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Leukocyte Heterogeneity in Adipose Tissue, Including in Obesity

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Abstract

Adipose Tissue (AT) plays a central role in both metabolic health and pathophysiology. Its expansion in obesity results in increased mortality and morbidity, with contributions to cardiovascular disease, diabetes, fatty-liver disease and cancer. Obesity prevalence is at an all-time high and is projected to be 50% in the US by 2030. AT is home to a large variety of immune cells, which are critical to maintain normal tissue functions. For example, gamma delta T cells are fundamental for adipose tissue innervation and thermogenesis, and macrophages are required for recycling of lipids released by adipocytes. The expansion of visceral white AT (vWAT) promotes dysregulation of its immune cell composition and likely promotes low-grade chronic inflammation, which has been proposed to be the underlying cause for the complications of obesity. Interestingly, weight loss following obesity alters the adipose tissue immune compartment, which may account for the decreased risk of developing these complications. Recent technological advancements that allow molecular investigation on a single-cell level has led to the discovery of previously unappreciated heterogeneity in many organs and tissues. In this review we will explore the heterogeneity of immune cells within the vWAT and their contributions to homeostasis and pathology.

Keywords

inflammation; immune system; obesity

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Introduction

Adipose tissue (AT) harbors a plethora of immune cells, including all major leukocyte types of the innate and adaptive immune systems, possibly due to the richness of fatty acids and other energy-related metabolites embedded within AT. Obesity changes the availability and abundance of energy sources in AT, since adipocytes both accumulate and release more lipids. Additionally, obesity alters the production of hormones (adipokines) and mediators of inflammation (cytokines, chemokines and lipid mediators) by adipocytes, which may influence immune responses locally and systemically. Therefore, the composition of AT immune cells, as well as their phenotype and gene expression signatures, are significantly altered in obesity. Low-grade inflammation that is promoted by obesity is thought to be the underlying cause for the associated comorbidities, including cardiovascular disease (reviewed in[1]). This low-grade inflammation has contributions from both the adipocytes themselves (e.g., by adipokine secretion) as well as from their neighboring immune cells, and includes the cross-talk between the sub-populations of cell types in adipose tissue (see[2] for a recent review).

Two major types of AT have been identified and segregated according to their location, morphology and function. The brown AT (BAT) is comprised of multilocular adipocytes that are rich in mitochondria and specialized in energy dissipation through thermogenesis[3]. White AT (WAT) mainly contains unilocular adipocytes, characterized by single lipid droplets that are rich in triglycerides[3] and whose central functions are the storage of energy in the form of fatty acids and regulation of systemic metabolic processes by the production of hormones and adipokines. The WAT is further divided into 2 subtypes: subcutaneous (scWAT), located under the skin, and visceral (vWAT), which is intra-abdominal. vWAT has been intensively studied, due to the association of high vWAT mass with increased risk for development of the metabolic syndrome and cardiovascular risk[4–6]. One major difference between scWAT and vWAT is that the former demonstrates a greater potential to undergo “beiging”, that is, the process by which white adipocytes become brown-like, with increased mitochondrial function and capacity for energy dissipation[7, 8]. This may explain the distinct contributions of the two WAT depots to metabolic health, while scWAT is more relevant to temperature control, vWAT has been characterized as playing a more preeminent role in metabolic control[9]. Of the vWAT sources, epididymal WAT (also known as eWAT or perigonadal) has been most studied in animal models, due to its prominent abundance and clear association with metabolic syndrome development in animal models[10]. Although other vWAT sources, such as perivascular (surrounding blood vessels), mesenteric (intestine associated) and perirenal (kidney adjacent) may have other distinct features, their immune landscape is less known.

The immune system has emerged as a key regulator of metabolic health. In obesity, several of the leukocyte populations that reside in the vWAT, such as macrophages (Møs)[11, 12], T cells[13, 14] and Dendritic Cells (DCs)[15, 16] have been shown to play a role in the development of insulin resistance in metabolic syndrome models[11]. Recent technological advances, including single cell RNA-sequencing (scRNA-seq), have enabled the discovery of previously unappreciated cellular heterogeneity. With this in mind, our main focus in this review is to explore the extensive heterogeneity of immune cells within the vWAT, the local

contribution of these cells to vWAT physiology and the implications to the development of obesity and metabolic syndrome. Some aspects of the roles of immune cells in AT and obesity are covered in a companion review, and we refer the reader to it for complementary information[17]. The contributions of other cell types, like adipocytes and stromal cells, and soluble factors such as hormones and adipokines, to the systemic control of body metabolism are extensively reviewed elsewhere[18, 19] and will not be further explored in detail here.

I. Innate immune cells-Drivers of the metabolic syndrome or protectors of the visceral white adipose tissue?

WAT is dominated by a rich variety of innate immune cells, which are crucial for tissue maintenance and homeostasis. Obesity changes the immune cell repertoire in the WAT, which is coupled with vast transcriptional alterations. While neutrophils and mast cells are considered harmful in obese conditions, eosinophils are generally regarded as promoting tissue homeostasis, at least in leanness. Møs and DCs have been proposed to have both harmful and protective roles. It is increasingly appreciated that different cell populations are more heterogeneous than previously anticipated, and that each of the major immune cell types (i.e. Møs, DCs, neutrophils, etc.) have multiple subsets. Hence, we postulate that subsets of innate immune cells contribute to the numerous functions attributed to the major cell type. In this section we will describe major innate immune populations and how sub-populations contribute to physiological and pathophysiological processes in the vWAT.

1. Macrophages—Møs are specialized phagocytes found in most, if not all tissues. Møs are the most abundant immune cells in AT and constitute ~5% of total human vWAT and mouse eWAT cells in lean conditions[20]. In the steady-state, the eWAT releases ~1% of its lipid content daily[21]. A major role of adipose tissue Møs (ATMs) is the recycling of these fatty acids released from adipocytes[21]. Møs uptake the lipids as exosomes[21] and catabolize them in the lysosome through Lysosomal Acid Lipase (Lipa). Mice deficient for *Lipa* are born without apparent defects; however, their adipose depots are depleted over time; they develop severe hepatosteatosis; and they die at an age of 7–8 months[22]. Interestingly, these phenotypes are rescued by expression of *Lipa* specifically in Møs[23]. These data suggest that ATMs uptake and recycle lipids secreted from adipocytes, indicating that they actively participate in WAT maintenance and functions. Another function of ATMs is to support adipocyte lipolysis and beiging in response to cold exposure in order to enhance heat production[24, 25]. Animal models submitted to a cold challenge had their capacity to adjust their body temperature compromised when Møs where not expressing the interleukin (IL)-4 receptor. IL-4 has been described as a fundamental factor for the maintenance of ATM phenotype in steady state[25–27]. These data indicate that ATMs contributes directly to the physiology of the WAT[24].

In a seminal paper published in 2003, Weisberg et al. showed that obesity resulted in a significant accumulation of Møs in the vWAT of humans and eWAT of mice[28]. ATM accrual is correlated with adiposity[28] and can be explained by enhanced monocyte recruitment through C-C Motif Chemokine Ligand 2 (CCL2)- C-C Motif Chemokine Receptor 2 (CCR2) axis[29–33], increased Mø retention[34], enhanced survival[35] and local

proliferation[36, 37]. This may be due, at least in part, to the increased secretion rates of fatty acids by obese adipocytes, which more than double compared to lean conditions[21]. The distribution of ATMs in the obese WAT is also altered, with ATMs accumulating in crown-like structures (CLSs) around dying adipocytes[38].

