

Adipose tissue macrophage dysfunction is associated with a breach of vascular integrity in NASH

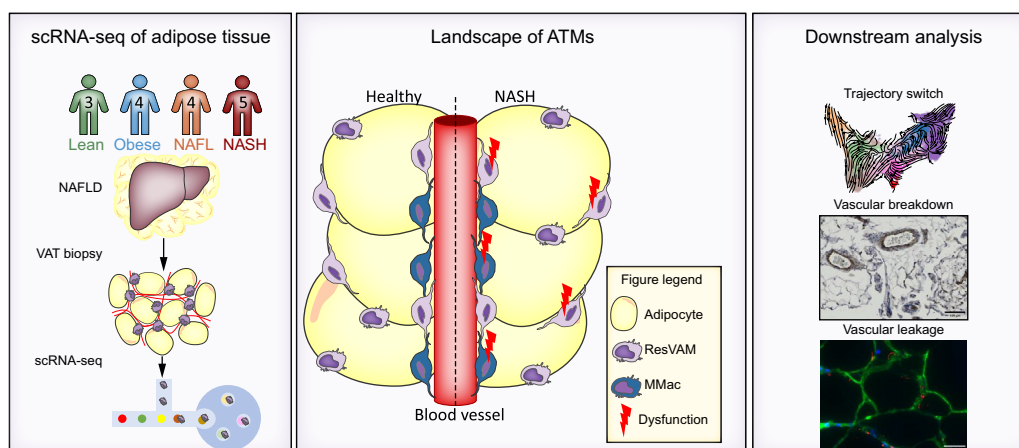
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Graphical abstract



Highlights

- The human visceral adipose tissue comprises multiple macrophage subtypes.
- Metabolically active and resident vascular-associated macrophages contribute to protection of the adipose tissue vascular barrier.
- These macrophage subsets feature a dysfunction in NASH that correlates with a breakdown of vascular integrity.

Impact and implications

Our study describes for the first time the myeloid cell landscape in human visceral adipose tissue at single-cell level within a cohort of well-characterized patients with non-alcoholic fatty liver disease. We report unique non-alcoholic steatohepatitis-specific transcriptional changes within metabolically active macrophages (MMacs) and resident vasculature-associated macrophages (ResVAMs) and we demonstrate their spatial location surrounding the vasculature. These dysfunctional transcriptional macrophage states coincided with the loss of adipose tissue vascular integrity, providing a plausible mechanism by which tissue inflammation is perpetuated in adipose tissue and downstream in the liver. Our study provides a theoretical basis for new therapeutic strategies to be directed towards reinstating the endogenous metabolic, homeostatic and cytoprotective functions of ResVAMs and MMacs, including their role in protecting vascular integrity.

Adipose tissue macrophage dysfunction is associated with a breach of vascular integrity in NASH

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Background & Aims: In non-alcoholic fatty liver disease (NAFLD), monocytes infiltrate visceral adipose tissue promoting local and hepatic inflammation. However, it remains unclear what drives inflammation and how the immune landscape in adipose tissue differs across the NAFLD severity spectrum. We aimed to assess adipose tissue macrophage (ATM) heterogeneity in a NAFLD cohort.

Methods: Visceral adipose tissue macrophages from lean and obese patients, stratified by NAFLD phenotypes, underwent single-cell RNA sequencing. Adipose tissue vascular integrity and breaching was assessed on a protein level via immunohistochemistry and immunofluorescence to determine targets of interest.

Results: We discovered multiple ATM populations, including resident vasculature-associated macrophages (ResVAMs) and distinct metabolically active macrophages (MMacs). Using trajectory analysis, we show that ResVAMs and MMacS are replenished by a common transitional macrophage (TransMac) subtype and that, during NASH, MMacS are not effectively replenished by TransMac precursors. We postulate an accessory role for MMacS and ResVAMs in protecting the adipose tissue vascular barrier, since they both interact with endothelial cells and localize around the vasculature. However, across the NAFLD severity spectrum, alterations occur in these subsets that parallel an adipose tissue vasculature breach characterized by albumin extravasation into the perivascular tissue.

Conclusions: NAFLD-related macrophage dysfunction coincides with a loss of adipose tissue vascular integrity, providing a plausible mechanism by which tissue inflammation is perpetuated in adipose tissue and downstream in the liver.

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Introduction

Due to the global obesity pandemic, the prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing at an alarming rate. Accordingly, NAFLD has become the most common cause of chronic liver disease in Western countries, affecting nearly 25% of the world's population.³ NAFLD refers to a disease spectrum that includes diverse hepatic manifestations ranging from steatosis (non-alcoholic fatty liver [NAFL]) to non-alcoholic steatohepatitis (NASH). NASH in particular is characterized by inflammation, hepatocyte loss and stellate cell activation, which in turn may propagate fibrogenesis. Importantly, NASH and advanced fibrosis develop in up to 12% of the NAFLD population and serve as a risk factor for further disease progression towards cirrhosis and the development of hepatocellular carcinoma. NASH has become one of the main

indications for liver transplantation in Western countries.^{4–6} The development of NASH therefore serves as a key transitional step in the clinical progression of NAFLD and understanding the mechanisms driving its progression will be essential in designing new therapeutic strategies in the future.

Although NASH develops on the background of altered metabolism, there is a strong immune-inflammatory component within the expanding adipose tissue compartment that significantly contributes to, and may even be a prerequisite for, NASH progression.² Adipose tissue macrophages (ATMs) play a particularly prominent role in this regard, since their pro-inflammatory cytokine repertoire contributes to the adipose tissue secretome. Macrophages have also been identified as key drivers of insulin resistance as well as local and hepatic fibro-inflammation.^{7–10} Furthermore, ATMs may directly

Keywords: Non-alcoholic fatty liver disease; macrophages; adipose tissue; single-cell RNA sequencing.

