T cells account for ≈21% to 27% of all T cells.[39,46] They become rarer and engage cell cycling genes and increase IL-7 signaling,[39] which supports survival, differentiation, and proliferation of T cells,[88] in more advanced atherosclerosis. This indicates that naive T cells either leave the plaque or transform into activated cell types represented by one of the other clusters. Because the core genes of this cluster are expressed by CD4+ and CD8+ cells,[89] this cluster likely contains naive CD4+, CD8+, and immature-like T cells. Notably, the naive (versus activated) gene signature seems to excel transcriptional differences between principal lineages (CD4+ versus CD8+). In CyTOF of human plaques, naive T cells did surprisingly not form a distinct cluster but were located within principal lineage clusters.[37] Numerically, naive T cells were relatively sparse in carotid plaques compared with blood,[37] corroborating the predominant activation of lesional T cells.

THE CXCR6+ MULTILINEAGE COMMITTED T-HELPER CELL—AN EXT_{REG}?

The most discrete cluster among non-*Cd8*+ T cells[39,46,47,52] expresses the transcripts *Cxcr6* coding for chemokine receptor CXCR6 (C-X-C chemokine receptor type 6) and *Rora* coding for the TF ROR-α, which is expressed by T_H17 cells and by a subsets of T_{regs}.[90] Other core genes include *Tmem176*, *Ramp3*, *Il7a,* and *Il1rl1* (Figure 1). This cluster was termed *Il17*+*Cxcr6*+ in a recent meta-analysis.[47] It accounts for ≈21% of T cells in *Ldlr*−/− mice (cluster 8 in the original dataset[46]) and ≈28 % in *Apoe*−/− mice (cluster 6 in the original dataset[39]). CXCR6 is expressed by CD4+ T cells and natural killer T cells and guides T-cell homing. Global deficiency of CXCR6 reduces atherosclerosis and T-cell accumulation,[67] particularly of IL-17A-producing CD4+ T cells,[91] which is consistent with the concomitant expression of *Rora* in this subset. Accordingly, *Tmem176a* and *Tmem176b* are known to be overexpressed in T_H17 cells.[92] Yet, the gene signature of this cluster does not seem to be entirely consistent over different studies and comprises a strong T_H17[46] and/or an additional T_H2 gene signature.[39,52] A selective analysis of scRNA-seq of filtered *Cd4*+*Cd8*− transcriptomes in the aorta showed the highest expression of *Cxcr6* in clusters with a mixed $T_H1/T_H2/T_{reg}$ and an T_H1/T_H17 transcriptome.[5] These findings argue for a more complex phenotype and T_H-type of this cluster.

Traditionally, differentiation into T_H-types is thought to occur unidirectional with only minimal overlap between distinct lineages. This concept was challenged by the discovery of CD4+ T cells co-expressing Foxp3, T-bet, and/or RORγT in various (auto-) immune pathologies.[3,10,29,93–95] Expression of >1 TF may argue for an enhanced plasticity of cells that are in transition into another T_H-type. On the other hand, in the intestine, RORγt+ T_{regs}[96] and T_H1-like T_{regs}[97] represent unique lineages that control mucosal immunity. Depending on the condition, these mixed phenotypes result in pro- and/or anti-inflammatory properties.[2,3,10,29,93–95] Based on flow cytometry with canonical gating strategies and immunohistochemistry 22% to 30% of all CD4+ T cells are pathogenic T_H1 cells.[29,35] T_{regs} are less abundant in the aorta (≈5% to 30%).[98,99] It should be noted that aortic T_{regs} numbers decline in atherosclerotic mice over time.[28] Despite the contrasting role of T_H1 and T_{reg} in atherosclerosis, 2 newer studies reported T_H1-like T_{regs} co-expressing FoxP3, T-bet, IFN-γ, and CCR5 in lymph nodes and atherosclerotic aortas of *Apoe*−/− mice.[6,10,29] Fate mapping experiments with adoptive transfers established that these T_H1/T_{regs} evolve from T_{regs}.[5,29] Consistently, in early scRNA-seq experiments, about 40% of all CD4+ T cells expressed T_{reg} and T_H1 lineage genes.[29] In bulk RNA-seq, T_H1/T_{regs} were transcriptionally closer to effector T cells than to T_{regs}.[10] Functionally, these cells failed to fully suppress T_{effs} and did not protect from atherosclerosis—despite the expression of FoxP3.[10,29] Recent evidence has validated that 50% of all lesional CD4+ T cells express *Cxcr6* and $T_H1/T_H17/T_{reg}/T_{FH}$ genes simultaneously.[5] The coexpression of T_H1- and T_H17-associated genes has also been shown in CD4+ T cells isolated from human carotid plaques.[37]