Several groups have investigated the direct involvement of Møs in obesity and AT inflammation. Deletion of *Ccr2*, a central chemokine receptor of monocytes and other leukocytes, blocked pro-inflammatory gene expression observed following high fat diet (HFD) feeding in mice[31]. Inhibition of CCR2 during HFD feeding attenuated weight gain and improved glucose and insulin tolerance in mice[39–43]. This indicates that the recruitment of monocyte-derived Møs is fundamental to the pathophysiology of the metabolic syndrome. Many of these recruited Møs express high levels of CD11c and this Mø population persists in the eWAT after weight loss[44]. By fluorescence-activated cell-sorting, it was shown that $CD11c^+$ ATMs in obesity express higher levels of the pro-inflammatory cytokines TNFα and IL1, relative to the $CD11c^-$ population[44], indicating a functional heterogeneity of ATMs. Moreover, depletion of $CD11c^+$ after the establishment of obesity caused an improvement of glucose and insulin tolerance[45]. Taken together, these data suggest that ATMs, especially those expressing CD11c, are drivers of obesity and insulin resistance. However, recent single-cell RNA-seq (scRNA-seq) data from atherosclerotic plaques showed that CD11c is a marker for lipid-laden Møs, and that the non-foamy Mø populations were the ones exhibiting a pro-inflammatory transcriptome46. This has called into question the notion that CD11c expression is a reliable marker of pro-inflammatory Møs, or that the populations marked by it in lipid-rich environments differ in important ways.

Because the approaches mentioned above use tools that are not specific to Møs, a few groups performed studies involving direct ablation of ATMs using chlodronate-containing liposome injections, which promote Mø "suicide"[47]. These studies show that ATM depletion during HFD feeding does not improve glucose and insulin tolerance[48] and may actually increase systemic inflammation and plasma levels of IL-6 and IL-1β[49]. Short-term (6-day) ATM ablation after obesity was established was shown to be beneficial and improved glucose and insulin tolerance[50]. With that said, the increased insulin and glucose sensitivity was improved to a greater extent following macrophage ablation in the lean eWAT[50]. Taken together, these studies suggest an important role of ATMs in obesity, with inconsistencies that may be attributed to the heterogeneous nature of Møs. It is possible that some Mø subpopulations are protective and others pathological in the context of obesity.

There are several hypotheses as to the functional alterations of Møs in obesity. The distribution of ATMs in the vWAT is drastically modified in obesity. While lean WAT is mainly populated by anti-inflammatory/homeostatic (classically termed M2 or alternatively activated Møs (AAMs)), obese/insulin resistant vWAT has a significant increase in the numbers of Møs with cell surface markers classically ascribed to pro-inflammatory Møs (M1 or classically activated). The M2 phenotype in lean eWAT is maintained by the Mø response to type 2 cytokines (IL-4 and IL-13) in a Signal Transducer And Activator Of Transcription (STAT) 6-dependent manner[26, 53, 54]. In some cases, studies reported that obesity decreased ATM expression of anti-inflammatory genes, such as *Il10*, Arginase (*Arg1)* and *Cd301*[55], while increasing the expression of pro-inflammatory factors, such as

tumor necrosis factor alpha (TNFα, gene name *Tnf*) and inducible nitric oxide synthase (iNOS, gene name *Nos2*)[28, 31, 55]. One explanation for the phenotypic difference between lean and obese ATMs is their divergent origins: In homeostatic conditions, most ATMs are of embryonic origin (also known as tissue-resident Møs), while in obesity there is substantial infiltration of monocytes that differentiate into Møs thereafter[51, 52].

Another predominant hypothesis is that ATMs undergo reprogramming under different metabolic perturbations leading to dysfunction. Of note, obesity was shown to activate lysosomal lipid metabolism pathways in ATMs, independent of their immune activation state[56]. As alluded to above, it has been recently reported that ATMs metabolize lipids secreted by adipocytes in the form of exosomes[21]. Obesity caused a doubling in the amount of adipocyte-derived exosomes, possibly suggesting that accumulation of Møs in obesity is necessary to handle excess adipocyte-derived lipids. Moreover, lipolysis in adipocytes induced rapid recruitment of monocytes to mouse eWAT[57], where ATMs further support differentiation of adipocyte progenitors[58], lipid handling[21, 59] and clearance of apoptotic adipocytes by ATMs[60, 61]. Taken together, these data show clearly that ATMs are a fundamental accessory cell that helps the vWAT to perform its primary function[62]. However, less is known regarding how different ATM subpopulations contribute to vWAT during homeostasis and obesity. Although some progress has been made recently, we believe that many Mø functions are yet unknown.

Next, we will discuss the heterogeneity of Mø subpopulations and how the understanding of the functional role of each subpopulation can be important to uncover mechanism that can modulate the metabolic syndrome. In the past few years, newer technologies, such as High Dimensional flow-cytometry and scRNA-seq, have allowed the identification and characterization of unappreciated subpopulations of many cell types, including Møs. In one paper combining very limited scRNA-seq with more comprehensive bulk RNA-seq of ATMs in obese eWAT, Hill et al.[63] reported 3 distinct ATM populations, $Ly6C^+$ and 2 distinct $CD11b^+Ly6C^-$ ($CD11b^+CD9^+Ly6C^-$and $CD11b^+CD9^-Ly6C^-$). One of these $Ly6C^-$ subsets had high expression of CD9, as well as genes related to lipid metabolism, and the other, a CD9 low population. The transcriptome of the $Ly6C^+$ population was deep-sequenced with RNA-seq and compared to that of $Ly6C^-$ cells. One general observation that the authors made was that “neither obesity-associated ATM subtype shares predominant features of classical M1 or M2 activation by comprehensive transcriptome analysis, consistent with an evolving view that the M1/M2 paradigm is not directly applicable to *in vivo* ATM populations”. The $CD9^+Ly6C^-$ ATMs expressed a variety of pro-inflammatory genes, with an enrichment in leukocyte activation and inflammatory response pathways, while the $Ly6C^+$ ATMs were enriched in genes related to angiogenesis and extracellular matrix organization. Both the $CD9^+Ly6C^-$ and the $Ly6C^+$ ATMs were shown to be monocyte-derived, with the $CD9^+Ly6C^-$ being the major population to accrue in obesity, reside in CLSs and accumulate lipids. The authors thus concluded that the $CD9^+Ly6C^-$ ATMs were pro-inflammatory cells that accumulate in the obese AT[63]. However, a subsequent report (discussed in more detail below) showed that the CD9-expressing ATMs that accrue in obesity also express TREM2 and protect against adipose hypertrophy and exacerbation of the metabolic syndrome[52].

In another study, Li et al.[64] compared the transcriptome of lean and obese purified eWAT Møs ($CD45^+CD11b^+F4/80^+$ cells) to bone marrow-derived Møs (BMDMs), stimulated *in vitro* with either lipopolysaccharide (LPS) and interferon (IFN) γ (M1), IL-4 and IL-13 (M2) or unstimulated (M0). Using controlled *in vitro* conditions, the authors developed a computation tool, termed MacSpectrum, to stratify Møs according to their "differentiation" and "polarization" states. The authors termed the differentiation index based on the expression of 435 genes previously implicated in terminal maturation, regardless of the polarization cue, and the polarization index as the regression line of transcriptomes to M1 or M2 gene sets (for specific gene sets used see[64]). Conventional methods of scRNA-seq clustering showed no overlap between BMDMs and ATMs, regardless of the different perturbations, with ATMs from lean and obese mice showing much greater similarities to each other than to any of the BMDM populations. Of note, the analysis found that classical M1/M2 genes were rarely expressed in ATMs, in agreement with the findings noted above. With that said, using MacSpectrum the authors showed higher similarity of lean ATMs to M2 Møs, with high differentiation and low polarization states. On the other hand, obese ATMs were more heterogeneous, and showed similarities to M0, M1 and M2 Møs[64].