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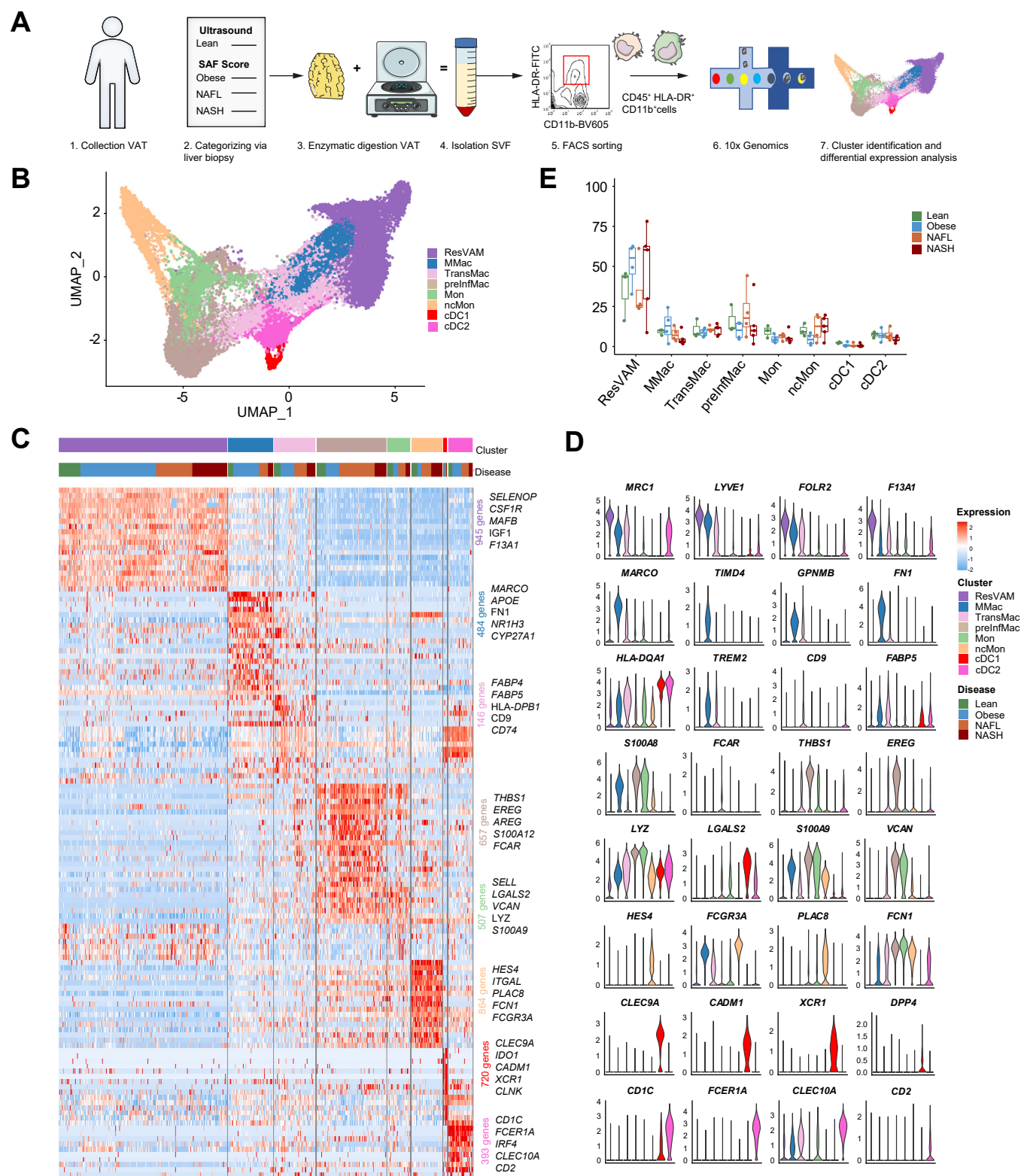


Fig. 1. Single-cell map of human VAT macrophages. (A) Workflow of the experimental pipeline. Human visceral adipose tissue was collected from patients undergoing cholecystectomy and bariatric surgery, NAFLD-stratified based on liver histology, and the stromal vascular fraction enzymatically isolated. The cells were sorted based on CD45, CD11b, and HLA-DR positivity and analyzed using 10X Genomics Chromium droplet single-cell RNA sequencing. The cells were then clustered based on marker genes and downstream analysis was performed. (B) Annotated UMAP map of 35,405 myeloid cells from the VAT of lean ($n = 3$) and obese patients with normal liver histology ($n = 4$), NAFL ($n = 4$) and NASH ($n = 5$), showing ResVAMs, MMac, TransMac, preInfMac, Mon, ncMon and cDCs. (C) Heatmap showing the top 20 marker genes for the myeloid populations. Indicated are individual cluster markers and the total number of genes characterized. The expression is indicated by color saturation, the cluster and disease identity are marked by individual coloring. (D) Violin plots showing the expression of selected marker genes in myeloid cells,

influence the recruitment of immune cells locally into adipose tissue, as well as into distant organs such as the liver, where their frequencies correlate with hepatic histopathological severity.^{11–13} While these studies implicate ATMs as potential therapeutic targets, recent single-cell RNA-seq (scRNA-seq) studies have revealed significant heterogeneity within the ATM pool.^{1,14–19} This warrants a more in-depth characterization of their phenotypic and functional traits to enable more specific targeting strategies. Such heterogeneity may arise from imprinting of tissue-associated cues, distinct ontogenetic origins or disease-induced differential cellular activation states.²⁰ For example, CCR2-independent TIM4⁺ resident macrophages have recently been reported to modulate adiposity and energy storage via the production of platelet-derived growth factors in murine white adipose tissue.¹⁹ Additionally, CD9⁺ ATMs that reside within crown-like structures in the adipose tissue, are lipid-laden and pro-inflammatory. In contrast, CD9[−] ATMs expressing the murine monocyte marker, Ly6C, localize outside crown-like structures and participate in angiogenic- and adipogenic processes.¹⁵ In turn, lipid-associated macrophages (LAMs) utilize the lipid receptor, TREM2, as an extracellular lipid sensor and institute protective functions to counteract adipocyte hypertrophy, inflammation and metabolic dysfunction.¹⁴ Finally, vasculature-associated macrophages (VAMs) have been identified that localize around blood vessels in adipose tissue, where they are strategically positioned to endocytose circulating macromolecules.¹⁶

While some human data is now emerging in patients stratified by grade of obesity,^{1,14,21} this does not always reflect NAFLD severity since patients can exhibit severe obesity but a metabolically healthy status and normal liver function. Inversely, mildly overweight patients can exhibit severe metabolic complications, including NASH.²² As such, our study design has been uniquely set up to include a liver biopsy at the time of visceral adipose tissue sampling to enable accurate scoring of the NAFLD severity state by expert pathologists. In this study, we identified several distinct visceral ATM populations using scRNA-seq within well-defined groups of patients with NAFLD. Importantly, we show changes in their trajectory paths, and clear transcriptional modifications that point towards a uniquely altered functionality in the NASH condition. In addition, we show that NASH-specific transcriptional modifications of ATMs likely affect NAFLD progression due to disruption of the adipose tissue vascular barrier.

Materials and methods

Study participants

Human visceral adipose tissue (VAT) was obtained from patients undergoing bariatric surgery or cholecystectomy in the University Hospital Leuven, Belgium. Peripheral blood was collected before the surgical procedure from all patients for standard biochemical analysis. A liver biopsy was performed during bariatric surgery and assessed by expert liver pathologists blinded to the biochemical and clinical information, based on the semi-

quantitative fatty liver inhibition of progression (FLIP) steatosis (S), activity (A), and fibrosis (F) scoring system.²³ Patients with underlying cirrhosis and diabetes were excluded. Lean controls were patients undergoing cholecystectomy who had a BMI under 28 kg/m² and no signs of steatosis following ultrasound examination. All patients provided written informed consent and the protocols were approved by the UZ Leuven ethical committee (S32380 & S63593).

