Molecules and Cells

Minireview

Adipose tissue macrophage heterogeneity in the single-cell genomics era

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ABSTRACT

It is now well-accepted that obesity-induced inflammation plays an important role in the development of insulin resistance and type 2 diabetes. A key source of the inflammation is the murine epididymal and human visceral adipose tissue. The current paradigm is that obesity activates multiple proinflammatory immune cell types in adipose tissue, including adipose-tissue macrophages (ATMs), T Helper 1 (Th1) T cells, and natural killer (NK) cells, while concomitantly suppressing anti-inflammatory immune cells such as T Helper 2 (Th2) T cells and regulatory T cells (Tregs). A key feature of the current paradigm is that obesity induces the anti-inflammatory M2 ATMs in lean adipose tissue to polarize into proinflammatory M1 ATMs. However, recent single-cell transcriptomics studies suggest that the story is much more complex. Here we describe the single-cell genomics technologies that have been developed recently and the emerging results from studies using these technologies. While further studies are needed, it is clear that ATMs are highly heterogeneous. Moreover, while a variety of ATM clusters with quite distinct features have been found to be expanded by obesity, none truly resemble classical M1 ATMs. It is likely that single-cell transcriptomics technology will further revolutionize the field, thereby promoting our understanding of ATMs, adipose-tissue inflammation, and insulin resistance and accelerating the development of therapies for type 2 diabetes.

Keywords: Adipose tissue macrophages (ATMs), ATM heterogeneity, Inflammation, Insulin resistance, Obesity

INTRODUCTION

Obesity is defined as an abnormal and excessive accumulation of fat (Blüher, 2019) due to excessive energy intake and/or decreased energy expenditure. This unbalanced energy homeostasis is promoted by diverse factors, including eating behavior, nutrition supply, working patterns, lack of exercise, social status, and genetics (Dixon, 2010). Since the prevalence of obesity is growing both worldwide and in Korea (Jung et al., 2020), and obesity associates strongly with the development of multiple diseases, including cardiovascular disease, cancer, chronic lung disease, and type 2 diabetes (T2D) (Conway and Rene, 2004), considerable research effort continues to be focused on the mechanisms by which obesity induces these pathologies.

The present minireview concentrates on the role of adipose-tissue macrophages (ATMs) in the development of obesity-induced inflammation and insulin resistance that eventually leads to T2D (Kahn et al., 2006). Insulin resistance is caused by dysregulated systemic glucose homeostasis. Normally, glucose metabolism is tightly regulated by the liver, pancreas, skeletal muscles, and adipose tissue. Thus, between meals, the liver finely tunes the glucose levels in the circulation by conducting glycogenolysis and gluconeogenesis. After a meal, the increased circulating glucose levels induce the pancreatic β cells to produce insulin, which causes the liver, skeletal muscles, and adipose tissue to take up the glucose, convert it to glycogen/triglycerides, and store it for future energy needs (Roden and Shulman, 2019). This systemic glucose homeostatic balance is disrupted by insulin resistance, which is a defect in intracellular insulin signaling. In this setting, muscle and adipose tissue become unable to take up glucose. In an attempt to maintain glucose homeostasis, the β cells produce increasing levels of insulin until they become inert and/or start dying from exhaustion and/or glucose toxicity. At this point, T2D develops (Kahn et al., 1993).

The significant health and economic impact of T2D has led to intense interest over the past 4 decades in the molecular mechanisms that underlie the development of T2D (Shoelson et al., 2006). Multiple mechanisms that have strong supporting evidence have been proposed. They include hyperlipidemia and ectopic fat accumulation, particularly in the skeletal muscle; endoplasmic reticulum (ER) stress; mitochondrial dysfunction;

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metabolite abnormalities, including those produced by the microbiome; and low-grade but chronic systemic inflammation that is induced by obesity-generating factors, including a high-fat diet (Roden and Shulman, 2019).

This minireview focuses on the latter mechanism, for which there is considerable evidence (Esser et al., 2014). The evidence for the pathogenic role of chronic inflammation in the development of insulin resistance and T2D first emerged in 2001, when an epidemiological study showed that circulating inflammatory markers predicted the development of T2D in humans (Pradhan et al., 2001) and our study concomitantly reported that inflammation activated via the IκB kinase β (IKKβ)/nuclear factor kappa B (NF-κB) pathway attenuated insulin resistance in rodent models (Yuan et al., 2001). Subsequent studies showed that this obesity-induced inflammation is regulated by classical immune cells such as macrophages, Tregs, T and B cells, and NK cells. Interest then rapidly focused on the roles of these cells in the adipose tissue (Lee et al., 2016). The data suggested that obesity shifts the native anti-inflammatory state in adipose tissue, which is maintained by Tregs and Th2 T cells, to a proinflammatory state. This is mediated by obesity-induced activation of proinflammatory M1 ATMs, Th1 T cells, CD8 T cells, and NK cells. Studies in both rodent models and humans then showed that this proinflammatory shift specifically the epididymal fat in mice and visceral fat in humans helps drive the development of insulin resistance and T2D (Lumeng et al., 2007; Viola et al., 2019). These findings thus elucidated a key paradigm in the obesity field, namely, that adipose tissue not only acts as an energy-storage organ, it also regulates systemic immunity and metabolism.

The ATMs have long been the main target of research in obesity because their numbers are greatly increased in obesity, they display a highly activated proinflammatory state in obesity, and ATM-suppressing measures ameliorate insulin resistance/T2D. Indeed, proinflammatory ATMs have long been thought to be key drivers of this disease (Lee and Lee, 2014). Interestingly, however, the recent advent of single-cell RNAseq technology has revealed considerable heterogeneity in ATMs, which suggests that the current pro-/anti-inflammatory M1/M2 view of these cells does not fully capture their roles in obesity-induced insulin resistance/T2D. This minireview will discuss the new single-cell RNAseq findings in the context of the current paradigm regarding ATMs.

ADIPOSE-TISSUE MACROPHAGES

Adipose tissues contain most of the classical immune cell types, including macrophages, CD4 and CD8 T cells, B cells, granulocytes (eg, neutrophils and mast cells), natural kille T (NKT) cells, and NK cells (Bonamichi and Lee, 2017; Exley et al., 2014). Among these immune cells, ATMs are the most abundant of the immune cells, and furthermore, the numbers and activities of ATMs are dramatically increased in obesity (Russo and Lumeng, 2018).