Overall, we can take from these studies two major points. First, the oversimplified M1/M2 dichotomy established long ago by *in vitro* experiments does not reliably reflect the *in vivo* Mø biology complexity. Second, the diversity of Mø subpopulations was largely underestimated and new approaches are needed to evaluate the relevance and contribution of each of these subpopulations to WAT physiology.

Another recent study using RNA-seq analyses of sorted cells examined the heterogeneity of mouse eWAT phagocytes and identified 4 distinct ATM populations, according to the expression of surface markers, such as Tim4, MHCII and CD206[51]. The authors observed that tissue-resident $CD206^{HIGH}$ ATMs are associated with vessels, which they termed vascular-associated ATMs (VAMs). These specialized Møs are intimately associated with blood vessels of the eWAT and efficiently endocytose macromolecules from the circulation. VAMs are sensitive to perturbations as demonstrated by their rapid decrease in numbers upon fasting and inflammation. However, in obesity, VAM abundance increased by 2–3 fold, while monocyte-derived Møs (defined by the authors as $CD11c^+CD64^+$ double positive, DP) increased by approximately 10-fold. Functionally, obesity reduced the endocytic capability of VAMs. Transcriptomic analysis of sorted populations revealed that obesity did not cause a pro-inflammatory program in any of the ATMs and that several anti-inflammatory genes were upregulated compared with lean ATMs. Despite the lack of tools to address the specific function of each population, this report revealed that different subpopulations of Møs are present in the eWAT depending on inflammatory and metabolic conditions and indicated that the subpopulations have distinct functions. Understanding the role of each subpopulation has a great potential for elucidating the roles of Møs in the metabolic syndrome pathophysiology and possibly establish relevant therapies.

Subsequent studies that investigated the heterogeneity of not only myeloid cells or ATMs, but all eWAT leukocytes, revealed that Møs are the most heterogeneous leukocyte type in the eWAT in the lean and obese state[52, 65, 66,] and following weight loss[65]. Unbiased clustering of total $CD45^+$ cells sorted from the eWAT resulted in three[52] to seven[65] distinct Mø

populations, with additional monocyte populations. In general, the aforementioned studies make the important point that there are unique functions for distinct ATM populations under different metabolic perturbations. In their study, Jaitin et al.[52] performed scRNA-seq on vWAT leukocytes from lean and obese mice and humans and found a monocyte-derived Mø population that is scarce in lean conditions, but that accumulates with obesity, similar to the DP population described by Silva et al[51]. This ATM population showed gene expression patterns enriched in pathways that are associated with oxidative phosphorylation and lipid metabolism. This provides more evidence that the M1/M2 classification is not appropriate to describe a possible function for Møs *in vivo*. The highly expressed genes in this population included *Cd9*, *Trem2* and *Cd36*, which were previously described as pro-inflammatory. Using immunostaining, the authors showed that this ATM population accumulated in CLSs and had elevated lipid content, thus these cells were termed lipid-associated Møs (LAMs).

Monocyte fate-mapping studies showed that LAMs are of monocytic origin[52]. Deletion of *Trem2* in mice resulted in adipocyte hypertrophy and worsening of the metabolic syndrome following high-fat feeding[52]. *Trem2* deficiency dramatically reduced the abundance of LAMs in the obese eWAT and reduced the lipid content of the remaining LAMs. These data suggest that the accumulation of monocyte-derived Møs in the eWAT in obesity is a protective mechanism against worsening of the metabolic syndrome, as opposed to the previous perception that these Møs exacerbate eWAT inflammation and cause insulin resistance. However, further studies using animal models with *Trem2* ablation specifically in Møs will be needed to clarify the importance of *Trem2* expression in LAMs. The $Trem2^{+}CD9^{+}$ ATM population was identified in 2 additional scRNA-seq studies as the major ATM population in the lean and obese eWAT[65, 66], as well as being the population that robustly accumulated in obesity.

In another study, Weinstock et al.[65] examined eWAT leukocyte heterogeneity by scRNA-seq from obese mice before and after weight loss, in which the same diet was continued but food intake was restricted by 30%[57]. The authors reported 7 distinct ATM populations. In line with other studies described here, the results showed that there was no particular enrichment of pro-inflammatory ATMs in obesity or an anti-inflammatory signature in lean eWAT. Conversely, based on gene expression signatures, the authors postulated that the ATM populations have distinct lipid-handling functions (such as glycerolipid metabolism, arachidonic acid metabolism and oxidative phosphorylation), which resembled Mø populations that were described in other tissues, such as the heart and atherosclerotic lesions. Consistent with this is the aforementioned finding of the TREM2 cluster in vWAT in Jaitin et al.[52] in atherosclerotic plaques[67]. Moreover, the data demonstrated that many of the transcriptional changes seen following different metabolic perturbations (e.g. obesity and weight loss) are shared among the ATM populations. Interestingly, the authors described a unique ATM population following caloric restriction-induced weight loss. This population was the largest among the leukocytes following caloric restriction and was enriched in pathways related to phagocytosis. The authors postulated that these phagocytic ATMs assist in clearing dying adipocytes and leukocytes in the resolution of obesity and its related eWAT inflammation. It is possible that the phagocytic ATMs affect insulin resistance as well, since the calorically restricted mice showed improved glucose tolerance compared to obese mice[65]. Taken together, these studies suggest the existence of a division of labor between

different Mø subpopulations within the vWAT. The development of new tools that allow the modification of specific Mø subpopulations can help in understanding the contribution of these cells to several pathologies, as well as to tailor new therapeutic approaches.

In summary, Møs are the most abundant and diverse of the leukocytes in the adipose tissue. The debate regarding their inflammatory nature in obesity in still ongoing; however, recent investigations of the heterogeneity of ATMs suggest that they undertake metabolic functions (further reviewed in the companion review[17]), such as lipid handling and recycling and dampen inflammation in obesity and following weight loss (Figure 1). Importantly, the influence of ATMs is systemic, through their secreted factors. For instance, eWAT Møs in obesity were shown to produce IL-1β, which caused proliferation of bone marrow progenitors, resulting in monocytosis and neutrophilia, which, collectively, exacerbated atherosclerosis progression[68]. Thus, understanding both the local and systemic effects of ATMs will be crucial to fully understand their contributions in obesity and cardiovascular risk.

2. Dendritic Cells—Dendritic cells (DCs) are phagocytes, constantly surveying tissues, sampling antigens and instructing adaptive immunity by activating and shaping T cell responses and by releasing chemokines and cytokines. Ralph Steinman and Zanvil Cohn[69] discovered these cells in the 1970s and in the past 50 years the ontogeny, function and subsets have been extensively studied and reviewed elsewhere[70–72]. DCs are conventionally classified as cDC1s ($CD11b^{-}CD103^{+}$), cDC2s ($CD11b^{+}CD103^{-}$) and plasmocytoid DCs (pDCs, characterized by their ability to synthesize large amounts of type I interferons, IFN, in response to viral stimulation)[71]. This section will be focused on cDC1 and cDC2, since pDCs are rare in the vWAT[51].