Statistics and reproducibility

Clinical characteristics were compared using the Kruskal–Wallis test. Immunohistochemistry results were compared with independent t-test and outliers per group removed based on Grubbs' test. Data are shown as mean ± SEM unless indicated otherwise. In the figure legends or in the figure themselves the *p* and *n* values are indicated. *P* < 0.05 was considered significant and in case of immense significant yield, only the lowest *p* values were utilized. Statistical analysis and graphs were produced using GraphPad Prism v8.0 (GraphPad Software) or the respective R packages.

Details on other materials and methods are provided in the [supplementary information](#).

Results

Human visceral ATMs during NAFLD development are transcriptionally distinct

To investigate myeloid cell heterogeneity during NAFLD, we performed scRNA-seq on VAT collected from obese patients (*n* = 13) undergoing bariatric surgery and lean patients (*n* = 3) undergoing cholecystectomy. According to biochemical parameters and histological evaluation of a liver biopsy collected at the time of surgery using the Steatosis-Activity-Fibrosis (SAF) score,²³ obese patients were stratified into three clinically relevant subgroups: obese with normal liver histology (*n* = 4), NAFL (*n* = 4) and NASH (*n* = 5) (see [Table S1](#) for the patient clinical characteristics). To maximize the transcriptional resolution of our analysis, we enriched for myeloid-derived cells by isolating single-, viable-, CD45⁺CD11b⁺HLA-DR⁺ cells followed by immediate processing for droplet-based scRNA-seq ([Fig. 1A](#)). We obtained 35,405 myeloid cells following exclusion of contaminating populations (lymphocytes, (pre)adipocytes, adipose-derived stromal cells and proliferating cells; [Fig. S1A,B](#) and [supplementary data 2](#)). Several distinct clusters of myeloid cells were revealed ([Fig. 1B](#)) that were well integrated across disease state and individual patients ([Fig. S1C,D](#) and [supplementary data 1](#)). As reported for other tissues,²⁴ we identified three distinct macrophage populations within VAT samples of all patients based on *C1QC* and *MRC1/CD206* RNA expression ([Fig. 1C,D](#) and [Fig. S1E](#) and [supplementary data 3](#)).

The first macrophage subset was characterized by high levels of *LYVE1* and *FOLR2*, as well as *F13A1*, *CD163*, *IGF1*, *SELENOP* and *CSF1R*. This transcriptional signature is typical for tissue-resident macrophages and conserved across different

the cluster identity is marked by individual coloring. (E) Bar plot showing the ratio (%) of myeloid cells derived from obese (*n* = 4), NAFL (*n* = 4) and NASH (*n* = 5) groups. cDCs, conventional dendritic cells; MMacs, metabolically active macrophages; Mons, monocytes; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; ncMons, non-classical monocytes; preInnMacs, pre-inflammatory macrophages; ResVAMs, resident vasculature-associated macrophages; TransMacs, transitional macrophages; VAT, visceral adipose tissue; UMAP, uniform manifold approximation and projection.

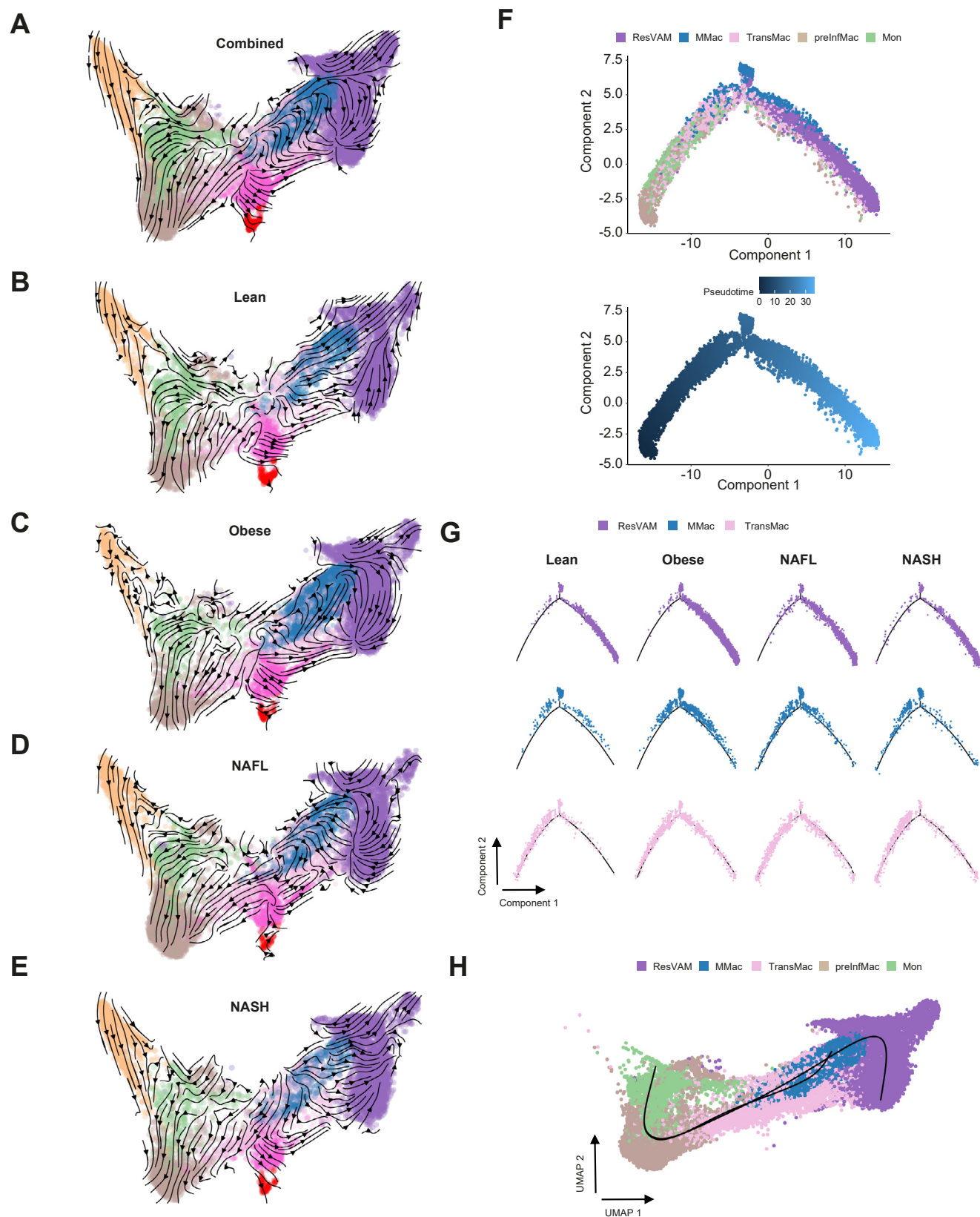


Fig. 2. Predicted trajectories of ATMs and how they are shaped during NAFLD. (A) UMAP plot visualizing RNA velocity of myeloid cells. The arrows indicate the predicted future cell state. (B-E) UMAP plot visualizing RNA velocity of myeloid cells in lean, obese, NAFL and NASH patient groups. The arrows indicate the predicted future cell state. (F) Monocle pseudotime analysis of macrophages and monocytes, including ResVAMs, MMacs, TransMacs, preInfMacs and Mons, indicating cell type

organs.²⁵ Additionally, *MAF* and *MAFB*, known as drivers of terminal macrophage differentiation, were highly upregulated in this cluster, while monocyte markers *FCN1*, *S100A8*, and *LYZ* were low or not expressed (Fig. 1C,D and Fig. S1E).²⁶ Functionally, resident macrophages have been implicated as key regulators of developmental and homeostatic processes in various tissues, and closely associate with the respective vasculature.^{27–29} Characteristically, this cluster featured an enrichment of differentially expressed genes involved in lysosomal function (*CTSC*, *LGMN* and *CD36*) and phagocytosis (*DAB2*, *STAB1*, *CD209*, *CD163* and *MRC1*) while gene ontology additionally indicated vasculature development and angiogenic processes (Fig. 1C,D and Fig. S1E,F). According to their functional traits and *LYVE1* expression, we labeled them resident vasculature-associated macrophages (ResVAMs).