The loss of the T_{reg}-defining TF FoxP3, particularly in antigen-specific T_H cells, in the context of hypercholesterolemia and intracellular cholesterol accumulation seems to contribute to the switch of a T_{reg} into an exT_{reg}.[5,22] In fact, in FoxP3-lineage-tracer mice, about 15% to 26% of all lesional CD4$^+$ T cells stem from initial T_{regs}.[5,22] The exact molecular or cellular stimuli that cause a loss or an inactivity of FoxP3 in the setting of atherosclerosis are largely unknown. A solid line of evidence has identified inflammatory cues as crucial drivers of such T_{reg} plasticity: Certain thymus-derived T_{regs} undergo a reprogramming to effector-like T cells that depends on the downregulation of the transcription factor EOS encoded by *Ikzf4*, an important co-repressor of Foxp3,[100] at sites of inflammation. This process seems to require IL-6, a proinflammatory cytokine that is elevated in atherosclerotic plaques.[101] IFN-γ, another proatherogenic key cytokine secreted by proinflammatory T_H1 cells[102] has the ability to promote T_{reg} destabilization.[103] Under chronic cellular hypoxia as detected in atherosclerotic plaques,[104] Foxp3 becomes polyubiquinated and degraded in lysosomes. A key transcription factor induced by hypoxia is HIF-1α (hypoxia-inducible factor 1-alpha), which can bind to Foxp3 and directly cause its degradation while simultaneously stabilizing T_H17 gene expression.[105] In addition, it has been established that other proinflammatory cytokines or lipopolysaccharide promote Foxp3 degradation.[106] Likewise, several signaling pathways such as these involving the TF NFκB (nuclear factor κ-light-chain-enhancer), IRF (interferon regulatory factor)-4, Runx (Runt-related transcription factor), FoxP (Forkhead box protein P)-1, the nuclear protein DBC (deleted in breast cancer)-1, and serine/threonine-Pak (protein kinase)-2 (reviewed in study by Korn and Muschaweckh[107]) as well as self-peptide induced TCR-signaling[108] have been linked to the plasticity of T_{regs}. Altogether, these findings demonstrate a strong association of inflammation and T_{reg}-instability, which sparks the yet unproven hypothesis that the multitude of factors that initiate and maintain atherosclerosis, such as local and systemic inflammation, LDL (low-density lipoprotein), vascular integrity and injury, immune cell accumulation, and antigen-recognition by T_{regs}, favor a stage- and context-specific conversion of an initial protective into a pathogenic immune response (Figure 2). This process is likely gradual. Yet, it remains to be determined, which of the known signaling pathways and upstream mediators have a dominant role specifically in the context of atherosclerosis.

The definitive function of exT_{regs} is controversial: while the mixed T_{reg}/T_H17 phenotype may represent an intermediate stage toward a T_H1-dominated composite phenotype with a residual atheroprotective function,[5] a mixed T_H1-T_{reg} aggravated atherosclerosis in a direct T-cell transfer in mice.[10,29] On the other hand, T_{regs} have the ability to acquire a T_{FH}-like phenotype with enhanced IFN-γ secretion: a deletion of the T_{FH}-transcription factor BCL (B-cell lymphoma protein)-6 seems to be atheroprotective, suggesting that the conversion of T_{regs} into T_{FH} may be proinflammatory.[22] However, in a transgenic mouse model, LDL and LDL-reactive CD4$^+$ T cells converted into T_{FH} cells, which protected from atherosclerosis though secretion of LDL-depleting IgG antibodies.[109] Thus, T_{FH} cells could be pro- and anti-inflammatory, and this effect may likely dependent on antigen-specificity and tissue localization. Depending on the development of cluster algorithms in dimensionality reduction, it is foreseeable that even more distinct clusters with mixed phenotypes will be discovered. Conclusively, it should be assumed that the plasticity of T_{regs} represent a gradual shift in phenotypes and effector functions triggered by a multitude of spatial and temporal events rather than a singular event that switches a cell from one to another definitive phenotype (Figure 2).