For many years the study of AT DCs was overshadowed by the attention directed at ATMs. Furthermore, the technical difficulties of discriminating DCs from Møs in the eWAT were a major hurdle. DCs and Møs share many surface markers, such as CD11b, F4/80, MHCII, CX3CR1 and CD11c. Some groups relied on the use of CD11c and MHCII as markers of DCs in the eWAT[15, 73], however, Møs can express these surface markers as well, which has confounded data interpretation. Another technical hurdle is that cells within the eWAT are intrinsically more autofluorescent, which can complicate the analysis of flow-cytometry data. The recent use of multi parametric flow-cytometry and more advanced genetic tools, such as Zbtb46GFP[74–76] (fluorescently marks cDCs specifically) and the CRE-lox[77] mice, has enabled direct studies of AT DCs.

The identification of DCs in the mouse eWAT has improved with the use of CD64 as a Mø marker[16, 51], since DCs do not express this gene in the eWAT. It was shown that the eWAT is populated by both cDC1s ($CD45^{+}CD19^{-}B220^{-}CD11b^{-}CD11c^{+}MHCII^{+}CD64^{-}CD103^{+}$) and cDC2s ($CD45^{+}CD19^{-}B220^{-}CD11b^{+}CD11c^{+}MHCII^{+}CD64^{-}CD103^{-}$)[16, 51, 78]. At steady-state, the eWAT is enriched in cDC2s, which are the third most abundant immune cell population after Møs and T cells[20], while cDC1s represent only a small fraction of immune cells[51]. These cells express a set of genes related to an immunoregulatory phenotype[51]. For example, cDC2s express high levels of *Aldh1a2*, the gene encoding retinol dehydrogenase 2, an enzyme fundamental to the synthesis of retinoic acid from retinaldehyde. Retinoic acid

has been shown to be a potent immunoregulatory factor, capable of inducing the differentiation of regulatory T cells[79]. Single-cell analysis of eWAT leukocytes identified three DC populations, one of which is proposed to be monocyte-derived[65]. However, the functional differences between the monocyte-derived DCs and the resident populations is unclear. Interestingly, during obesity, the number of DCs per gram of eWAT is not altered while there is a massive expansion in the amount of monocyte-derived Møs[51], leading to a proportional decrease in AT DCs. This indicates that DCs contribute to the immunoregulatory environment in steady-state; however, during obesity it is likely that this immunoregulatory function is diluted by the deluge of other leukocyte subpopulations that accumulate in obesity.

Recent studies have begun to shed more light on DC function in the eWAT. Pamir and collaborators have observed that DCs can contribute to adipogenesis and insulin sensitivity[80]. Using CSF2$^{-/-}$ mice (that presumably has a greater negative impact on DCs than on Møs) they observe that CSF2-deficiency leads to a reduction in the numbers of cDC2s. This reduction led to an increase in total body adiposity and adipocyte size. Furthermore, the researchers observed that metalloproteinase 12 (MMP12) and Fibronectin 1 (FN1), which are highly expressed by cDC2s in the vWAT, impaired adipogenesis *in vitro* indicating a possible mechanism of regulation of adipogenesis *in vivo* by DCs. However, CSF2 is also an important cytokine for the development of neutrophils, eosinophils, basophils and monocytes, which can be a confounding factor for the data observed with CSF2$^{-/-}$ mice. However, another report using the FMS like tyrosine kinase 3 ligand (*Flt3l*)-knockout mice, in which there are low levels of DCs[81, 82], showed opposite results, displaying reduced adiposity and increase insulin sensitivity[82]. Thus, further studies with conditional knockout animals targeting specific subpopulations of DCs are necessary to expand the knowledge about the role of DCs in vWAT function.

It is unclear, however, in these studies if littermate controls and co-housed animals were used to exclude an effect of the microbiome. Furthermore, Macdougall and colleagues have shown that DCs have a tolerogenic phenotype associated with the activation of β–catenin in cDC1s and peroxisome proliferator-activated receptors (PPAR) γ in cDC2s. Deficiency of these transcriptional regulators in DCs rendered mice subjected to HFD more predisposed to the development of insulin resistance. Moreover, it was observed that obesity inhibits the β–catenin and PPARγ pathways in DCs, modulating them towards a more pro-inflammatory phenotype[78].

Despite these advances, many outstanding questions remain regarding the role of DCs in the eWAT. Little is known about the trafficking of DCs from the eWAT to the draining lymph nodes and how this influences the differentiation of T cells in homeostasis and obesity. Additionally, how DCs are distributed in eWAT and the cells with which they interact is largely unknown. Do DCs contribute to the maintenance of AT regulatory T cells (adTregs)? If so, which subpopulation of DCs mediates the interaction with adTregs? We expect that in the near future, with the development of better genetic models to specifically manipulate DCs, the contribution of DCs to eWAT homeostasis will be revealed.

3. Monocytes—Monocytes are traditionally stratified into two main populations, based on their expression of Ly6C in mice or CD14 in humans (reviewed in[83]). Classical monocytes, also termed inflammatory monocytes, highly express Ly6C/CD14 and are the main monocyte type rapidly recruited to sites of inflammation and insult. Non-classical monocytes, characterized by low expression of Ly6C/ CD14 patrol the blood vessels and closely interact with the endothelium[65, 83]. It has been consistently shown that at least a fraction of the non-classical monocytes arise from classical monocytes[84]. They exhibit a short lifespan in the circulation (1–7 days)[85, 86], but can be recruited to tissues and reside there for long periods of time as tissue monocytes, or more commonly, differentiate into Møs or DCs[87]. scRNA-seq experiments of mouse peripheral blood monocytes revealed that within each population, Ly6C^{hi} and Ly6C^{lo} monocytes are homogeneous under steady-state conditions. However, a subset of monocytes with intermediate expression of Ly6C was shown to be heterogeneous[88]. Because circulating monocytes do not show great heterogeneity under physiological conditions, it will be interesting to understand whether their heterogeneity changes with inflammation or in different tissues. Moreover, since tissue microenvironments are critical in shaping the transcriptome of Møs[89] and recruited monocytes[90], monocyte heterogeneity may be enhanced within eWAT.

To date, scRNA-seq of eWAT leukocytes has not revealed much monocyte heterogeneity (represented by one-two populations), in either lean or obese conditions[52, 65, 66]. That said, some evidence suggests that the eWAT monocytes give rise to multiple populations of Møs and DCs[65], indicating potential functional heterogeneity. Furthermore, pseudotime analysis of scRNA-seq has shown that obesity and subsequent weight loss alters the monocyte's differentiation trajectories[65]. While in lean eWAT most monocyte-derived Møs retained transcriptional profiles similar to monocytes, in obesity and caloric restriction the ATMs appeared more differentiated and further away in pseudotime space from monocytes[65]. Monocytes recruited to the eWAT in obesity primarily differentiate into Møs that co-express CD11c and CD64[51]. It is likely that these double-positive ATMs consist of a heterogeneous population. Using a fate-mapping mouse model, Jaitin and colleagues showed that LAMs are monocyte-derived[52]. However, further single cell analysis of fate-mapped monocyte-derived ATMs may shed more light into the function and differentiation capacity of AT monocytes. We postulate that cues in the different microenvironments (e.g. bone marrow, spleen, circulation eWAT) shape the differentiation capacity of monocytes, thus regulating the abundance and function of the monocyte-derived cells- Møs and DCs.