The second macrophage population that we identified featured low levels of *LYVE1* and *FOLR2* and could be further defined by the expression of *FN1*, *MARCO*, *FCGR3A*, *TIMD4*, *NR1H3*, *GPNMB*, *CYP27A1* and *TREM2*.^{29,30} Although this subset expressed *MAF*, these cells also exhibited residual expression of *FCN1*, *LYZ* and *S100A8*, suggesting a possible monocytic origin (Fig. 1C,D). Functional enrichment analysis detected similar homeostatic functions to ResVAMs together with increased involvement in oxidative phosphorylation (OXPHOS), endocytosis and lipoprotein clearance (Fig. S1F). Additionally, several identified marker genes showed involvement in lipid- and cholesterol metabolism, resembling the LAM population previously described by Jaitin *et al.* (Fig. S1F and supplementary data 3).¹⁴ Importantly however, our data indicate that different aspects of lipid metabolism could be detected across all three ATM subsets and were not only confined to one population (Fig. S1F). In addition, a much broader range of metabolic functions were activated within the second macrophage subset (see section below, Fig. S1F). Based on these important differences we termed this subset metabolically active macrophages (MMacs). Interestingly, MMac frequencies across the NAFLD severity spectrum showed a downward tendency (Fig. 1E and Fig. S1G). A potential reduction in MMac frequency in the more severe human disease phenotype, as well as recent data showing that *TREM2* ablation aggravated hypercholesterolemia, inflammation and glucose intolerance in mice, indicate a potential protective role of the MMac population.¹⁴

The third macrophage cluster exhibited dampened expression of *LYVE1* and *FOLR2*, but expressed higher levels of antigen presentation molecules, *HLA-DQA1*, *HLA-DPB1* and *CD74*.²⁴ This subset further expressed high levels of *FCN1*, *LYZ*, *S100A8* and not *MAF*, suggesting an early or transitional state and hence we termed these transitional macrophages (TransMacs). Additionally, TransMacs expressed low levels of *TREM2* and *GPNMB*, and could be distinguished further by *CD9* and fatty acid-binding protein (*FABP4* and *FABP5*) expression, reminiscent of the recently described immature LAMs in the liver (Fig. 1C,D).¹⁴ This subset shows some

transcriptional overlap with MMacs and, as such, it is plausible that this subset has been merged and jointly characterized as a *LYVE1*^{low}*MHCII*^{high} population, residing outside of perivascular spaces and interspersed in various tissues including human white adipose tissue.³¹

Besides the three macrophage subsets, we identified a subset of monocyte-like macrophages (preInflMac) characterized by the abundance of inflammation-triggering alarmins, *S100A8*, *S100A9*, *S100A12*, which are chemo-attractants for neutrophils and responsible for monocyte production of *TNF α* .³² This subset could be further distinguished by the expression of *FCN1*, *FCAR*, *THBS1*, *EREG*, *AREG* and *VCAN*, indicating that it constitutes the most recently elicited subset (Fig. 1C,D). Gene ontology terms indicated regulation of cellular response to hypoxia (Fig. S1F). Finally, we identified a classical monocyte subset (*LYZ*, *SELL*, *LGALS2*, *S100A8*, *S100A9*, *FCN1*, *VCAN*), a non-classical monocyte subset (*HES4*, *PLAC8*, *ITGAL*, *FCN1*), a subset of conventional type 1 dendritic cells (*CLEC9A*, *CADM1*, *XCR1*), and a subset of conventional type 2 dendritic cells (*CD1C*, *FCER1A*, *CLEC10A*) (Fig. 1C,D).³³

To validate our clustering analysis, we integrated our data with a recently published single-nuclei sequencing dataset of VAT obtained from lean or BMI-stratified patients with obesity.¹ Canonical correlation analysis showed that the macrophage transcriptomes from the two datasets were largely overlapping (Fig. S2A–C). Notably, while it is unclear whether patients with NASH were included in this study, there were no differences in the hMac3/MMac proportions in lean vs. obese conditions (Fig. S2D,E). We further applied our previous microarray data of VAT obtained from patients with well-defined NAFLD states and analyzed how macrophage frequencies were affected using a deconvolution algorithm.² The data suggests a similar downward tendency in MMac frequencies in patients with NASH (Fig. S2G). Together, these data provide an extensive characterization of human ATMs and provide insights on how the myeloid subset proportions are shaped in the setting of NAFLD.

NASH skews the developmental trajectories of adipose tissue macrophages

To unravel the origin and transcriptional fate of the identified myeloid cell clusters, an RNA-velocity analysis was performed. When analyzing all experimental conditions together, the velocity vectors among the myeloid cell clusters traced ResVAMs and MMacs as terminal endpoints that emanate from monocytes at the root. Interestingly, both subsets were preceded by TransMacs, as a putative transitional state (Fig. 2A). When we dissected differentiation pathways per disease condition, the velocity analysis revealed a striking change in the TransMac differentiation pathway. In the NASH condition, MMacs were no longer the major subset replenished by TransMacs but instead, TransMacs predominantly fed the ResVAM pool (Fig. 2B–E).

(top) and pseudotime directionality (bottom). (G) Monocle pseudotime analysis of macrophages and monocytes, showing the pseudotime directionality change of ResVAMs, MMacs and TransMacs in the lean, obese, NAFL and NASH condition. (H) Slingshot results of macrophages and monocytes transposed on the UMAP plot to validate the trajectory and pseudotime analyses. ATMs, adipose tissue macrophages; MMacs, metabolically active macrophages; Mons, monocytes; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; preInflMacs, pre-inflammatory macrophages; ResVAMs, resident vasculature-associated macrophages; TransMacs, transitional macrophages; UMAP, uniform manifold approximation and projection.