THE APOB-SPECIFIC T_H CELL

The existence of ApoB-specific T_H cells has been indirectly inferred from a restricted (oligoclonal) TCR repertoire in the plaque[37] and of ApoB-specific T cells in lymph nodes,[5] cloning of oxLDL (oxidized low-density lipoprotein)-reactive CD4$^+$ T cells from human and mouse atherosclerotic plaques[4,110] as well as from inflammatory cytokine secretion by CD4$^+$ T cells restimulated with ApoB or LDL.[5,65] Several fundamental characteristics of the autoreactive T-helper cell response have remained unanswered because tools to detect single ApoB-specific T cells (ApoB$^+$) were not available. To overcome these limitations, we have recently developed recombinant MHC-II multimers loaded with ApoB-peptides for the direct fluorochrome-based detection of single ApoB-reactive CD4$^+$ T cells in mice and humans.[3,5] These studies helped to define fractions, phenotypes, and dynamics of ApoB-specific cells in the atherosclerotic plaque and systemically: even in the absence of relevant atherosclerosis, we detected small fractions of ApoB$^+$ cells in mice and humans that transcriptionally and phenotypically resembled T_{regs}.[3,5] In disease, ApoB$^+$ cells gradually acquired a proinflammatory phenotype close to that of $T_H1/T_H17/T_{FH}$ cells (Figure 2). In single cell gene set enrichment analysis, about 50% of aortic CD4$^+$ T cells (*Cd4*$^+$*Cd8*$^-$) had a gene signature similar to that of ApoB$^+$ T cells. ApoB$^+$ but not ApoB^{neg} T cells in lymph nodes expressed moderate to high levels of *Cxcr6* in bulk RNA-seq,[5] suggesting that CXCR6 may serve as the homing receptor of antigen-specific CD4$^+$ T cells. Although integrative analysis of this cellular subset and aortic T cells is not available, the gene signature strongly suggests that *Cxcr6*$^+$ T cells in the plaque may represent T cells specific for an atherosclerosis-relevant antigen.

While the function of the heterogeneous population of ApoB$^+$ CD4$^+$ T cells in atherogenesis has not been clarified completely, the distinctive function of individual T_H-lineages has been validated extensively in the last decades.[6,7] Notably, scRNA-seq of ApoB$^+$ T cells has