Macrophages are innate immune cells that play important roles in removing dead or infected cells and in regulating other cells, including immune cells, by producing various cytokines and chemokines (Lee and Lee, 2014). Macrophages also play key roles in wound healing/tissue remodeling after inflammation is resolved. ATMs exhibit many of these classical macrophage phenotypes (Gordon, 2003).

The Chen and Ferrante groups were the first to show that obesity increases ATM numbers and that ATMs are the largest producers of diabetogenic proinflammatory cytokines (eg, tumor necrosis factor-α (TNF-α)) in obesity (Weisberg et al., 2003; Xu et al., 2003). This countered the prevailing notion at the time that adipocytes were the main sources of proinflammatory cytokines (Hotamisligil et al., 1996). It was then found that blocking ATM migration into adipose tissue in obesity by inhibiting the C-C motif chemokine receptor 2 (CCR2) receptor improved insulin sensitivity in obesity (Weisberg et al., 2006). Similarly, obesity-induced insulin resistance improved when IKKβ, which activates NFκB and thereby induces inflammation, was deleted in myeloid cells (Arkan et al., 2005). Numerous studies then showed with various genetic mouse models that upregulating and downregulating ATM inflammation respectively promote and ameliorate insulin resistance (Lee and Lee, 2014). However, it should be noted that most of these studies could not show that ATMs specifically drive insulin resistance because cell-specific knockout mice are usually generated by crossing the floxed target gene-knockout mice with LysM-cre mice, which deletes the target genes in all myeloid cells (ie, dendritic cell (DC) subpopulations and granulocytes such as neutrophils as well as macrophages) in not only adipose tissue but also all other tissues (Abram et al., 2014). This inclarity remains unresolved because an ATM-specific Cre mouse line has still not been developed. Nonetheless, it can be concluded that ATMs play important roles in the development of obesity-induced inflammation and insulin resistance/T2D.

ATM HETEROGENEITY BEFORE SINGLE-CELL GENOMICS

While the studies described above were being conducted, another area of intense research started emerging, namely, macrophage heterogeneity. Studies in the classical immunology field have increasingly shown that many immune cell types are heterogeneous, and it became increasingly clear that this was true for macrophages as well (Fang et al., 2018). This in turn promoted research on ATM heterogeneity, which led to several ATM classifications, as follows.

Proinflammatory M1 and Anti-inflammatory M2 Phenotypes

The best-known, and most paradigmatic, macrophage classification in immunology is the dichotomous categorization of macrophages into proinflammatory M1 and anti-inflammatory M2 cells. This categorization was originally established on the basis of an in vitro system that generates M1 and M2 macrophages by treating bone marrow-derived macrophages (BMDMs) with the proinflammatory stimuli interferon-gamma (IFN-γ)/lipopolysaccharide (LPS) and the anti-inflammatory cytokines interleukin (IL)-4 ± IL-13, respectively (Orecchioni et al., 2019). Hence, M1 macrophages are Th1-biased and considered to be proinflammatory macrophages, and M2 macrophages are regarded as Th2-biased anti-inflammatory macrophages (Sica and Mantovani, 2012). The binary nature of this classification means that macrophages are exclusively categorized as M1 or M2, and intermediates or alternative phenotypes are not considered.

ATMs have also been classified as M1 and M2 macrophages: M2 ATMs are thought to predominate in lean adipose

tissue while M1 ATMs emerge in large numbers in obesity. A typical marker of M1 ATMs is CD11c, which is a classical marker of DCs (Russo and Lumeng, 2018). Obesity dramatically increases the frequency and absolute numbers of $CD11c^+$ ATMs (typically gated on $CD45^+$ $CD11b^+$ $F4/80^+$ cells) (Lumeng et al., 2007). These cells also express higher levels of proinflammatory cytokines such as TNF-α. Consequently, it is widely believed that obesity induces ATM polarization from the M2 to the M1 phenotype, which strongly elevates adipose-tissue inflammation, which in turn promotes the development of insulin resistance in obesity (Lumeng et al., 2007).

However, already before single-cell genomics, there was some evidence that the M1/M2 classification may not fully explain the roles of ATMs in insulin resistance. For example, Xu et al. (2013) showed that $CD11c^+$ ATMs from obese mice and $CD11c^-$ ATMs from lean mice did not differ in terms of their messenger RNA (mRNA) expression of classical M1 and M2 genes. Moreover, our study found with quantitative real-time PCR (qRT-PCR) that both M1 and M2 genes were upregulated in adipose tissue by obesity (Kim et al., 2013). In addition, microarray analysis of ATMs sorted from lean and obese adipose tissue indicated that while some M1 genes were upregulated and some M2 genes were downregulated by obesity, other M1 and M2 genes showed no difference or the opposite patterns. An example is the M1 genes encoding TNF-α and IL-6: while the former is upregulated in ATMs from obese mice, the latter is decreased (Kim et al., 2013). Thus, while the M1/M2 classification has been useful for elucidating the important role of ATMs in insulin resistance/T2D, it is likely to be too simplistic for understanding at a deeper level how ATMs interact with each other or other cells to drive or ameliorate this disease.

Crown-like Structure

Another ATM classification is based on microscopy of adipose tissues, which shows that the macrophages in obese adipose tissues cluster around dead, dying, or damaged adipocytes in a crown-like structure (CLS). This is readily shown by hematoxylin and eosin staining or immunohistology with antibodies against macrophage-specific markers such as MAC-2 (Galectin-3) and F4/80. These structures are observed in both human and murine adipose tissue and are much more common in obese adipose tissue, particularly the epididymal/visceral fat of rodents and humans (Sano et al., 2003). Moreover, the ATMs in CLSs often form multinucleated giant cells; these cells are a hallmark of chronic inflammation in not only obesity (Cinti et al., 2005) but also other chronically inflamed states (eg, in persistent infections and rheumatoid arthritis) (Giles et al., 2018). However, CLSs can be only identified by microscopy, meaning that it is difficult to isolate and further characterize the ATMs in these structures by fluorescence-activated cell sorting (FACS) or conventional genomic tools.