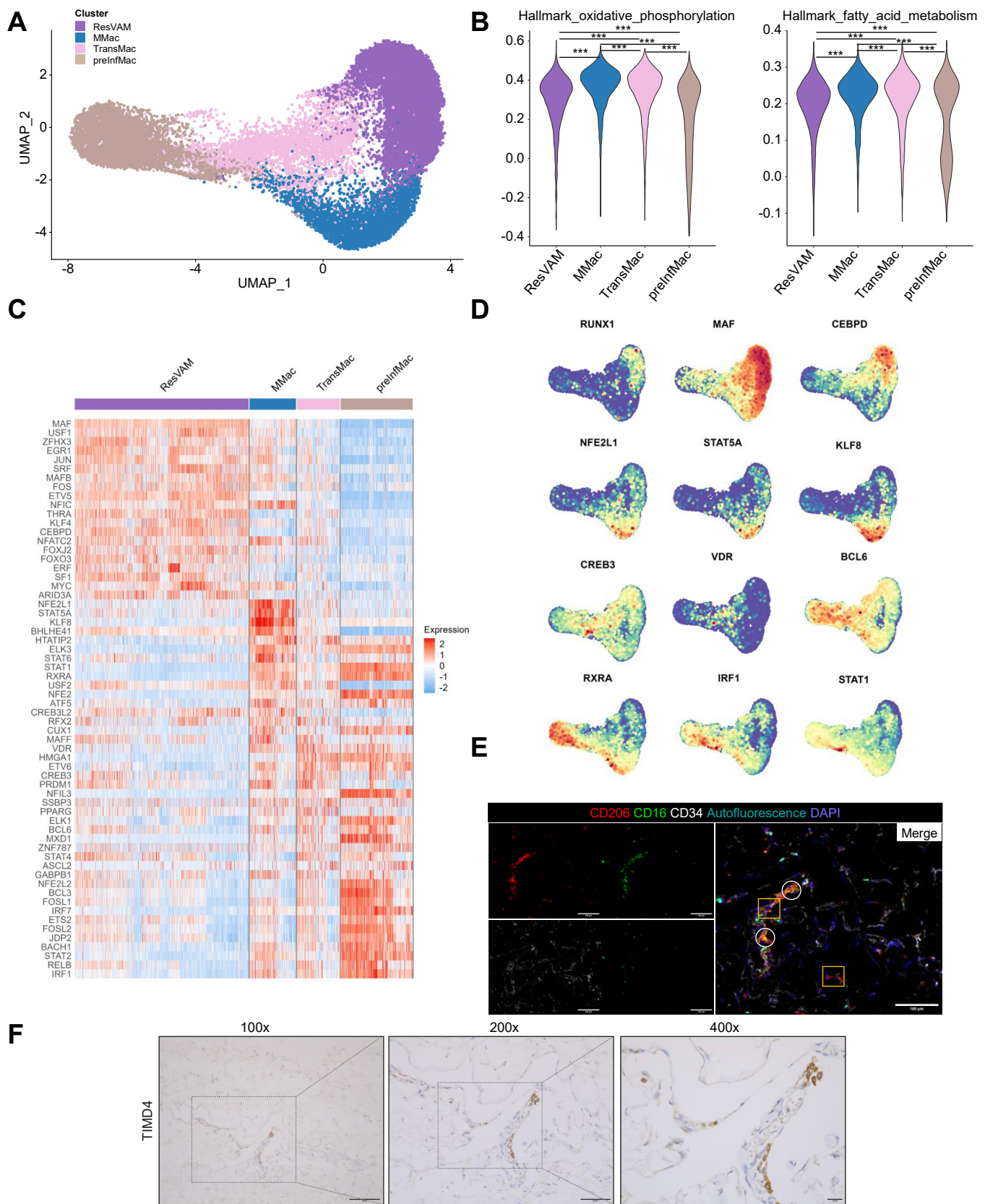


Fig. 3. In-depth characterization of human ATMs in steady state and the micro-anatomical location of ResVAMs and MMac surrounding the vasculature. (A) Recalculated UMAP based on gene expression of the ATMs (ResVAM, MMac, TransMac and preInfMac) in all patients. (B) Violin plot depicting the GSVA score of indicated signatures in each ATM subset. *** $p < 0.001$ and absolute t -value > 10 by a linear model. (C) Heatmap illustrating the rescaled SCENIC area under curve-identified top 20 marker regulons in the ATMs. The expression is indicated by color saturation. (D) UMAP showing the activity of the indicated transcription

To support these findings, we implemented Monocle's pseudotime analysis to analyze monocytes and macrophages. The results showed that the trajectory began at the monocyte cell node (darkest blue) and ended at either the MMac (medium blue) or ResVAM nodes (lightest blue) (Fig. 2F). Branched expression analysis modeling of differentially expressed genes between the nodes showed clear transcriptional gradients as genes are lost or gained and cells moved from a monocyte to a mature state (Fig. S3A). For example, genes typical for monocytes were downregulated following this trajectory (*FCN1*, *S100A8*), whereas typical tissue-resident genes were elevated (*FOLR2*, *LYVE1*) (Fig. S3B). Additionally, the TransMacs distributed throughout all branches, and their transition into either MMacs or ResVAMs was further confirmed (Fig. 2F,G). Importantly, this multidirectional fate decision was not observed in other cells (Fig. S3C). Calculating the proportion of TransMacs on each branch also revealed a preferential developmental switch of TransMacs into ResVAMs, rather than MMacs, in VAT from patients with NASH (Fig. 2G and Fig. S3D). An additional slingshot analysis showed a clear lineage separation of the various macrophage subtypes that transition through the TransMac population (Fig. 2H). Finally, we were able to reproduce our findings using the dataset from Emont *et al.*¹ Hereby, starting from the monocytes, hMac1 (ResVAM) and hMac3 (MMac) populated mainly their own mature node, while hMac2 (TransMac) were distributed across all trajectories, corroborating our findings (Fig. S2F). By harnessing scRNA-seq, the trajectory analysis sheds new light on the developmental trajectories of human ATMs and how these are altered in the NAFLD environment.

Accessory roles for MMacs and ResVAMs in restraining inflammation, regulating (lipid) metabolism and protecting the vascular barrier

To characterize human visceral ATMs in steady state, we performed an in-depth analysis of the molecular features that distinguish the macrophage subsets identified. Subclustering showed a clear separation between the ResVAM-, MMac- and preInnMac-subsets with the TransMacs centrally orientated (Fig. 3A). An additional inter-population comparison further identified a larger set of discriminating markers that could potentially be used in future studies for prospective isolation and functional characterization (Fig. S4A and supplementary data 4–5). A more extensive pathway analysis revealed that MMacs featured upregulation of genes involved in diverse metabolic pathways, including OXPHOS, and mTORC signaling, pointing towards a highly metabolically active state within this subset in addition to their role in adipogenesis, and cholesterol and fatty acid metabolism (Fig. 3B and Fig. S4B). Although the TransMac's transcriptome overlaps most closely with MMacs, they also showed some resemblance to ResVAMs. Conversely,

preInnMacs exhibited an enrichment for inflammatory pathways (Fig. S4B).