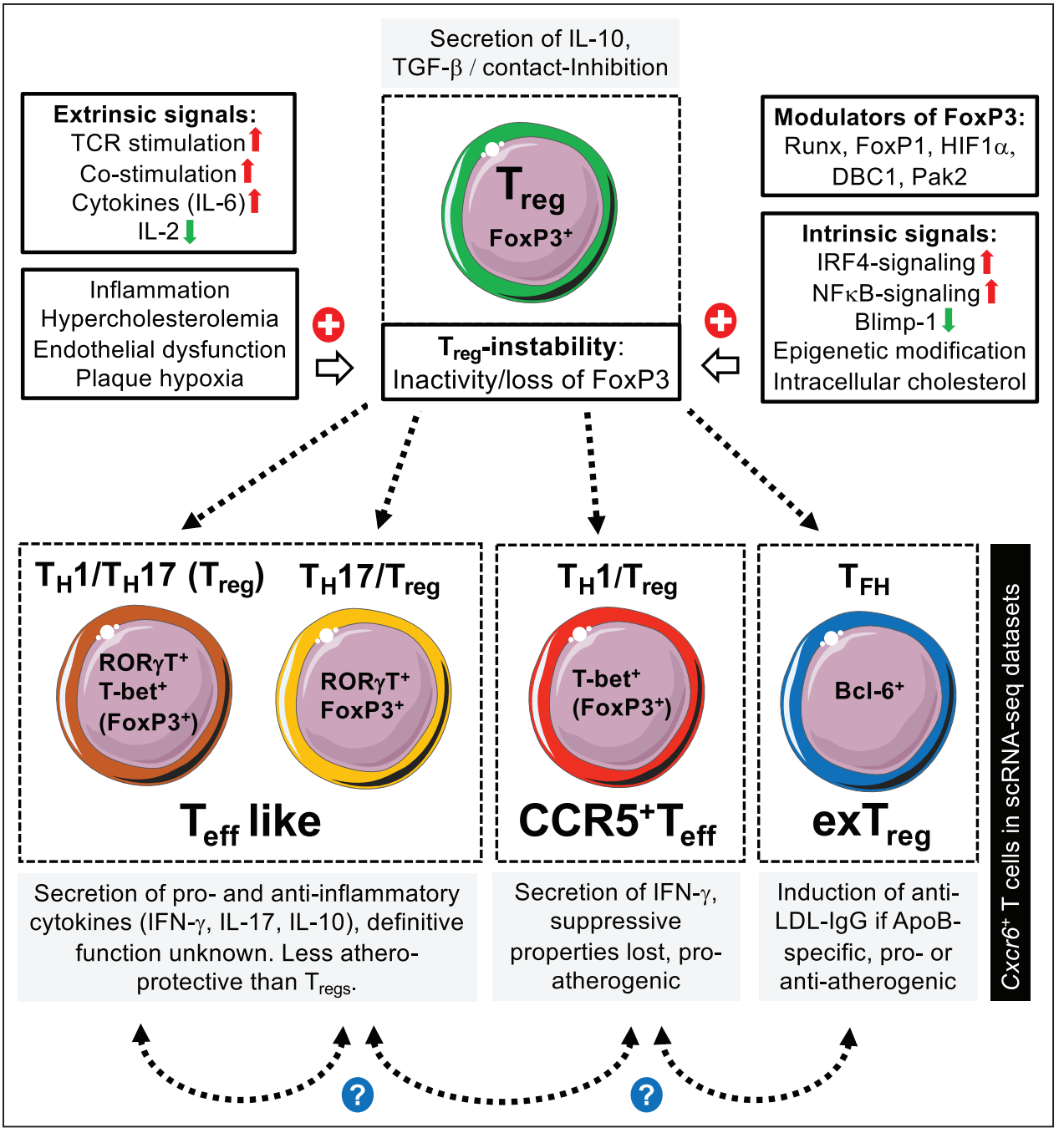

Figure 2. Proposed fate of T regulatory cells in atherosclerosis.
In the course of atherosclerosis, regulatory T cells (T_{reg}) with an immunosuppressive and atheroprotective function lose their protective properties. In atherosclerotic plaques or lymph nodes, former T_{regs} are characterized by an entire loss (exT_{reg}) or an inactivity of FoxP3 (T_{eff}-like, CCR5$^+$ T_{eff}). We propose that several classes of signals may induce such inactivity or loss of FoxP3. These include extrinsic signals (hypercholesterolemia, chronic low-grade inflammation, endothelial dysfunction, and plaque hypoxia) and overwhelming immune-signaling caused by repetitive (auto-) antigen presentation and costimulation. Intrinsic proinflammatory signaling events and epigenetic modifications may partially be promoted by such extrinsic stimuli and/or intracellular cholesterol accumulation. In addition, several modulators of FoxP3 have been identified. Destabilized T_{regs} can develop into distinct phenotypes, which can express several opposing TFs and canonical T-cell cytokines simultaneously: (1) T_H1/T_H17 T_{regs}, (2) T_H17 T_{regs}, (3) T_H1 T_{regs}, and (4) follicular T helper cells (T_{FH}). Because of the composite $T_H1/T_H17/T_H2/T_{FH}$ phenotype and the expression of CXCR6 (C-X-C chemokine receptor type 6), it has been speculated that ApoB (apolipoprotein B)-specific T cells (ApoB$^+$) correspond to the *Cxcr6*$^+$ T-cell subset in single-cell RNA sequencing studies. However, it is not clear if an ApoB$^+$ cell evolves from pure a FoxP3$^+$ T_{reg} (shown in green) or only exists in the reported composite phenotype. Likewise, the relationship of the 4 switched T_{reg} phenotypes and the existence of more intermediate phenotypes remains unclear. Schematics adapted from "Smart Servier Medical Art." Transcription factors displayed in brackets indicates only a low level of residual gene or protein expression. Bcl-6 indicates B-cell lymphoma protein-6; DBC1, deleted in breast cancer-1; FoxP, Forkhead box protein P; IFN-γ, interferon-gamma; IL, interleukin; IRF4, interferon regulatory factor-4; LDL, low-density lipoprotein; NFκB, nuclear factor κ-light-chain-enhancer; Pak, protein kinase; Runx, Runt-related transcription factor; TCR, T-cell receptor; and TGF-β, transforming growth factor-β.