Yolk Sac-derived (Tissue-Resident) and Bone Marrow-derived (Recruited) Macrophages

In 1968, van Furth's group proposed that monocytes differentiate from promonocytes in the bone marrow, migrate to the tissues, and then differentiate further into tissue-resident macrophages (TRMs) (van Furth and Cohn, 1968). This led to the widespread and longstanding view that TRMs largely derive from bone marrow-derived circulating monocytes. However, it was then observed that macrophages can arise from yolk-sac promonocytes in the embryonic stage, long before the bone marrow develops (Takahashi et al., 1989). Several studies then showed that the TRMs in postnatal mouse pups are in fact mainly derived from yolk sac-derived monocyte progenitors. Since the specific markers for yolk sac-derived macrophages have not been identified, this was mainly confirmed by lineage tracing and parabiosis techniques. Nonetheless, events after birth, such as the induction of an inflammatory state, can induce bone marrow-derived monocytes to enter the circulation, travel to tissues, and replace the yolk sac-derived TRMs in these tissues. Thus, newly recruited macrophages can eventually predominate in the inflamed tissues. These observations may also be true for adipose tissue. First, it has been shown that in lean adipose tissue, ~80% of the ATMs are yolk sac-derived macrophages (Hassnain Waqas et al., 2017). Second, there is evidence that macrophages are recruited into the adipose tissue during obesity: for example, Weisberg et al. (2006) showed that inhibiting the CCR2 receptor blocks macrophage migration into adipose tissue and improves insulin resistance in obesity. Thus, bone marrow-derived ATMs may replace the yolk sac-derived residential ATMs as obesity develops. However, it should be noted that obesity can also induce ATMs to proliferate (Amano et al., 2014). Thus, it is possible that obesity elevates ATM numbers in various ways, namely, by inducing yolk sac-derived ATM proliferation, BMDM recruitment, and/or the proliferation of recruited bone marrow-derived ATMs. Since macrophage ontogeny can significantly shape subsequent macrophage immune responses, it is of interest to determine whether single-cell technology can identify specific markers for yolk sac-derived macrophages, and thereby to determine whether yolk sac-derived and bone marrow-derived ATMs play different roles in obesity.

Thus, pathogenic ATMs have been variously classified as proinflammatory M1 ATMs, CLS ATMs, and potentially, bone marrow-derived ATMs. This together with the fact that M1 ATMs in obesity bear M2 markers suggests that ATMs are probably quite heterogeneous in terms of functions and characteristics and thus could well play highly nuanced, diverse, and possibly obverse roles as obesity develops. To unravel this diversity, it is necessary to move away from the approaches that were used up to this point, namely, qRT-PCR, FACS, western blot, and/or microarray/bulk RNA-seq analyses of whole ATM populations or subpopulations that were sorted by FACS on the basis of one or a few surface markers (Kim et al., 2013). The advent of single-cell genomics technologies means that it is now possible to identify the ATM subpopulations and thereby determine their functions and roles in obesity-induced adipose-tissue inflammation and insulin resistance/T2D. Below, we will first summarize the current single-cell technologies and then describe the findings of these technologies that relate to the field of ATM inflammation in obesity.

SINGLE-CELL GENOMICS TECHNOLOGIES

Microarray was the first commercially available high-throughput genomic analysis technique. It allows the user to identify the

differentially expressed genes (DEGs) in one or more experimental samples relative to a reference sample (Trevino et al., 2007). This technique has dramatically advanced and expanded biological knowledge in many areas, including basic and translational research and drug development (Fernandes et al., 2009). However, the technique is based on predesigned probes for targeted gene (Miller and Tang, 2009). Thus, it does not examine the full spectrum of genes, let alone changes in noncoding RNAs. This limitation led to the emergence, starting in 2000, of next-generation sequencing (NGS) technologies. These technologies examine all RNA sequences and provide normalized absolute gene expression counts. Over time, they have become more readily available, easier to use, and more reproducible. These developments have hugely expanded many research fields (Jung and Lee, 2023; Stark et al., 2019). However, the early NGS technology was based on bulk RNA-seq of whole tissues or isolated cell populations, meaning that its ability to identify cell subsets was also limited (Li and Wang, 2021). To overcome this limitation, various single-cell NGS technologies have been developed (Table 1) (Metzker, 2010; Van Dijk et al., 2014). Below, we will describe some of the techniques that are currently commonly utilized.

Single-Cell RNA Sequencing

Single-cell RNA sequencing (scRNA-seq) technology is the first single-cell genomics technology (Wen et al., 2022). It basically involves dissociating tissues into single cells and capturing each individual cell in a vehicle that contains the necessary reagents, including a tagged primer that labels all sequences from the cell and thus acts as a barcode that differentiates these sequences from those in other sequenced cells. A tagged library is then generated from each cell, and all libraries are sequenced together (Fig. 1a). The genomic information of each cell is analyzed and the cells can be grouped on the basis of the commonalities of their DEGs. This process is called annotation (Hedlund and Deng, 2018).

Several scRNA-seq methods have been developed. Although they share the basic workflow described above, they vary in some aspects, particularly the methods for capturing single cells. One method involves sorting the single cells individually by FACS into microwells, followed by sequencing each cell (Nguyen et al., 2018). This technique permits full-capacity sequencing (< 12,000 genes), but the number of cells that can be sequenced is limited to the number of cells that can be sorted with FACS. Moreover, generating the libraries is very laborious and technically challenging. The other method for capturing cells involves placing each cell in a lipid-based droplet or single chamber based on the fluidics of the flowing cells (Nguyen et al., 2018). Since this technique utilizes an automatic system, it is easier and relatively reproducible. However, far fewer genes can be sequenced (~3,000 genes), and the droplet/chamber sizes limit the size of the captured cells to ~50 μm. In particular, the size limitation is a critical issue for analyzing single cells in adipose tissues because the sizes of adipocytes and a subpopulation of ATMs (lipid-laden adipose tissue foam cells) are over 50 μm and the fluidic system will exclude these cells during the capturing processes. Hence, alternative methods are FACS-based scRNA-seq or single-nucleus RNA sequencing (snRNA-seq), which have their own advantages and disadvantages (Table 1).

Like bulk RNA-seq, scRNA-seq reveals the DEGs and allows pathway and other analyses. Unlike bulk RNA-seq, it allows the determination of cell subclusters, thus revealing in many cases novel cell subtypes. Trajectory analyses can also show how and when particular clusters differentiated from other cell types in the sample. Moreover, cell-to-cell interaction analyses can show how the different cell types interact with each other (eg, adipocytes-immune cells including ATMs, immune cells-immune cells, neuron-immune cells etc) and this can help to understand adipose tissue biology. This scRNA-seq-mediated profiling of single cells has greatly expanded the biology field, in particular in the immunology and cancer fields (Papalexi and Satija, 2018).