4. Eosinophils—Historically eosinophils were viewed as effector cells mainly involved in allergic inflammation and responses to parasites, but their roles in AT homoeostasis became appreciated in the last decade. The stromal vascular fraction of vWAT contains ~5% eosinophils[27]. The importance of WAT eosinophils was emphasized in a seminal publication from Locksley and colleagues[27], where the authors found that eosinophils are the major source of IL-4 in the WAT, supporting alternative activation of ATMs. As described in the Mø section, IL-4 produced by eosinophils is necessary for heat production upon cold exposure by promoting beiging of white adipocytes[25] and increasing the availability of energy from fatty acids by enhancing lipolysis[24]. Furthermore, IL-4/STAT6 signaling regulates nutrient metabolism and insulin sensitivity. Although STAT6 deficient mice gain

less weight on a HFD than their WT counterparts, due to increased energy expenditure, they are more glucose intolerant because of decreased insulin action[26].

IL-5 is an important cytokine required for the maintenance of eosinophils and its overproduction resulted in accumulation of eosinophils in the eWAT[27], while easonophil recruitment to tissues is mainly governed by eotaxins (reviewed in[91]). Innate lymphoid cells (ILCs) have been identified as the source of IL-5 in the eWAT that acts to maintain the eosinophil population[92, 93] (the contribution of ILCs to AT biology will be further discussed below). eWAT eosinophil counts dropped in a mouse model of diet-induced obesity and eosinophil deficiency enhanced glucose intolerance[27] and worsened the metabolic syndrome[94]. This may be due to impairment of lipid accumulation and adipocyte differentiation in the obese eWAT upon eosinophil deficiency[94]. Conversely, long term caloric restriction of lean mice resulted in accumulation of eosinophils in the WAT, increased IL-4 production, promoted adipocyte beiging and enhanced glucose tolerance[95].

Eosinophil heterogeneity has been recognized for approximately 35 years[96, 97], with peripheral blood eosinophils known to have both different appearances microscopically and functionality in patients suffering from allergies[98] and parasites[99]. This altered eosinophil phenotype, termed hypodense, occurred following degranulation of their cytotoxic vesicles. However, it is unclear if hypodense eosinophils represent a distinct population, or whether this is indicative of their activation state. Notably, although eosinophils can be easily detected using flow-cytometry[51], all 3 recently published scRNA-seq studies of eWAT leukocytes failed to identify any eosinophil populations[52, 65, 66]. This may indicate that AT eosinophils do not have a substantially different transcriptome from other leukocytes, thus preventing their identification as a distinct population. Alternatively, there could be a bias introduced by the procedures and instrumentation involved in scRNA-seq[100], which precludes the identification of eosinophils in eWAT. It is possible that as more advanced methods are introduced to the field, such as Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE)-seq that combines proteomics and transcriptomic data, more light will be shed on the phenotypes and function of eosinophils in eWAT.

5. Neutrophils—Neutrophils are rare in the lean eWAT (<0.01% of the stromal vascular fraction)[101], where they have been proposed to be engulfed by Møs and maintain the tissue integrity[102]. Neutrophils rapidly accumulate in the eWAT upon HFD feeding and their numbers in the stromal vascular fraction increases by ~20 fold within 7 days, after which their proportion is steadily maintained[101, 103]. The accumulation of neutrophils in the eWAT following HFD is dependent on neutrophil elastase (NE), and its deficiency improves metabolic parameters, such as glucose tolerance and insulin resistance. Mechanistically, it was shown that NE may degrade Insulin receptor substrate 1 (IRS1) in hepatocytes and adipocytes, therefore promoting lipogenesis and insulin resistance[101]. In the circulation of obese patients, neutrophils produced more pro-inflammatory mediators, such as myeloperoxidase (MPO), IL-8 and CCL2[104]. MPO deficiency was later shown to enhance insulin sensitivity and induce heat production via upregulation of uncoupling protein 1 (UCP1)[105].

It has been proposed that similar to Møs and T cells, neutrophils are heterogeneous in their activation states and responses, with “N1” population referring to a pro-inflammatory neutrophil and “N2” an anti-inflammatory population[106–108]. Although this stratification of neutrophils is over simplistic, as with M1/M2 Møs, it has remained a convenient “high level” classification.

Similar to eosinophils, neutrophils have been shown to be of variable densities. For instance, three neutrophil populations with varying cellular densities were characterized in the circulation of tumor-bearing mice and humans[109]. The suggested model for this phenomenon is that immature, low-density neutrophils arise from immature neutrophils in the bone marrow and serve as myeloid-derived suppressor cells (MDSCs), whereas mature low-density neutrophils are derived from conventional circulating mature neutrophils. Both low-density neutrophil populations were found to be immunosuppressive and tumor promoting[109].

A recent scRNA-seq analysis of cells positive for $Gr1^+$ cells (also known as Ly-6G/Ly-6C, and expressed on granulocytes and monocytes) in steady-state conditions showed that neutrophils are even more heterogeneous than appreciated previously[110]. The authors report 3 major neutrophil populations in the bone marrow, and 3 transcriptionally distinct neutrophil populations in peripheral blood and spleen. Interestingly, there was little overlap between neutrophil populations in the bone marrow and peripheral tissues; however blood and spleen neutrophils showed significant overlap[110]. One caveat of this study is the purification of cells with Gr1 expression, since this is a shared marker for neutrophils, monocytes, natural killer (NK) cells and other cell types. Thus, it is possible that the observed heterogeneity reflects different leukocyte types. It is also possible that the distinct populations reflect varying differentiation states and neutrophil-biased progenitors[111]. Other support for neutrophil heterogeneity comes from a recent scRNA-seq study of heart neutrophils following myocardial infarction (MI)[112]. The authors isolated cells from the infarcted heart 0, 1, 3, 5 and 7 days post MI and performed CITE-seq and found 6 distinct neutrophil populations. In the MI model, neutrophil heterogeneity was observed temporally, as different neutrophil clusters populated the heart in different stages post-MI. These data further suggested that neutrophil heterogeneity is, at least in part, due to local phenotypic transition in the ischemic heart[112]. Since similar numeric changes of neutrophils occur in the eWAT upon high-fat diet feeding, it will be interesting to determine whether populations similar to those observed in the infarcted heart are also present in the eWAT. Neutrophil heterogeneity possibly regulates vWAT inflammatory status via production of Neutrophil Extracellular Traps (further reviewed in[17]) on one hand, and the suppressive nature of MDSCs on the other.

6. Mast cells—Mast cells home to peripheral tissues as immature cells and undergo their final maturation under the specific control of mucosal and epithelial tissue microenvironments[113]. The WAT contain mast cell progenitors and can produce mature cells that home to the intestine, skin and WAT upon hematopoietic cell transplantation[114]. The heterogeneity of mast cells was proposed in the 1980’s. Two major populations of mast cells were described in humans, based on the protease content of their cytoplasmic granules. Connective tissue mast cell granules contain tryptase, chymase and carboxypeptidase,

whereas mucosal tissues mast cells produce only tryptase[115]. A common hypothesis is that mast cell heterogeneity is related to the fact that their final maturation occurs in tissue microenvironments[116], and their phenotypes are thus dictated by the specific milieu in which they mature.