 Arterioscler Thromb Vasc Biol. 2021;41:549–563. DOI: 10.1161/ATVBAHA.120.312137

revealed several distinct clusters with mixed T_{reg}, T_H17, and T_H1 phenotypes in mice. In some of these clusters, a supposedly protective while in others a pathogenic gene expression profile dominated.[5] Flow cytometry of human ApoB$^+$ T cells has revealed similar phenotypic patterns.[3,5] In another study, LDL-reactive T cells from a mouse expressing a transgenic TCR directed against LDL/ApoB developed into $T_{FH.}$ These were atheroprotective by inducing antibodies that cleared LDL.[109] It is therefore to assume that ApoB$^+$ T cells likely exist in several flavors and functions that range from immunosuppressive to pathogenic phenotypes. It should also be noted that in several pathologies, antigen-specific T_{regs} are prone to mechanisms of plasticity as outlined above.[108] In general, effector functions of T-helper cells in atherosclerosis are manifold: they have the ability to interact with other immune and stromal cells and thereby instruct other leukocytes to promote or inhibit plaque regression,[50] and to secrete pro- or anti-inflammatory cytokines, myeloid cell growth factors, or matrix-destabilizing proteases that can induce an instable plaque phenotype. In addition, they may act immunosuppressive by direct cellular contact inhibition or cytotoxic by granzyme-induced cell death.[111] The exact mechanisms by which ApoB$^+$ T cells potentially modulate atherosclerosis, however, are currently unknown.

EVIDENCE FOR IMMATURE THYMOCYTE-LIKE T CELLS IN THE PLAQUE?

Several studies employing CyTOF, scRNA-seq, or CITE-seq have demonstrated the existence of aortic and plaque-resident T cells that do not express protein or gene transcripts coding for CD4 and CD8 (DNs) or that express both (DPs).[37,39] It has been speculated that aortic/plaque DP and DN may represent immature T cells (thymocyte-like),[47] but this hypothesis is controversial: In early scRNA-seq studies and a recent meta-analysis, one cluster had a high cluster-wide expression for *Cd4*, *Cd8,* and *Cd3g/Cd3d* which was termed mixed, possibly because of a mix of single-positive *Cd4*$^+$ and *Cd8*$^+$ T cells in the same cluster. This cluster accounts for ≈13% to 26% of aortic T cells in mice[39,46] and expresses high levels of the thymocyte genes coding for TCF1 (*Tcf7*), Rag-1 (*Rag1*), and C-C motif chemokine receptor 9 (*Ccr9*) (Figure 1). The existence of DPs (*Cd4*$^+$*Cd8*$^+$) on a single cell level, however, was not directly proven in these data sets because of the technical limitations of scRNA-seq described above. In addition, immature DP-thymocytes express only extremely low levels of extracellular TCR-β/CD3-complexes. CyTOF of human lesional T cells, however, detected a cell cluster with surface expression of CD3, CD4, and CD8,[37] which is arguing against a real thymocyte-like phenotype. Interestingly, mature CD3$^+$CD4$^+$CD8$^+$ T cells that do not express thymic-stage markers have been identified in cancer tissues[112,113] and blood of patients with rheumatoid arthritis.[114] CD4$^+$CD8$^+$ DP aortic T cells may in fact be closer to these peripheral DP cells than to immature (thymic) DP cells.

Other clusters in one CyTOF dataset expressed TCR[39] (in mice) or CD3[37,39] (in humans) at a low level, but not CD4 or, which is suggestive of thymocyte DN-like cells or a chronically activated T cell that has downregulated CD4 or CD8, respectively.[115,116] In one study, some of these CD4$^-$CD8$^-$CD3$^+$ cells additionally expressed the NK cell marker CD56, and CD45RA, which may argue for natural killer T cells[117] rather than for DN T cells. CD4$^+$CD8$^+$ and CD4$^-$CD8$^-$ cells could therefore contain several subsets (DP-like, DN-like) with distinct functionality and origin. While some expression patterns argue for immature cells, their exact phenotypes, transcriptomes, and functions are not resolved yet. The most intriguing and controversial question will have to be addressed in future work: Can extrathymic T-cell maturation occur in atherosclerotic plaques?

EVIDENCE FOR TISSUE-RESIDENCY AND EXHAUSTION IN ATHEROSCLEROSIS?