Single-Nucleus RNA Sequencing

While scRNA-seq is highly useful for single-cell profiling, it has some limitations. In particular, the dissociation of the tissues into single cells can damage cells, especially fragile cells, which include ATMs and lipid-filled adipocytes (Sárvári et al., 2021). This means that gentle digestion methods must be developed for each tissue. This consideration also means that fresh tissues should be used, which can be highly problematic in fields where experimental logistics require some tissue storage. An example of this is human samples. To overcome this limitation, snRNA-seq emerged, and thus snRNA-seq has been extensively used for the single-cell analyses for adipocytes and ATMs from both human and mouse models. This method involves isolating the nuclei of the cells in the sample and then tagging and sequencing each nucleus. This reveals the mRNA expression in the nucleus, thus identifying the most recently expressed transcripts in the cell. The workflow and analytical methods in snRNA-seq are almost identical to those of scRNA, but the method sidesteps the problems of scRNA-seq with regard to large cells, fragile cells, and the need to use fresh tissues: this is because nuclei are more resistant to the physical stress imposed by tissue dissociation than cells (Kim et al., 2023) (Fig. 1b). Consequently, snRNA-seq can be used with frozen samples, including those from humans (Piwecka et al., 2023). Moreover, snRNA-seq achieves higher throughput with fewer cells compared to scRNA-seq (Wu et al., 2019). However, the limitation of snRNA-seq is that it only measures the pre-mRNA in the nucleus, meaning that the scope of gene expression is more limited than in scRNA-seq. This is particularly problematic for immune cells (Maniyadath et al., 2023).

Single-Cell ATAC Sequencing

Chromatin-immunoprecipitation sequencing has been widely used to determine the molecular mechanisms that regulate gene expression. It involves incubating samples with antibodies that immunoprecipitate specific target transcription factors or coregulators. Since those antibodies are not always available, an Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) or similar techniques was developed. ATAC-seq identifies accessible DNA regions by artificially inserting an adapter of an active mutant Tn5 transposase into the open chromatin regions of the genome (Sun et al., 2019). Sequencing of those DNA regions and analysis and deduction of the promotor-binding regions via bioinformatic analyses then reveal potential transcription factors

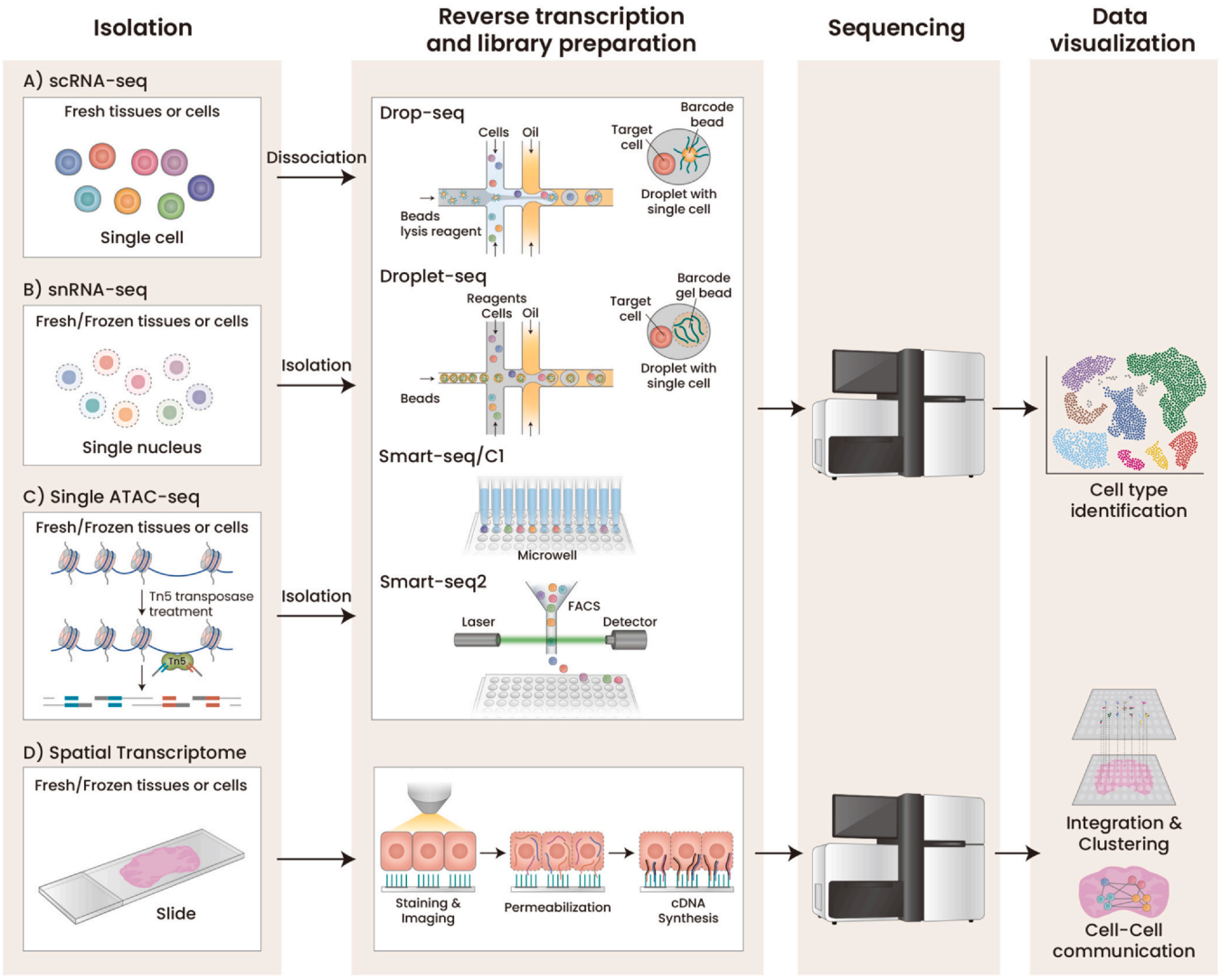


Fig. 1. The basic workflow of the 4 RNA-seq technologies. While scRNA-seq can only be used with fresh samples, snRNA-seq, scATAC-seq, and spatial RNA-seq can be used with both fresh and frozen samples.