We next implemented the SCENIC workflow to identify the transcription factor-mediated gene regulatory networks (regulons) that drive the transcriptional state of ATMs. Potential master regulators for ResVAMs included MAF and RUNX1 (Fig. 3C,D), which have previously been implicated in regulating the identity of resident macrophages.²⁰ ResVAMs were additionally characterized by regulons curbing inflammation, such as *ETV5*.³⁴ Additionally, the CEBPD regulon was active in ResVAMs, supporting the notion that lipid metabolic functions are not restricted to MMacs (Fig. 3C,D).³⁵ Interestingly, MMacs showed activity within regulon RXR α , encoding the heterodimerization partner of LXR α (liver X receptor α), which controls lipid and iron metabolism and independently functions in innate immune regulation.^{36,37} In addition, NFE2L2, a major antioxidant transcription factor, was active in MMacs.³⁸ Furthermore, KLF8, an apoptosis repressor and pro-angiogenic transcription factor, was highly specific for MMacs (Fig. 3C,D).^{39,40} Finally, STAT6, a master regulator driving the anti-inflammatory transcriptional program in macrophages, was also upregulated in MMacs.⁴¹ As expected, the gene regulatory network of TransMacs featured a close overlap with MMacs, however, they could be specifically characterized by the VDR regulon. Finally, regulons involved in the stress response and inflammation (e.g., STAT1, and IRF1) appeared in preInnMacs (Fig. 3C,D).

For downstream analysis, we focused on the two most terminally differentiated subsets (*i.e.*, ResVAMs and MMacs) and further probed their accessory role in tissue-supporting functions. In this regard, we used the data from Emont *et al.*, and performed a receptor-ligand interaction analysis between identified cell types.¹ Interestingly, the data indicated that both MMacs were enriched for putative interactions with endothelial cells (Fig. S4C,D and supplementary data 6). We next evaluated whether these results would correlate with a micro-anatomical location close to vascular endothelial cells within human VAT samples. Hereto we performed immunofluorescent staining using CD206 (*MRC1*) as a marker expressed by both subsets, along with CD16 as a selective MMac marker. Additionally, CD34 was included in the panel to reveal the location of the blood vessels (Fig. 3E, see Table S2 for the patient clinical characteristics). Strikingly, CD206^{pos}CD16^{pos} MMacs (white circle) were positioned exclusively around the blood vessels while CD206^{pos}CD16^{neg} ResVAMs were located both around the blood vessels and interspersed in the tissue (Fig. 3E and Fig. S4E, yellow square). To corroborate these results, we performed immunohistochemistry whereby TIMD4 was used as another distinctive MMac marker. Of note, we performed the staining on the consecutive tissue section and relocated to the same position as indicated in Fig. 3E to verify a potential association of both these markers to the same cell. The results clearly implicate TIMD4 positivity exclusively on cells with

factors/regulons. The ATMs in which the indicated transcription factor is active and reaches a certain threshold are indicated by color saturation (low (blue) to red (high)). (E) Representative images of fluorescent staining for CD206 (red) macrophages, CD16 (green), CD34 (white) blood vessels, and DAPI (blue) in VAT samples from obese patients without NAFLD ($n = 2$). The CD206^{pos}CD16^{pos} MMac population (yellow) clusters in proximity to the blood vessels (white circle). CD206^{pos}CD16^{neg} ResVAM were localized around the blood vessels and were interspersed in the tissue (yellow squares). Scale bar 100 μ m, magnification 10x, autofluorescence is indicated in cyan. (F) Consecutive immunohistochemistry images of TIMD4 macrophages in VAT samples of patients ($n = 8$) to identify TIMD4^{pos} MMacs. Scale bar 50 μ m magnification 100x, and scale bar 100 μ m, magnification 200x and 400x. ATMs, adipose tissue macrophages; GSVA, gene set variation analysis; MMacs, metabolically active macrophages; NAFLD, non-alcoholic fatty liver disease; preInnMacs, pre-inflammatory macrophages; ResVAMs, resident vasculature-associated macrophages; TransMacs, transitional macrophages; VAT, visceral adipose tissue; UMAP, uniform manifold approximation and projection.

macrophage morphology, that align the vasculature and the positioning of TIMD4 staining with the previously depicted CD206^{pos}CD16^{pos} MMacS (Fig. 3F). Of interest, Silva *et al.* demonstrated the presence of two distinct vasculature-associated macrophage subsets in murine adipose tissue that are depleted under acute inflammatory or metabolic stress.¹⁶ It is therefore plausible that ResVAMs and MMacS represent two transcriptionally distinct subsets that jointly regulate the vascular niche and protect the barrier, preventing spillover of lipotoxic molecules to the systemic circulation and the liver.

NASH-induced transcriptional modulation of the ResVAM and MMac pool is associated with breaching of the adipose tissue vasculature

Due to their perivascular localization, ResVAMs and MMacS are continuously exposed to circulating macromolecules; as such, these subsets may act as important immune sentinels at the interface between adipose tissue and the circulation. We first probed whether macrophage subsets exhibit disease-mediated functional defects. Notably, the disease-specific transcriptional alterations within the TransMacS and preInfMacS have been depicted in Fig. S5 (and supplementary data 9-10). Hereto, NASH-ResVAMs featured elevated levels of heat shock proteins (*HSPA1A*, *HSPA90AA1*), anti-inflammatory parameters (*MRC1L1*, *SEPP1*), as well as neutrophil- and monocyte chemoattractant molecules (*IL8*, *CCL2*, *CCL8*) (Fig. 4A and supplementary data 7).

Regarding the disease-specific changes within the MMac subset, NASH-MMacS showed elevated expression of the pro-inflammatory alarmins (*S100A4*, *S100A6*), parameters modulating cell-cell and cell-matrix interactions (*LGALS1*, *PFN1*, *VCAN*, *LSP1*), scavenger receptor (*CD36*), and the fatty acid-binding protein (*FABP5*) (Fig. 4A and supplementary data 8). Interestingly, NASH-induced transcriptional alterations seem to be ATM subset specific (Fig. S5B). Taken together, these findings point towards an aberrant functional status of both ResVAMs and MMacS within the adipose tissue of patients with NASH.

Perivascular macrophages have been shown, in murine models of obesity, to play an important role in protecting adipose tissue vascular integrity.⁴² We postulated that the perturbed transcriptional state of ResVAMs and MMacS may lead to disruption of the vascular barrier, driving NASH progression. In order to verify the existence of such a breach we performed immunohistological staining on paraffin embedded human adipose tissue sections (n = 5-10/group) of the endothelial cell residing plasmalemma vesicle-associated protein 1 (PV1), a key marker for endothelial permeability.⁴³ The data indicated increased intensity of PV1 staining in patients with NASH compared to those with NAFL and controls (Fig. 4B,C and Fig. S5C). Importantly, PV1 intensity positively correlated with the fibrosis score in patients tested within this independent cohort (Fig. 4D and Fig. S5D-H).