In contrast to scRNA-seq, which allows screening of the entire transcriptome at the cost of low-expressed genes, high-parametric protein immunophenotyping by CyTOF helps to interrogate phenotypic heterogeneity based on known surface markers. CyTOF was particularly powerful to subdivide human lesional T cells into distinct states of activation, tissue residency, and exhaustion. The predominant differentiation of lesional T cells into activated (antigen-experienced) effector-memory T cells and central-memory cells (CCR7^{low}CD45RA^{high}CD69$^+$) was observed and suggested the existence of tissue-resident memory T cells (T_{RM}) in the atherosclerotic plaque.[37] T_{RMs} are a unique lineage of T cells that reside in tissues but do not recirculate and thereby provide local tissue protection in infection. CD69 and CD103 are key markers for T_{RMs}, although not all T_{RMs} express these markers.[37,69] In atherosclerotic plaques, a subcluster of CD8$^+$ T cells coexpressed CD69$^+$ and CD103+0.37 While the exact role of this T-cell subpopulation remains to be defined experimentally, it is intriguing to speculate whether T_{RMs} in atherosclerotic lesions are antigen specific or not: a large body of work has suggested that chronic and acute infection caused by cytomegalovirus, HIV, influenza virus, *Chlamydia pneumoniae*,[118] and severe acute respiratory syndrome coronavirus 2[119] may directly interfere with the development and progression of atherosclerosis. The in-depth evaluation of T_{RMs} may help to better understand potential interactions between pathogens, the healthy vessel wall, and atherosclerotic disease.

Several populations of plaque-resident T cells were detected by CyTOF[37] that express the coinhibitory molecule PD-1 (programmed cell death protein 1), which marks T-cell exhaustion,[120] a state of terminal differentiation, dysfunctionality, and hyporesponsiveness induced by chronic antigen-specific stimulation.[120] PD-1$^+$ cells coexpressed other known markers of T-cell exhaustion: CD57, which indicates terminal differentiation and senescence in T cells,[121] EOMES (eomesodermin), and Lag-3 (lymphocyte-activation gene 3). In mice, the ectonucleotidase CD39 inhibits expression of the effector cytokines TNF-α and IFN-γ in a subset of CD8$^+$ T cells in atherosclerotic aortas.[122] Physiologically, T-cell exhaustion seems to arise from a chronic exposure to autoantigens. Whether this state of dysfunctionality is driving or protecting from atherosclerosis is not entirely clear yet. A genetic deficiency of PD-1 or antibody-mediated blockade of PD-1 increased atherosclerotic lesion size,[123,124] which is consistent with the clinical observation that cancer patients receiving checkpoint inhibition therapies that block PD-1 and, thus, reverse exhaustion are at an increased risk of atherosclerotic disease.[125]

AUTOIMMUNITY—A TRANSLATABLE TARGET FOR HUMAN DISEASE?

Preventive concepts for patients with clinical atherosclerosis are currently limited to a modulation of classical risk factors and lipid-lowering strategies, although an increasing body of evidence argues for a role of the immune system and inflammation.[6] High parameter immunophenotyping has proven its ability to identify novel and unexpected candidate markers and mediators of atherosclerosis. For instance, scRNA-seq from human carotid atherosclerosis has surprisingly suggested a predominance of proinflammatory IFN-γ-, IL-12-, and TNF-signaling across different cell types in asymptomatic patients—a finding that contradicts the traditional view that proinflammatory pathways trigger plaque instability and cardiovascular complications.[126] Likewise, pathways associated with IL-1β, a validated therapeutic target in coronary atherosclerosis in the CANTOS trial (Canakinumab Antiinflammatory Thrombosis Outcome Study),[127] were less engaged in T cells from rupture-prone symptomatic carotid plaques. Although these findings may be explained by fundamental differences in carotid versus coronary atherosclerosis or an only partially proatherosclerotic role of IL-1β,[128] more extensive research is required to understand the clinical impact of T-cell diversity in humans.