or coregulators of specific genes. Since Chromatin-immunoprecipitation sequencing technology that permits single-cell analyses has not yet evolved, single-cell ATAC sequencing (scATAC-seq) has been developed to identify the molecular mechanisms of gene regulation in heterogeneous chromatin regions at the single-cell level (Baysoy et al., 2023) (Fig. 1c). Compared to the other single-cell technologies, scATAC-seq is relatively easy to use: the materials can be frozen or fresh, small samples can be used, and the sample preparation time is short. Moreover, scATAC-seq can identify Differentially Accessible Regions and determine interactions between different genomes via co-accessibility analyses (Baek and Lee, 2020; Li et al., 2021). However, the data-analysis pipeline for scATAC-seq is still not as well established as that of scRNA-seq or snRNA-seq (Baek and Lee, 2020). Since ATMs are very heterogeneous, scATAC-seq is potentially very useful to understand how different ATM clusters are developed/differentiated. Unfortunately, until now, there is no study to conduct scATAC-seq analyses of ATMs yet, although, with the current trajectory of adipose tissue single-cell research developments, it is expected that the data for the scATAC-seq of adipose tissue cells including adipocytes and ATMs will be available soon.

Spatial Transcriptomics

The spatial organization of the individual cells in a tissue is often crucial for their functions. Consequently, isolating single cells for scRNA-seq means that this spatial information is lost (Longo et al., 2021), including where in the tissue pathological differences in gene expression occur. To address this, a new method called spatial transcriptomics has been developed. This technology measures the gene-expression profiles of specific single cells in intact tissues, thereby preserving their spatial information (Williams et al., 2022). Unlike the single-cell sequencing methods, spatial transcriptomics involves fresh or frozen tissues with hematoxylin and eosin staining. In addition, immunofluorescence staining is typically preconducted to identify specific cells that express the chosen marker proteins. Thereafter, a library of each located cell is created with spatially barcoded oligonucleotides and sequenced (Fig. 1d). Thus, spatial transcriptomics identifies spatially variable genes within the context

Table 1. Comparison of multiple RNA sequencing technologies

	Bulk RNA-seq	Single-cell RNA-seq	Single-nucleus RNA-seq	Single-cell ATAC-seq	Spatial RNA-seq
Detection target	Differentially expressed genes	Differentially expressed genes, cell populations	Differentially expressed genes, cell populations	Differentially accessible regions, cell populations, co-accessibility	Spatially variable genes, dynamic tissue architecture
Advantage	Discovery of biomarker, cost-effective	Possible to check the gene profile of each heterogeneity cell type, cell trajectory analysis	Frozen samples, more resistance to cell lysis than scRNA-seq preparations, possible to check the gene profile of each heterogeneity cell type, cell trajectory analysis	Available with low cell counts, short preparation times, find key transcription factors and *cis*-regulatory elements (eg, enhancers)	Spatial profiling, revealing spatiotemporal gene expression patterns and tissue morphogenesis
Limitation	Not possible to know the expression pattern of genes in each cell, not determine the heterogeneity, missing the expression of small genes	Cell death, changes in the properties of the cell itself, spatial information is unknown, high cost	Measure almost only nuclear transcripts, spatial information is unknown	Data analysis pipelines are less established, highly noisy and sparse	Low resolution
Reference	Li and Wang, (2021)	Hedlund and Deng (2018)	Kim et al. (2023) Piwecka et al. (2023)	Baek and Lee (2020)	Longo et al. (2021)

of the 2-dimensional architecture of the tissue slides (Charitakis et al., 2023). This can reveal spatiotemporal gene expression patterns and tissue morphogenesis (Du et al., 2023). Although this technique has not been extensively used with adipose tissues, its ability to specifically isolate certain areas means that it could be used to determine the transcriptomes of the CLS ATMs, for example. Thus, its application in the adipose-tissue inflammation field is likely to rapidly expand our understanding of this field.