We next performed immunofluorescence labeling of albumin to demarcate sites of vasculature breakdown as a result of leakage of this intrinsic tracer into the tissue, to support the data on PV1 staining. By visualizing the endothelium with collagen IV, albumin leakage into the extravascular space was observed in both NAFL and NASH (Fig. 4B,E). Importantly, elevated albumin crossing was associated with advanced steatosis in this patient cohort (Fig. S5I-N). Collectively, these results support the breakdown of the adipose tissue vascular

barrier as well as transcriptional modulation of macrophage subsets within the vascular niche during NASH.

Discussion

NAFLD has become the most prevalent liver disease worldwide, affecting a quarter of the world's adult population. Even more alarmingly, studies evaluating the prevalence of NASH estimate that it may involve up to 6.5% of the general population and as many as 37% of those with type 2 diabetes.³ Why only some patients with NAFL progress to NASH remains poorly understood. We and others have previously shown that adipose tissue inflammation is more pronounced and the frequency of CD206⁺CD11c⁺ macrophages increased in the adipose tissue of patients with NASH compared to NAFL.² In addition, ablating ATMs or removing visceral white adipose tissue in animal models attenuated insulin resistance and liver inflammation, which suggests that adipose tissue inflammation may be a prerequisite for the development of NASH.⁴⁴⁻⁴⁶ Conceptually, adipose tissue contributes to the secretome, which reaches the liver through the portal circulation, while circulating factors in obesity may promote adipose tissue inflammation.⁸ Our study describes for the first time the diversity of ATMs in well-defined patients with NAFLD. Our data reveal unique disease-related alterations in various ATM subsets that suggest that these cells may actively participate in NASH progression.

Specifically, we detected the presence of two terminally differentiated macrophage populations, where one subset expressed high levels of *LYVE1* and *FOLR2*, in addition to a set of conserved markers reminiscent of resident macrophages.^{25,29} We termed this population ResVAMs. Unlike resident macrophages in other tissues, trajectory analysis predicted ResVAMs not to be strictly embryonically derived (or self-renewing) but to be replenished from the circulating monocyte pool. This finding is supported by a recent study of adipose tissue using a murine fate-mapping model.⁴⁷ Furthermore, our data suggests that the disease transition towards NASH is associated with an aberrant functionality of resident macrophages whereby they lose their capacity to perform cytoprotective homeostatic functions, leading to disease progression in NAFLD.⁴⁸⁻⁵⁰

We further confirmed the presence of a second subset of terminally differentiated monocyte-derived macrophages that expressed elevated levels of *TREM2*, *GPNMB*, *MARCO* and *TIMD4*, which we termed metabolically active macrophages or MMacS. "Metabolic" macrophages have also been noted in other niches such as the liver and in atherosclerotic plaques.^{51,52} The transcriptional profile of MMacS exhibited some similarities to LAMs. However, MMacS were enriched for genes that regulate diverse metabolic pathways and not just lipid processing, a trait shared by all subsets.¹⁴ Importantly, by leveraging the data of Emont *et al.* and our own, we further defined that this subset is not specific to obesity or NAFLD in adipose tissue or in murine NASH livers, respectively, as proposed by others.^{1,14,53-55} Instead, we also observed the MMac/LAM transcriptional profile in the healthy adipose tissue of lean individuals. Corroborating this concept, the core LAM signature has recently been shown to be conserved across different healthy human tissues.²⁴ Using trajectory analysis, we demonstrated that monocytes predominantly give rise to MMacS via an intermediate TransMac population. However, in NASH, this trajectory is diverted to replenish ResVAMs instead of MMacS. NAFL-MMacS further