The translation of murine T-cell immunology remains an ongoing challenge. Basic concepts of T-cell heterogeneity have largely been defined in the mouse with an increasing number of single cell studies in mouse atherosclerosis defined to either describe leukocytes[39,40,46–52] or stromal cells.[42–44] In contrast, only a limited number of studies exploring single cell phenotypes of T cells in human atherosclerosis is available yet.[37,45] The latter have established that—in contrast to mice—phenotypic differences in humans are driven mostly by transcriptional programs of activation, exhaustion, and inflammation and not by traditional T_H-types. How phenotypes in both species overlap is currently unknown. In addition, human plaques seem to be dominated by T cells, although atherosclerotic plaques from mice contain relatively more macrophages. Beyond differences in fundamental T-cell phenotypes, mechanisms and dynamics of immune cells in mice and humans differ considerably and render the direct comparison difficult. First, T cells in the mouse are generated throughout life, while the production of new T cells in humans ceases over time. This, together with the lack of repetitive infections and a mature microbiome in sterile-housed mice, partially explains the higher degree of exhaustion and activation in human T cells.[129,130] Second, inbred mouse strains favor certain types of immune responses, which is exemplified by C57BL/6J mice that have a bias toward T_H1 and BALB/C mice that are more likely to elicit T_H2 responses. The genetic background itself has a direct impact on atherosclerosis as recently demonstrated by the hybrid mouse diversity panel.[131] Third, mice represent an ideal model organism to explore peptide specific T-cell responses: common laboratory mouse strains express only one MHC-II variant (eg, I-A^b in C57BL/6J mice), which allows direct and feasible in silico screening strategies for potential self-peptides. On the contrary, humans exhibit a considerable variability with over 200 MHC-II alleles that form over 10 000 different functional MHC-II complexes. Because the susceptibility of an autoimmune response against self-peptides is determined by the capacity of peptides to bind to the MHC-II complex, antigen-specific responses in humans are far more difficult to predict. Only a few studies have validated the existence of ApoB-specific T cells in humans,[3,5,110] and it remains elusive whether the sequences and MHC-II affinities of different self-peptides predispose for distinct phenotypes and outcomes in humans. It is now acknowledged that a more fine-grained analysis of dynamics, phenotypes, and specificities of atherosclerosis-relevant or ApoB-specific T cells is instrumental to establish novel immunomodulatory strategies for human atherosclerosis in the future.

CONCLUDING REMARKS

High-parametric leukocyte analysis of atherosclerotic aortas and plaques has led us to a deeper understanding of leukocyte diversity and activation. Several studies have applied transcriptional, proteomic, and immunologic approaches, often in a combination of these techniques, to describe yet unknown layers of T-cell heterogeneity