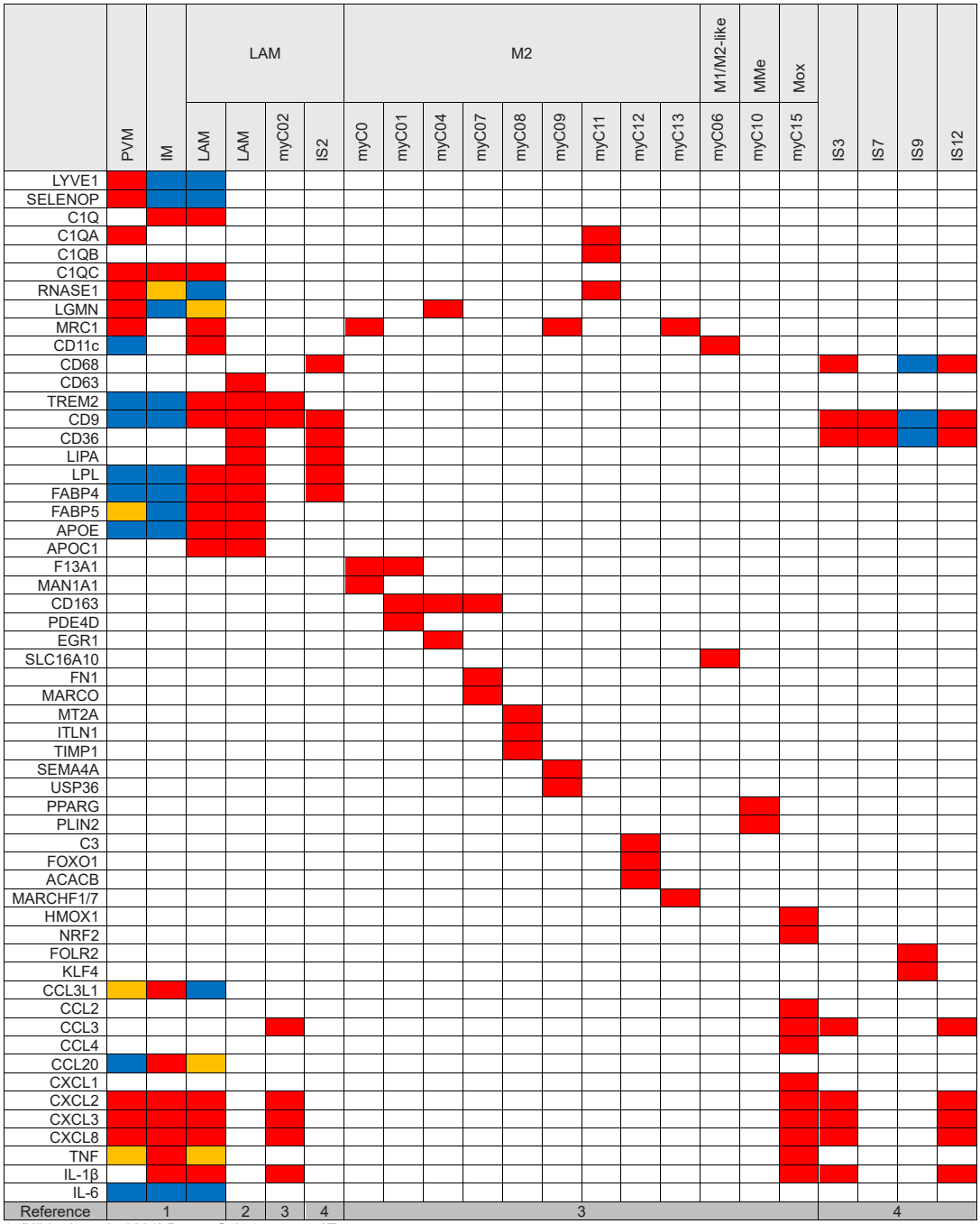
DETERMINATION OF ATM HETEROGENEITY WITH SINGLE-CELL GENOMICS

The emerging power of the new single-cell genomics technologies means they can shed light on many ongoing questions in the adipose-tissue inflammation field, including the limitations of the M1/M2 paradigm of ATM heterogeneity, the roles of CLS ATMs, and whether yolk sac-derived and/or BMDMs are responsible for obesity-induced inflammation and insulin resistance/T2D. Indeed, a number of important studies using these technologies have been published recently. Here, we will discuss the studies on ATM heterogeneity in mouse models and humans. Given the youth of the field, and the fact that each study has employed different experimental approaches (eg, different adipose-tissue sources, conditions, data analyses, and especially annotation markers), it is challenging at this point to unify the resulting data into a cohesive view of the field. In particular, each study has used its own annotation markers for clustering, which makes it difficult to directly compare the clusters between studies. This problem can be solved by reanalyzing the archived data. However, such analyses are out of the scope for this review. Instead, we will summarize the clusters and the genes in each cluster as described by the published literature (Tables 2a and 2b), with a particular focus on the clusters that could potentially play important roles in obesity.

Lipid-associated Macrophages and Proliferative Lipid-associated Macrophages

As mentioned above, obesity greatly increases the number of proinflammatory ATMs, which are often defined as $CD11c^+$ ATMs (Lumeng et al., 2007). Hence, a key focus of the scRNA-seq analyses published to date has been to identify the ATM cluster(s) that are expanded in obesity and whether they can demonstrate proinflammatory phenotypes. When Jaitin et al. used scRNA-seq on epididymal adipose tissue from lean and obese mice, they found 3 ATM clusters. One was reduced by obesity and resembled perivascular macrophages (PVMs). The other 2 clusters were strongly expanded by obesity. One of these expressed the CD9, Trem2, and CD63 markers and many lipid-metabolism genes such as lipoprotein lipase (LPL), lipase A (LIPA), and CD36. The latter cells were very rare in lean white adipose tissue (WAT) and were dramatically increased by obesity. They also contained high levels of intracellular lipids and in fact are lipid-laden adipose-tissue foam cells. Consequently, these cells were termed lipid-associated macrophages (LAMs). The other expanded ATM population may be a precursor of the LAMs since it also expressed CD9 but not Trem2 or lipid-metabolism genes. scRNA-seq analysis of lean and obese visceral adipose tissue from humans then showed that LAMs were also present in humans (Jaitin et al., 2019). Other

Table 2a. Annotation marker genes for human ATM clusters



1; (Hildreth et al., 2021) Depot; Subcutaneous AT

2; (Jaitin et al., 2019) Depot; Ometal AT

3; (Massier et al., 2023) Depot; Combined subcutaneous, visceral, and perivascular AT

4; (Vijay et al., 2020) Depot; Subcutaneous and visceral AT

Expressed
Not expressed
Mildly expressed
Not reported

Table 2b. Annotation marker genes for mouse ATM clusters

	PVM		NPVM	LAM		P-LAM	CEM	RM	Mac2
	PVM	Mac1		LAM	Mac3				
Mrc1									
Lyve1									
Cd163									
Cd209f									
Cd74									
Fcrls									
Ear2									
Cd63									
Lpl									
Trem2									
Cd9									
Spp1									
Lipa									
Nceh1									
Pola1									
Kif11									
Kif15									
Tgfbr3									
Col5a2									
Col3a1									
Prg4									
Tgfb2									
Ltbp1									
Reference	1	2	1		2	1			2

1; (Hildreth et al., 2021) Depot; Epididymal AT

2; (Jaitin et al., 2019) Depot; Epididymal AT

Expressed Not expressed

Mildly expressed Not reported

lines of evidence suggest that LAMs may play important roles in obesity-induced inflammation. First, Jaitin et al. and other studies showed that the CD9 marker of LAMs is also present in the ATMs in CLSs (Daemen and Schilling, 2020; Hill et al., 2018; Jaitin et al., 2019). Second, the Trem2 marker of LAMs is a lipid receptor that participates in phagocytosis, inflammatory responses, and energy metabolism (Khantakova et al., 2022). Third, deleting Trem2 in the whole body and then feeding the mice with a high-fat diet increases obesity, the proinflammatory responses of the ATMs (but not their TNF-α levels), and insulin resistance (Liu et al., 2019). This suggests that LAMs play a protective effect in insulin resistance and may not be the main emitter of TNF-α, which plays a key role in insulin resistance. On the other hand, Hildreth et al. conducted scRNA-seq of the subcutaneous adipose tissue of lean and obese humans. They detected 3 ATM clusters, namely, the Trem2-, CD9-, and LPL-expressing LAMs, an inflammatory macrophage (IM) cluster that expressed C-C motif chemokine ligand 3 (CCL3L1), TNF, and C-X-C motif chemokine ligand 3 (CXCL3) at high levels, and the PVMs. The LAM and IM ATM clusters were expanded in obesity while the PVMs were downregulated. Whether the IM cluster expressed M2 marker genes is currently unclear (Hildreth et al., 2021; Jaitin et al., 2019; Sárvári et al., 2021).