Similar to other types of immune cells, mast cells accumulate in the vWAT in obesity[117]. Mast cell precursors showed enhanced migration following treatment with eWAT conditioned media from pre-obese db/db mice, suggesting that mast cells accumulate in the eWAT before obesity is established[118]. Genetic and pharmacologic interventions in mice that impair mast cell maturation and function resulted in decreased weight gain on a HFD and subsequently enhanced glucose tolerance[117, 119]. Strikingly, treatment of obese mice with an inhibitor of mast cell degranulation induced weight loss, even when the high-fat diet feeding continued[117]. Adoptive transfer of mast cells to mice deficient in mast cells partially restored the obese phenotype, but only if the transferred cells were sufficient for IL-6 and IFNɣ, suggesting a role for these factors in the deleterious effects of mast cells in obesity[117]. Furthermore, mast cells were shown to produce prostaglandin J2 (PGJ2), an activator of the adipogenic transcription factor PPARɣ, and may promote adipogenic differentiation via this mechanism[119]. Additionally, obese WAT mast cells seem to be degranulated[120] and serum levels of the mast cell protease trypase positively correlates with BMI[121]. Overall, the preponderance of evidence indicates that vWAT mast cells exacerbate obesity and the metabolic syndrome; however, their heterogeneity in the WAT is yet to be established. It is plausible that, similar to Møs, there are distinct mast cell populations that are responsible for different functions.

II. The adaptive immune system in WAT: lymphoid cells and their tugs of war

Despite the vWAT being a tissue predominantly populated by myeloid cells, it is also home to a diverse population of lymphoid immune cells. At steady state, Tregs, ILC2s, γδ T cells and invariant natural killer T cells (iNKTs) contribute to vWAT homeostasis by inhibiting inflammatory processes and promoting an optimal environment for adipocyte function to control systemic metabolism. On the other side of the spectrum, effector CD4+ T cells (mainly type 1 T helper cells –Th1), CD8+ T cells and natural killer cells (NKs) and/or type 1 innate lymphoid cells (ILC1s) play important roles in inducing an inflammatory environment and consequently, compromising vWAT metabolic function leading to insulin resistance in obesity. This section will explore how these myriad cell populations participate in the activity of vWAT at steady state and in obesity.

7. αβ (conventional) T cells—T cells are the second largest immune population in the WAT[20] and can be divided into 2 major subtypes, based on cell surface expression of CD4 or CD8. Upon antigen stimulation in the context of presentation on MHC molecules, T cells are activated and become effector cells, which are crucial for mounting a primary response against pathogens, as well as establishing immunological memory. In obesity, T cells were shown to accumulate in the vWAT in both mice and humans[122, 123], possibly through recruitment via CCR5-CCL5 interactions[122].

Nagai and colleagues performed a time course examination of the mouse eWAT stromal-vascular fraction[124], which revealed that while Mø frequencies begin to increase at 10 weeks post HFD feeding, $CD8^+$ T cells increased proportionally within 6 weeks of HFD[124]. Other studies showed that T cell accumulation in the eWAT is subsequent to Mø accrual during HFD feeding[125], and recent scRNA-seq data showed no marked difference in T cell frequencies in obesity[52] or weight loss[65]. In absolute numbers, $CD4^+$ T cells are much more abundant in the vWAT[123] and they increase further in obesity[126]. However, the distributions of the different $CD4^+$ T cell sub-populations change with obesity, which will be discussed later in this section. In addition, obesity induced the accumulation of memory T cells in the eWAT following viral infection, and mediated acute pancreatitis and eWAT necrosis upon re-challenge[14]. Weight cycling was also shown to increase T cell abundances in the eWAT, without influencing the number of ATMs[127].

Because there is an increase in T cells in the obese eWAT, it is reasonable to speculate that some T cells undergo clonal expansion. Recent T cell Receptor (TCR) sequencing studies found that both $CD8^+$ and $CD4^+$ T cells from obese eWAT are enriched for clones that are shared across many individual mice, indicating a process of selection by similar auto-antigens[13, 128, 129]. Furthermore, MHCII expression was increased at early times post-HFD feeding (2 weeks) and mice deficient in MHCII showed similar adiposity, with greater insulin sensitivity compared to WT controls[130]. That said, MHCII inhibition in mice during HFD feeding did not improve glucose tolerance, although it did reduce the eWAT $CD4^+$ T cell abundance[131]. Importantly, deficiency in conventional T cells (in $TCR\beta^{-/-}$ mice) led to improvement of glucose and insulin sensitivity compared to WT mice, albeit lipid accumulation increased in the eWAT and adipocytes hypertrophied after HFD feeding[132]. Similarly, treatment of obese mice with anti-CD3 antibody (for T cell depletion) led to improvement in glucose and insulin sensitivity[129]. On balance, these findings suggest that conventional T cell activation is deleterious in obesity. However, other experiments showed that T cell deficiency of the co-stimulatory molecule CD40 exacerbated obesity and insulin resistance[133], indicating that some T cell responses may be beneficial.

In general, $CD8^+$ T cells are considered harmful in obesity. Depletion of $CD8^+$ cells throughout HFD feeding or after the establishment of obesity, using a depleting antibody, decreased the expression of IL-6 and TNFα in the eWAT, and modestly improved glucose and insulin tolerance[124]. Moreover, CD8+ T cell-deficient mice fed HFD were more glucose and insulin tolerant than control mice, a phenotype that was overcome by adoptive transfer of WT $CD8^+$ T cells[124]. These data suggest that $CD8^+$ T cells are drivers of inflammation in the eWAT. The recruitment of $CD8^+$ T cells to the eWAT was shown to be largely mediated through CD11a, since adoptively transferred CD11a-deficient $CD8^+$ T, but not WT, cells failed to home to the eWAT[134]. In the obese eWAT, expression of perforin by $CD8^+$ T cells restricted their inflammatory capacity by inhibiting their proliferation and inducing apoptosis[135, 136].

$CD4^+$ T cell subtypes, also known as T helpers (T_h), have been characterized based on the cytokines they produce and the type of immune response they promote. Of the T helper sub-types, the central population responsible for maintaining WAT integrity is the Tregs, which will be discussed in the following section. The main T helper cell that is thought to have

deleterious effects in obesity is the T_h1 sub-type, which is regulated by the transcription factor T-bet and produces pro-inflammatory cytokines, including TNFα and IFNγ. Their frequency in the obese human and mouse vWAT is 10–20 fold greater than other T helper populations[129, 137, 138], while in lean conditions their proportion is similar to Tregs[129]. T_h1 cells were shown to accumulate in the eWAT and scWAT of mice fed a HFD, as well as in vWAT in humans, which strongly correlated with BMI[129].

IFNγ production from eWAT T_h1 cells increases in obese compared to lean mice, and IFNγ deficiency moderately improves glucose tolerance following HFD feeding[139]. TCRβ$^{-/-}$ mice were protected from diet-induced obesity compared to WT mice, and this protection was abolished by adoptive transfer of T_h1 cells, providing evidence that T_h1 cells are deleterious in obesity. A subset of T_h1 cells, which express PD1 and CD44, was shown to produce osteopontin in the obese eWAT that may contribute to worsening of glucose and insulin tolerance. This was confirmed by transferring PD1$^+$CD44$^+$CD4$^+$ T cells from spleens of obese mice into eWAT of lean recipients. This resulted in increased expression of inflammatory markers in the eWAT and in impaired glucose and insulin tolerance, but only when the transferred T cells were sufficient for osteopontin[126].