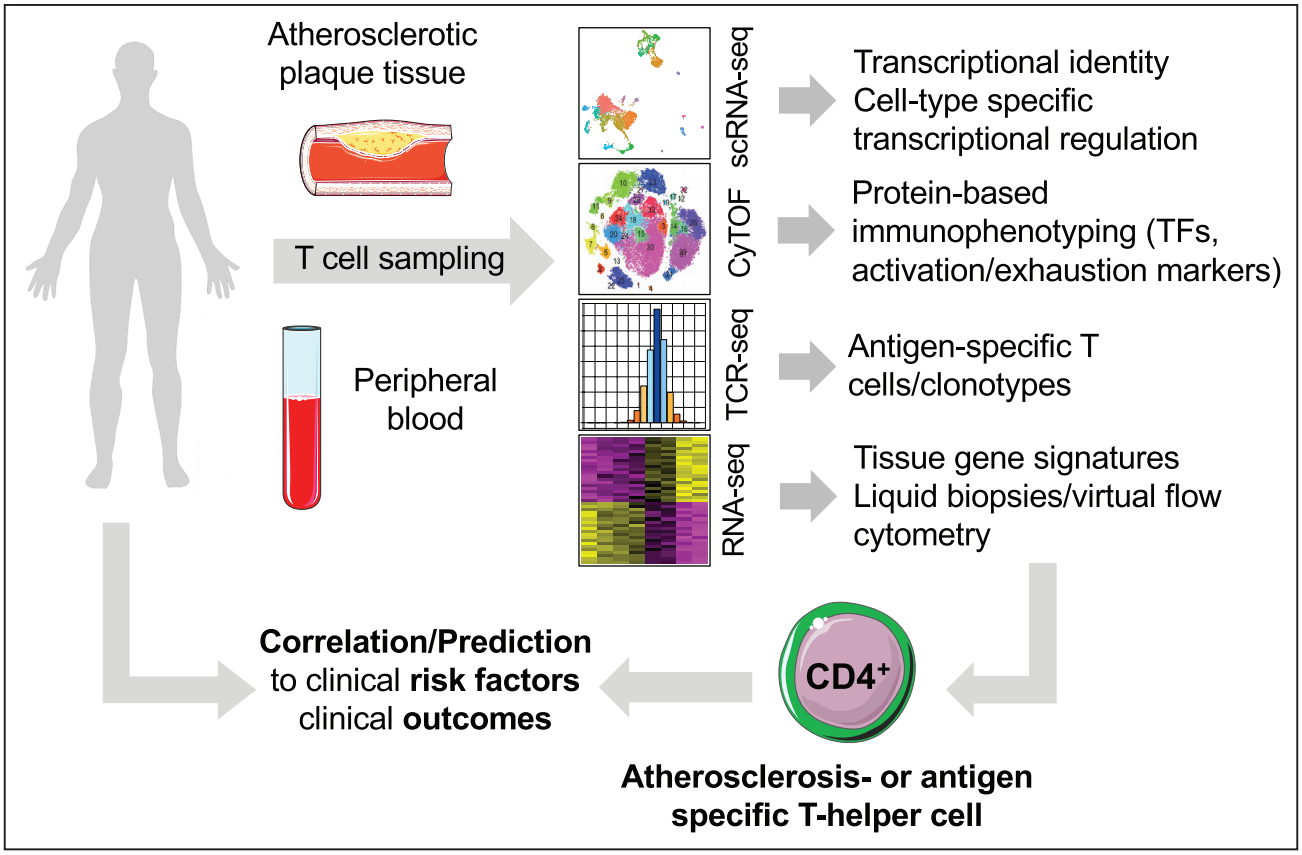

Figure 3. Novel high-parametric and functional immunophenotyping to develop future risk prediction tools.
In humans, T cells can be routinely sampled from surgically excised carotid, peripheral arterial atherosclerotic plaques, and peripheral blood. Mass cytometry (cytometry by time of flight [CyTOF]), single cell RNA-sequencing (scRNA-seq), or a combination of antibody-staining and scRNA-seq in cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) have the potential to uncover unique, even rare, cell populations with distinct identities and potentially specialized roles in atherosclerosis (atherosclerosis-specific T cell). The addition of functional layers by staining with tetramers of MHC-II (major histocompatibility complex II) loaded with peptides from autoantigens and with T-cell receptor (TCR) sequencing in a combination with CyTOF or scRNA-seq integrates the cell surface proteome and transcriptome with antigen-specificity. Such integration generates cell type-specific gene signature, which–vice versa–are detectable in silico in bulk RNA-sequencing data sets, an approach referred to as RNA-deconvolution, liquid biopsy, or virtual flow cytometry. The correlation with clinical outcome data will be helpful to discover clinically relevant cell types. Their transcriptomes or frequencies in blood or tissue may predict the clinical outcomes or identify novel targets for future immunomodulatory cell therapy. TF indicates transcription factor. Schematics adapted from "Smart Servier Medical Art."

in mouse and human atherosclerosis. Together, these studies pinpoint T cells as powerful modulators of plaque associated inflammation: they represent the largest population of viable leukocytes in the plaque, in particular in humans, with a great and largely unexpected phenotypic diversity. Several novel concepts have arisen from these first studies: first, traditional immunologic concepts that are based on T_H-lineages fail to describe the actual heterogeneity of T cells in the atherosclerotic plaque that is rather driven by states of activation, exhaustion, development, and inflammation. Second, a substantial proportion of pathogenic T cells stems from regulatory T cells that are highly plastic and have the ability to pass through different T_H-lineages. Third, a relevant fraction of $CD4^+$ T cells in the plaque can be considered antigen-specific and recognizes autoantigens such as LDL/ApoB. Ongoing antigen-recognition and TCR-signaling events, and the proinflammatory microenvironment in the plaque, are the likely causes for a unique T-cell phenotype that combines highly flexible pro- and anti-inflammatory attributes at the same time. Fourth, appearance and gene expression of leukocyte populations in the plaque and in the circulation seem to correlate with disease progression. At the same time, the field of high parameter immunophenotyping has just been evolved and many important questions have to be addressed in future work. It is critical to understand which exact stimuli drive the destabilization of T_{regs} and the appearance of pathogenic cell clusters and whether these can be targeted by medical therapies. Therefore, it is required to more precisely link phenotypes to clinical outcomes. The in silico enumeration of functional T-cell clusters in peripheral blood or tissues (liquid biopsies, virtual flow cytometry)[132] holds great promise to establish high-throughput prediction tools.[39] Future immunophenotyping will also have to account for disease-specific tissue gene signatures and the

identification of antigen-specific T cells by MHC-II multimers or TCR-sequencing (Figure 3). Only the detailed understanding of underlying mediators of the inflammatory and immune response will allow us to develop tailored immunomodulatory strategies as atheroprotective vaccination in the future.

ARTICLE INFORMATION

Received July 14, 2020; accepted November 17, 2020.

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Sources of Funding

This work was supported by a fellowship to D. Wolf from the Berta-Ottenstein-Program for Advanced Clinician Scientists at the Faculty of Medicine, University of Freiburg. This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No. 853425). H. Winkels was supported by the Deutsche Forschungsgemeinschaft Sonderforschungsbereich/Transregio 259/1 and the Neven-DuMont Foundation.

Disclosures

None.

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