Similarly, when Massier et al. conducted their own single-cell genomics analyses of human subcutaneous and visceral fat, combined these data with public scRNA-seq and snRNA-seq datasets, and conducted a meta-analysis of these data, they found LAMs (cluster myC02) as well as 7 other ATM populations, namely, 4 M2-like clusters, a mixed M1/M2 cluster, a metabolic-regulated cluster (designated Mme), and a redox-regulatory metabolic cluster (designated Mox). A secondary analysis of subcutaneous adipose-tissue bulk RNA-seq data of human patients for whom metabolic data were available then showed that the LAMs and metabolic-activated (MMes), but not the Mox cells, were expanded in patients with a metabolically deleterious profile (Massier et al., 2023).

Vijay et al. conducted scRNA-seq with obese visceral and subcutaneous adipose tissues from humans with and without T2D. They identified 5 ATM clusters. Cluster IS2 resembled LAMs in terms of its expression of CD9 and lipid metabolism genes, including CD36. However, clusters IS3, IS7, and IS12 also expressed CD9 and CD36. The authors did not specify whether any of these clusters were the LAMs reported by others (Vijay et al., 2020).

Sárvári et al. used snRNA-seq to analyze the epididymal-adipose tissue of obese and lean mice. They observed 6 macrophage populations, 2 of which were expanded by obesity

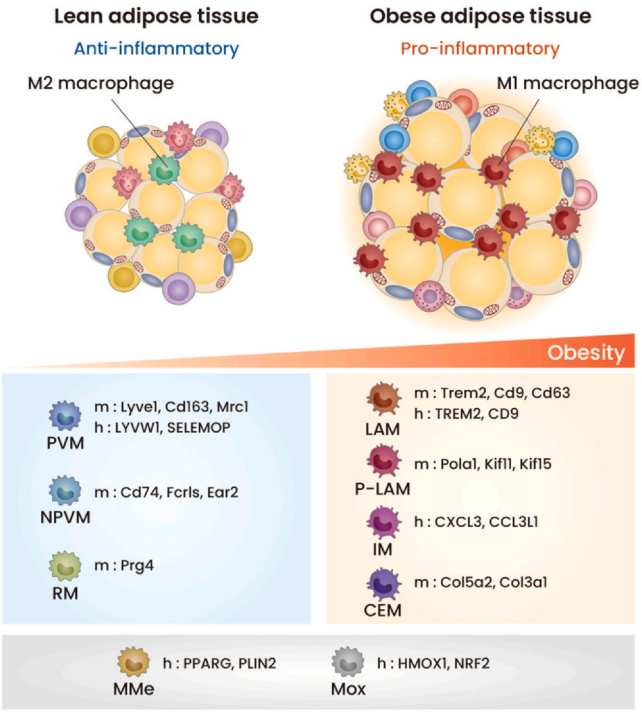


Fig. 2. The subpopulations and markers of ATMs that have been discovered in adipose tissues from humans and mice by single-cell genomics technologies to date. PVMs and NPVMs are abundant in lean adipose tissue and are significantly reduced by obesity. IMs are highly expanded by obesity, as are LAMs and P-LAMs. Although it is a very small population, RMs also appear to be increased by obesity. PVMs appear to be significantly decreased by obesity. CMs tend to be elevated by obesity, although this does not achieve statistical significance. Efferocytes were present at very similar frequencies in lean and obese adipose tissue. PVM, perivascular macrophage; NPVM, nonperivascular macrophage; IM, inflammatory macrophage; LAM, lipid-associated macrophage; P-LAM, proliferative-LAM; RM, regulatory macrophage; CEM, collagen-expressing macrophage; CM, Cycling macrophage.

and 2 of which were downregulated. None of the 6 ATM populations resembled classically activated M1 populations. The downregulated clusters were PVMs and a cluster termed non-PVM, namely, ATMs that occupied the nonperivascular space. LAMs were 1 of the 2 expanded ATM clusters. The second was a subcluster of LAMs termed proliferative LAMs (P-LAMs). Along with LAM markers, P-LAMs also expressed several proliferation markers, including Pola1, Kif11, and Kif15, which suggests that they are strongly proliferative. Both LAMs and P-LAMs were very rare in lean mice but strongly expanded in obese mice (Sárvári et al., 2021). Whether P-LAMs exist in humans is not yet known (Maniyadath et al., 2023; Sárvári et al., 2021). These cells have not yet been further characterized, including with regard to their M1/M2 markers, and it remains unclear whether they do indeed proliferate in obesity in vivo.

Perivascular Macrophages

As mentioned above, Jaitin et al. (2019), Sárvári et al. (2021), and Hildreth et al. (2021) all found that in both mice and humans, PVMs are present in high numbers in lean condition but are markedly reduced by obesity. PVMs are specialized TRMs that are found in close proximity to blood vessels (Maniyadath et al., 2023). Indeed, they maintain tight physical junctions with endothelial cells that allow them to regulate vessel permeability. They also prevent potential pathogens in the blood from invading the tissues by phagocytizing them (Lapenna et al., 2018). This also allows them to serve as antigen-presenting cells for T cells (Wen et al., 2024). Notably, while PVMs can promote inflammation in conditions such as uveoretinitis (Gullapalli et al., 2000), they can also play anti-inflammatory roles by producing IL-10 (Lapenna et al., 2018). Although the PVM markers vary depending on the tissue, mannose receptor C-type 1 (MRC1) and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) are common activation markers. The 3 studies showing that obesity reduces PVMs in murine and human adipose tissue all used these 2 markers for this clustering. Moreover, murine PVMs in epididymal fat were found to specifically express Cd163 while human PVMs in human subcutaneous fat expressed selenoprotein P (SELENOP) and RNASEQ (Hildreth et al., 2021; Jaitin et al., 2019; Sárvári et al., 2021). While adipose-tissue PVMs do not express Trem2, Cd9, and Lpl in the lean state, these genes are mildly upregulated by obesity. Nonetheless, the PVMs still demonstrated distinctively different clustering relative to the LAMs. The fact that obesity decreases PVMs while increasing their expression of lipid-metabolism genes suggests that PVMs may differentiate into LAMs in obesity (Hildreth et al., 2021). However, this requires verification with in vivo systems.

Inflammatory Macrophages

A major focus in single-cell ATM analyses has been to identify inflammatory ATM clusters. Interestingly, similar *bona fide*

inflammatory ATM clusters have not yet been identified in mice. By contrast, as mentioned above, Hildreth et al. identified the IM cluster in human subcutaneous fat with scRNA-seq: these cells were greatly increased by obesity and expressed high levels of CCL3L1, TNF, and CXCL3. Moreover, the IMs from obese humans showed greater IL-1β and TNF production on flow cytometry compared to their counterparts in lean humans (Hildreth et al., 2021). However, the M1/M2 marker expression of these cells remains to be comprehensively assessed.