T_h17 cells, which are regulated by the transcription factor RAR-related Orphan Receptor (ROR) γt and are the main source of IL-17, are another T helper subtype implicated in pro-inflammatory processes. Their role in obesity has not yet been extensively studied. Current data show that obese, insulin resistant individuals have an accumulation of T_h17 in their scWAT, compared with metabolically healthy obese or lean people[140]. The same study also demonstrated *in vitro* that IL-17 reduced insulin sensitivity in human liver cells and inhibited glucose uptake by rat skeletal muscle cells[140]. IL-17 deficient mice had better glucose and insulin tolerance than their WT counterparts, however, this effect was abrogated following HFD feeding and aging[141], indicating that IL-17 and T_h17 cells may not play a role in obesity. Further investigations using adoptive T cell transfer may shed more light on the role of T_h17 cells, since it was also shown that the major producers of IL-17 in eWAT are not T_h17, but rather γδ T cells[141].

Another T helper subtype is the T_h2, which is regulated by the transcription factor GATA Binding Protein (GATA) 3. These cells produce the STAT6 responsive cytokines IL-4 and IL-13, among others. T_h2 cells have been shown to be beneficial in mouse models of obesity: Recombination Activating Gene (Rag) deficient mice (lacking T and B lymphocytes) fed HFD were more obese and insulin resistant than their WT counterparts, a phenotype that was reversed via CD4$^+$, but not CD8$^+$, T cell transfer[129]. Most CD4$^+$ T cells that accumulated in the Rag$^{-/-}$ eWAT post-transfer expressed GATA3, and the improvement in metabolic parameters was impaired when the transferred cells were deficient for STAT6[129]. With that said, further examination of the contribution of T_h2 cells in obesity is needed, since it is currently thought that the main producers of IL-4/13 in the WAT are eosinophils and the target cell population for these cytokines are Møs (see sections above). Moreover, GATA3 can also be expressed by Tregs[142], which are critical for maintaining the WAT integrity (see Treg section below).

Despite many studies, the role of T cells in the WAT and their contribution to obesity are still incompletely understood. A plausible unbiased approach to unveiling the different T cell populations in the WAT is scRNA-seq (and preferably CITE-seq) of purified $CD3^+$ cells. Current scRNA-seq datasets of eWAT simply do not have enough T cells to investigate their heterogeneity. Another possibility is to aggregate all of the scRNA-seq data published thus far, in hopes that there is enough information to distinguish between the T cell subtypes.

8. Regulatory T cells—The discovery and characterization of Tregs[143, 144], whose differentiation is regulated by the transcription factor Forkhead Box P3 (FOXP3)[145–147], have revealed a plethora of functions for these T cells in health and disease. Tregs are essential to maintain homeostasis of most mammalian tissues. Mutations in the *Foxp3* gene have been linked to the development of several inflammatory diseases[148–151]. In addition to their contribution to controlling immune responses, Tregs have been described more recently by several groups as having an active role in the physiology of several organs[152], such as muscle[153] and eWAT[154–157].

Feuere et al. were the first to show that Tregs are highly enriched in eWAT in the steady state[155]. It was further observed that Tregs accumulate in the WAT of mice with aging, whereas they decline in obesity in both mice and humans, resulting in an exacerbated inflammatory environment that promotes the development of type 2 diabetes[155, 158]. Recent scRNA-seq data of mouse eWAT confirmed that the relative abundance of Tregs, which clustered together with ILC2 cells, decreased in obesity[65]. Interestingly, following caloric restriction-induced weight loss, adTreg proportions reverted back to their lean frequencies[65].

Following their initial report in WAT, several groups contributed to the characterization and functional analysis of adTregs. It is now well established that adTregs display a unique phenotype. AdTregs express the transcription factors PPARγ[154, 159] (the master regulator of adipocyte differentiation), Basic leucine zipper Transcription Factor (BATF), GATA3 and Interferon Regulatory Factor (IRF) 4[157]. These cells are thymic derived (expressing thymic Treg markers neuropilin-1[160] and Helios[161]), and express high levels of the receptors for the cytokine IL-33 (ST2, IL1RL1 or IL33R) and the Killer cell Lectin like Receptor (KLR) G1[154, 156, 157]. Elegant work by Vasanthakumar and colleagues demonstrated that adTreg accumulation in the eWAT relies on IL-33 signaling. Tregs deficient for IL-33R, Myeloid differentiation primary response (MyD) 88, BATF and IRF4, all of which are activated by IL-33, displayed impaired development of adTregs and increased susceptibility to the development of insulin resistance when submitted to a hyper-caloric diet[157]. On the other hand, mice administered IL-33 had their adTreg pool expanded and this correlated with an improvement in insulin sensitivity and glucose tolerance[156, 157].

AdTregs are long lived, self-dividing and harbor a restricted TCR repertoire, with the expansion of a few clones, which indicates that these cells are pre-selected to identify limited antigenic repertoire, and do not need constant thymic supply to maintain cell numbers in the eWAT[156]. More recently Li and colleagues discovered a stepwise acquisition of the adTreg phenotype[162]. This study clearly showed that FOXP3 and an AT pre-selected TCR are required for adTreg development and accumulation in the eWAT[162]. They further showed that after homing to the eWAT, adTregs upregulated the expression of the IL33R and

that higher abundances of IL-33 in this tissue drove the expansion of adTregs in the eWAT[162]. Moreover, it was reported that ILC2s (described below) also support the expansion of adTregs *in vivo* through Inducible T Cell Costimulator (ICOS)- Inducible T Cell Costimulator ligand (ICOSL) interactions[163]; however, experiments demonstrating this interaction in the eWAT by imaging are still missing.

One of the open questions in the field is how precisely adTregs contribute to the establishment of an immunoregulatory environment in the eWAT that favors insulin sensitivity. Several publications have shown an inverse correlation between adTregs numbers in the eWAT and the appearance of $CD11b^{+}CD11c^{+}$ Møs in the eWAT[154, 156, 157]. AdTregs are characterized by the high expression of the immunoregulatory cytokine IL-10[157] that has been shown in different settings to contribute to the maintenance of more homeostatic/ tissue-resident Møs[164–167]. In addition, adTregs express high levels of amphiregulin, a ligand of the epidermal growth factor receptor (EGFR), which promotes tissue repair and resolution of inflammation[152, 153]. It is also predicted that these cells deploy their regulatory functions to inhibit the activation of $CD4^{+}$ and $CD8^{+}$ effector T cells within the eWAT and, therefore, blunting of the initiation of inflammatory responses.

Despite the aforementioned mechanisms, little is known about how this is executed *in vivo*. A recent study showed that adTregs localize close to mesenchymal stromal cells (MSCs) that are the producers of IL-33 in the eWAT[168]. However, the interaction of adTregs with other cell types in healthy eWAT is still largely unknown. For example, the fact that adTregs harbor a restricted TCR repertoire suggests an active selection by antigen presenting cells (APCs) within the vWAT, and that these Tregs probably recognize vWAT specific antigens. However, little is known about which APC is responsible for selecting adTregs. As described earlier in this review, the vWAT is populated by diverse and complex populations of antigen presenting cells. The observation that adTregs are located close to MSCs could indicate that these cells could be responsible for the adTreg selection, however, they do not present antigen in a MHCII context. This question goes hand-in-hand with the lack of data regarding antigen recognition by adTregs. What are the antigens recognized by these cells? Are these antigens canonical peptides or could they even be modified peptides harboring lipidated branches due to the high concentrations of triacylglycerol and derivatives present in this tissue? Another open question is how the fat draining lymph node and APCs contribute to the development of adTregs. Li et al. claim that adTregs originate in the thymus, pass through the spleen, and finally home to the vWAT[102]. However, the vWAT draining lymph node could be a strategic place for drained antigens and/or circulating APCs that had passed through the vWAT and are shaped by the microenvironment to initiate the adTreg program and present vWAT specific antigens.