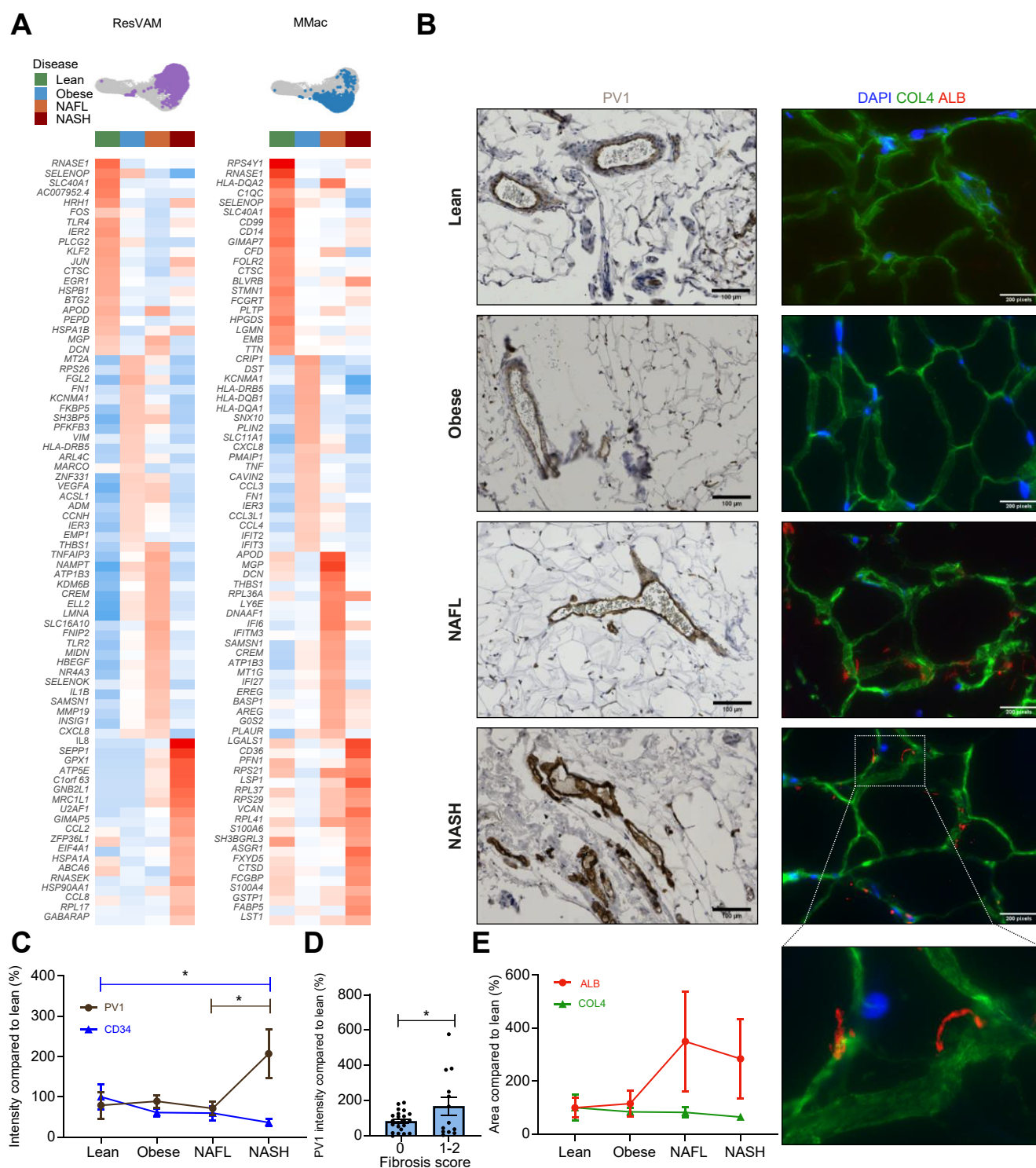


Fig. 4. ResVAM and MMac dysfunction in NASH is associated with adipose tissue vasculature breach. (A) Heatmaps illustrating the average scaled expression of the top 20 marker genes in the ResVAMs and MMac. The expression is indicated by color saturation, from high (red) to low (blue). (B) PV1 (brown) widefield microscopy images in FFPE VAT samples of lean controls (n = 5), obese (n = 10), NAFL (n = 10) and NASH (n = 10) patient groups. Scale bar, 100 μ m, magnification 20x. Representative images of ALB (red), blood vessels (COL4 – green) and cell nuclei (DAPI – blue) of lean control (n = 4), obese (n = 10), NAFL (n = 9) and NASH (n = 9) patient groups. Scale bar, 200 pixels (77.5 μ m), magnification 40x. (C) Measured DAB staining intensity of PV1 and CD34 (blood vessels) compared to the lean control. (D) PV1 DAB intensity compared to the lean control in patients with histological fibrosis score of 0 (n = 22) and 1-2 (n = 13). (E) Quantification of ALB- and COL4-positive areas in comparison to the lean control. Statistical comparisons were performed with independent t-test and outliers per group removed based on the Grubbs test. *p < 0.05. ALB, albumin; COL4, collagen IV; MMac, metabolically active macrophages; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; PV1, plasmalemma vesicle-associated protein 1; ResVAMs, resident vasculature-associated macrophages; VAT, visceral adipose tissue.

featured a release of anti-proliferative-, antioxidant- and cyto-protective properties and seemed to be overwhelmed in their capacity to metabolize lipids, properties that were similarly dysregulated in NASH-ResVAMs. Combined, these aspects may be responsible for the tendency towards a decrease in MMac frequency in NASH. Hereto, further research is warranted to establish the shaping of cellular proportions in NASH. Of interest, the loss of TREM2⁺ ATMs has previously been shown to result in increased obesity, persistent inflammation, insulin resistance, elevated hepatic triglyceride content and compromised recovery following liver damage, assigning a protective role to this subset.^{14,56,57} It is therefore tempting to speculate that redirecting TransMacs to replenish MMac may reinstate and maintain the altered adipose tissue- and whole body (metabolic) homeostasis in NAFLD.⁵⁸ Alternatively, small molecules may be utilized to correct OXPHOS to improve chronic inflammation in obesity-related disorders.⁵⁹

Our in-depth transcriptional analysis further characterized MMac by their co-expression of vascular-associated markers, *LYVE1*, *FOLR2* and *CD206*. This distinctive signature overlapped with the VAM markers previously described by Silva *et al.*¹⁶ Of note, CD206 positive- or OXPHOS macrophages have been demonstrated to actively phagocytose blood-borne cellular material and protect the vascular barrier, respectively.^{60,61} Indeed, using immunohistological labeling we confirmed that ResVAMs localized around the adipose tissue vasculature and were interspersed in the tissue, while MMac

were exclusively localized in the perivascular region. We further identified CD16 and TIMD4 as additional markers at a protein level to distinguish ResVAM (CD16^{neg}, TIMD4^{neg}, CD206^{pos}) and MMac (CD16^{pos}, TIMD4^{pos}, CD206^{pos}) subsets. Due to their perivascular localization and distinct transcriptional profiles, we propose the concept that MMac support the function of ResVAMs in regulating metabolism and homeostasis, as well as performing tissue protective functions, including the maintenance of the vasculature barrier. This led us to hypothesize that the dysfunction of MMac and ResVAMs, observed in association with more severe NAFLD phenotypes, may compromise vasculature integrity. Indeed, we could verify such a breach in the adipose tissue barrier by documenting an elevated expression of a key marker of vasculature permeability, PV1, and extravasation of albumin into the perivascular space. Since perivascular macrophages have been shown to play an important role in protecting murine adipose tissue vascular integrity, these results would support the notion that the dysfunction of MMac and ResVAMs could result in extravasation of lipotoxic and inflammatory molecules to the liver, thereby driving NASH progression.^{16,42}

In conclusion, our findings suggest that new therapeutic strategies to treat NASH or halt disease progression towards more severe phenotypes should be directed towards reinstating the endogenous metabolic, homeostatic and cytoprotective functions of ResVAMs and MMac, including their role in protecting vascular integrity.

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Abbreviations

ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; APC, (pre) adipocytes; ASC, adipose-derived stromal cells; AST, aspartate transaminase; ATM, adipose tissue macrophage; AUC, area under the recovery curve; BMI, body mass index; cDC, dendritic cells; COL4, collagen IV; FLIP, fatty liver inhibition of progression; FN1, fibronectin; GEO, Gene Expression Omnibus; GO, Gene ontology; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; LAM, lipid-associated macrophage; LDL, low-density lipoprotein; LXR α , liver X receptor alpha; MMac, metabolically active macrophage; Mon, monocyte; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ncMon, non-classical monocytes; OXPHOS, oxidative phosphorylation; preInMac, monocyte-like macrophages; PV1, plasmalemma vesicle-associated protein 1; ResVAM, resident vasculature-associated macrophage; SAF, steatosis, activity, and fibrosis; scRNA-seq, single-cell RNA-seq; ssGSEA, single sample gene set enrichment analysis; TG, triglyceride; TransMac, transitional macrophages; UMAP, uniform manifold approximation and projection; VAM, vasculature-associated macrophages; VAT, visceral adipose tissue; VIM, vimentin.

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Conflict of interest

The authors of this study declare that they do not have any conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

SVDM and HK conceived the study, directed and supervised the research. TR and PB reviewed the histopathologic diagnoses. ED, ML, JJ, HT and BT contributed to the enrolment of patients and the collection of patient information and samples. MB, RFA, MW, LS and LVM contributed to the tissue dissociation and single-cell suspension preparation. RFA, MB and HK performed the flow cytometry analysis. TV supervised the scRNA-seq sample preparation and scRNA-seq data pre-processing. MB and ASIF performed the scRNA-seq analysis. AL, PB, AS, TO, AH, OG, MR and MG performed immunohistochemistry and immunofluorescent experiments and image acquisition. AL, ASIF, MB and HK generated the figures and tables for the manuscript. All the authors contributed to the manuscript preparation. MB and HK wrote the original manuscript. SVDM, HK, JV, SF, MR and MG reviewed and edited the manuscript.

Data availability statement

The scRNA datasets are available from the corresponding authors on request. Emont *et al.* data is publicly available via <https://gitlab.com/rosen-lab/white-adipose-atlas> and accessible at GEO (GSE176171).¹ The utilized microarray data of NAFLD patient derived VAT by du Plessis *et al.* is accessible at GEO (GSE58979).²

Code availability

On reasonable request, the R scripts to follow the main steps of analysis are available from the corresponding authors.

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Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2023.10.039>.

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Author names in bold designate shared co-first authorship

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