Hildreth et al. also found that PVMs may indirectly promote inflammation in obesity because they express high levels of the monocyte chemokines CCL3, CCL3L1, CCL4, and CCL2. In addition, Massier et al. found that LAMs (myC02) express low but identifiable levels of CCL3, CCL4, CXCL2, CXCL3, and IL-1β, and that these levels were markedly higher in Mox ATMs (Massier et al., 2023). However, the inflammatory functions of these clusters have not been further characterized.

Redox-regulatory Metabolic Macrophages

As mentioned above, Massier et al. also identified the Mox ATMs. This cluster has been found only in human subcutaneous fat and not in mice. They are identified by their expression of NFE2-like bZIP transcription factor 2 (NRF2) and heme oxygenase 1 (HMOX1) and express high levels of many inflammatory cytokines, including CCL2-4, CXCL1-3 and CXCL8, IL-1β, and TNF (Massier et al., 2023). This is interesting because Mox were previously identified in the atherosclerosis field, where they were found to cluster independently from M1- and M2-classified macrophages and to comprise 30% of all macrophages in the advanced atherosclerotic lesions of low-density lipoprotein receptor (LDLR) knockout mice. It is thought that Mox macrophages are activated by oxidized phospholipids, which causes them to express proinflammatory phenotypes and promote redox-regulatory metabolism, thereby contributing to atherosclerosis (Kadl et al., 2010).

Other ATM Clusters

Massier et al. also identified the metabolic-regulated/MMe ATMs in humans. These cells are expanded in patients with poor metabolic profiles and exhibit unique characteristics. Specifically, they express peroxisome proliferator-activated receptor gamma (PPARG), perilipin 2 (PLIN2), CD36, and ATP-binding cassette, sub-family A member 1 (ABCA1) (Massier et al., 2023). MMes resemble the “metabolically activated” ATMs that were identified by proteomics, in vitro, and in vivo experiments by Kratz et al. These cells express a mixture of M1 and M2 signature genes and express high levels of TNF-α, IL-6, and IL1-β. Naïve ATMs develop this phenotype when they are exposed to glucose, insulin, and palmitate, and these cells are expanded in the adipose tissue of diabetic mice (Coats et al., 2017; Kratz et al., 2014). Thus, MMes may promote inflammation and control lipid metabolism in obesity. However, their in vivo functions and their counterparts in mice remain to be determined.

The snRNA-seq study of Sávári et al. on lean and obese epididymal fat revealed 2 other ATM clusters that they designated collagen-expressing macrophages (CEMs) and regulatory macrophages (RMs). Both are present at low numbers in lean fat, and these numbers are not markedly altered by obesity. The CEMs are interesting because while obesity induces fibrosis in adipose tissues (ie, the excessive deposition of extracellular matrix proteins such as collagen and fibronectin) (Sun et al., 2023), adipocytes are thought to be the primary source of these proteins (Sun et al., 2013). The fact that CEMs express high levels of collagens such as Col5a2 and Col3a1 (Sárvári et al., 2021) suggests that these ATMs can also contribute to fibrosis in obesity. This is supported by in vitro studies that show macrophages can express most extracellular-matrix proteins (Galli et al., 2011; Schnoor et al., 2008). The RMs identified by Sávári et al. are also interesting because they express proteoglycan 4 (Sárvári et al., 2021), and whole-body deletion of proteoglycan 4 improves obesity and obesity-induced inflammation and insulin resistance (Nahon et al., 2019).

CONCLUSIONS AND LIMITATIONS

The NGS technologies have been eagerly adopted globally, with the result that they are now widely used in biology and the clinical and medical fields for disease diagnosis. The advent of single-cell technologies further greatly expands basic and applied research because they provide large amounts of information about the individual cells within tissues, including their finely defined transcriptomic phenotypes, their shapes, and their tissue location. These technologies have been applied to adipose tissue and have confirmed that the proinflammatory M1:anti-inflammatory M2 macrophage classification does not adequately reflect the remarkable heterogeneity of ATMs in both lean and obese adipose tissue. Indeed, depending on the study, between 3 and 8 ATM clusters have been identified. Moreover, while one study found ATMs that produced TNF and IL-1β (the IMs), it is not clear whether they also express M2 markers (Hildreth et al., 2021; Jaitin et al., 2019; Massier et al., 2023; Sárvári et al., 2021; Vijay et al., 2020). Indeed, another study found that MMes are also expanded by obesity but have a mixed M1/M2 phenotype (Massier et al., 2023). LAMs, which are CLS ATMs, are also expanded by obesity but do not produce TNFα, but deletion of their marker Trem2 worsens insulin resistance; thus, these cells may play protective roles. P-LAMs, which may derive from LAMs and have a proliferative phenotype, are also expanded by obesity, but their M1/M2-related phenotypes remain to be determined. Moreover, some studies identified the Mox, CEM, and RM ATM clusters, which are not expanded by obesity but nonetheless have proinflammatory or other phenotypes that could promote insulin resistance. There is some evidence suggesting that TRMs give rise to the ATM populations in obesity: the numbers of adipose-tissue PVMs (which are likely to be TRMs) are high in lean subjects but drop with obesity. It is thus possible that these cells are converted into other ATM subtypes by obesity, although the trajectory study could not conclusively confirm this (Hildreth et al., 2021). Thus, there are many ATM subtypes, each of which could play distinct roles in homeostasis, obesity, and obesity-associated pathophysiology (Fig. 2). It is likely that further use of single-cell NGS technologies and identification of standard annotation/tissue location markers will greatly improve and harmonize our understanding of ATMs in obesity-induced inflammation and

insulin resistance/T2D, especially when these analyses are combined and validated by biological experiments. The latter experiments will require isolating specific surface markers and/or generating cluster-specific gene modulation tools (eg, cluster-specific Cre mouse lines), which may be challenging. Nonetheless, it is clear that single-cell genomics technologies have greatly advanced the field and are likely to accelerate the development of new avenues for the treatment of obesity, insulin resistance, and T2D.

AUTHOR CONTRIBUTIONS

H.K. and J.L. wrote the manuscript.

DECLARATION OF COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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