Finally, one of the most puzzling questions is why the number of adTregs is severely reduced in obesity and whether this is the cause or consequence of obesity/ metabolic syndrome? There are reports in the literature indicating that the fast increase in adipocyte size and numbers cause a hypoxic environment, promoting sterile inflammation[169–171], and possibly reducing adTreg cell numbers. However, it is already established in the literature that Tregs are more tolerant of hypoxia and that Hypoxia Inducible Factor (HIF) 1α actually favors Treg development[172]. Thus, the reason behind adTreg reduction in obesity is still a

mystery. If these questions can be addressed, it is possible to foresee the development of new therapeutic approaches to expand adTregs via administration of AT specific antigens and IL-33, to reduce vWAT inflammation and mitigate insulin resistance/ type 2 diabetes.

9. γδ T cells—Although not as prominently studied as αβ T cells, γδ T cells are an important immune cell population involved in response to both microbial and sterile inflammation[173]. γδ T cells comprise only 5% of the circulating T cells; however they are particularly enriched in barrier surfaces like the skin, intestinal epithelia and reproductive organs[174–176]. One peculiar characteristic of γδ T cells is that they harbor a restricted TCR repertoire, especially if compared to αβ T cells, with specific clonal expansion in each epithelial barrier that they populate[174–176]. Interestingly, until today, the antigens recognized by γδ T cells are largely unknown. In the lean eWAT, γδ T cells are as abundant as CD8 T cells, monocytes, mast cells and neutrophils[51], although they were not recognized as a separate cell population in recent scRNA-seq experiments[65,66]. With that said, it was previously shown that scRNA-seq technologies cannot effectively separate T cell subsets, and that integration of proteomic and transcriptomic approaches, such as CITE-seq, are better at distinguishing between different T cell populations[177].

Thus far a few groups have investigated the function of γδ T cells in the vWAT. A preliminary study has claimed that these cells promote inflammation and insulin resistance when animals are subjected to HFD. Mice deficient in γδ T cells displayed better insulin sensitivity after 10 weeks of HFD[178]. However, the fact that TCRδ knockout animals displayed several discrepancies from Vγ4/6 knockout mice (although both mouse models lack γδ T cells), and whether the animals were properly cohoused to normalize the microbiota, precludes a clear conclusion about the role of γδ T cells in the development of metabolic syndrome.

More recently, elegant work by Kohlgruber and collaborators showed that the eWAT is home to two different, self-renewing populations of γδ T cells. One population is characterized as Promyelocytic Leukaemia Zinc Finger protein (PLZF)$^{-}$, CD3ε^{low}, CD27^{+}, RORγT^{-}, T-bet^{+} that produces IFNγ, and the second population is PLZF^{+}, CD3ε^{high},CD27^{-}, RORγT^{+}, T-bet^{-} and able to produce high levels of IL-17A and TNFα[179]. Interestingly, the authors observed that animals deficient in γδ T cells, or specifically the PLZF^{+} γδ population, or IL-17A knockout mice all fail to accumulate adTregs in the eWAT. The authors show that presumably the IL-17A and TNFα produced by PLZF^{+} γδ T cells positively modulate the number of IL-33 producing stromal cells, which contributes to the accumulation of adTregs. Finally, it was observed that PLZF^{+} γδ T cells and IL-17A are fundamental for thermogenesis and mice deficient for these cells or cytokine, respectively, fail to control total body temperature[179]. These phenomena was confirmed in a new report showing that γδ T cells expressing IL-17A are key drivers of organ neuronal innervation that regulates thermogenesis[180].

This phenomenon may be linked to the fact that IL-33 is essential for adipocyte beiging and thermogenesis[181, 182], hence reduction in IL-17A producing γδ T cells leads to fewer IL-33-producing stromal cells, which in turn reduces the capacity of the AT to control core body temperature. Unfortunately, this study did not explore the impact of PLZF^{+} γδ T cells in the

development of the metabolic syndrome. In addition, studies involving the deletion of IL-17A specifically in γδ T cells can help clarify the direct role of this cytokine produced by γδ T cells. Another question that arises from this study is whether γδ T cells regulate the numbers of ILC2 cells, since it is already established that IL-33 can regulate both adTreg and ILC2 cell numbers[157, 181]. Overall, it seems that γδ T cells have a clear contribution to vWAT physiology and new studies will clarify their roles.

10. Invariant Natural Killer T cells—Natural Killer T cells (NKTs) are a major evolutionary link between the innate and adaptive immunity. They share several effector innate markers with NK cells but also are able to recognize antigens through an αβ TCR. However, these cells do not recognize peptides. NKTs recognize lipids and glycolipids bound to the MHC-like glycoprotein, CD1d[183, 184]. Invariant NKT cells (iNKTs) are a subset of NKTs that harbor restricted TCR consisting of one α chain (Vα14Jα18 in mice and Vα24Jα18 in humans) and a limited number of β chains[183]. Even though the endogenous antigenic ligands are still an enigma, it was discovered that the lipid α-galactosylceramide (α-GC), obtained from marine sponges can strongly stimulate human and mouse iNKT cells when presented in a CD1d-dependent context[185]. Moreover, a few endogenous ligands have been reported, such as isoglobotrihexosylceramide (iGb-3)[186].

iNKT cells have been reported to be important in mediating immunity against tumors[187] and bacterial infection, as well as in autoimmunity and allergy[183]. In addition, due to the nature of the AT, a lipid rich environment harboring a number of cells expressing CD1d (mainly Møs, DCs and adipocytes), several groups have recently evaluated the contribution of iNKTs to vWAT homeostasis. These cells were shown to be enriched in the vWAT, compared to their circulating levels[188, 189]. Similar to γδ T cells, iNKTs did not form a transcriptionally distinct population in recent scRNA-seq data of mouse eWAT, and were clustered with the conventional T cells[65]. Interestingly, cell frequency of the T/NKT cluster was the most stable across treatment (i.e. lean, obese and caloric restriction) among all leukocyte clusters in this study[65]. Because it is difficult to distinguish T cell subsets solely based on transcriptomic data, further investigations are needed to establish transcriptional and functional heterogeneity of vWAT T cells.

In 2011 and 2012 several reports attempted to clarify the contribution of iNKTs in vWAT physiology. The lack of consensus among these studies was obvious: some indicated that iNKTs are protective and help to improve insulin sensitivity[190–194], others indicated the opposite- that iNKTs can accelerate the development of insulin resistance[195–197], and some claimed that iNKTs do not play a role in systemic metabolic functions[198]. This variability in the results among different groups was already reviewed elsewhere[199–201] and several factors can contribute to it. Overall the studies display several differences in the experimental conditions such as the type of diet used and its duration, mouse age, and the genetic model used to manipulate NKT cells (some used CD1d knockout mice that eliminated all NKTs, others used Jα18 knockout mice that abolished only iNKTs, while others activated NKTs by injecting CD1d-α-GC tetramers). In some studies, it is not clear whether mice were cohoused to normalize their microbiota. In mice, NKT accumulate predominantly in the liver. It is possible that the metabolic phenomena observed upon NKT

manipulation are related to their effects on the liver, rather than the vWAT. Although this issue poses a tremendous technical challenge in general.

Overall it was shown that CD1d knockdown (leading to total NKT deficiency) or Jα18 knockdown (iNKTs deficiency) resulted in a more pro-inflammatory environment within the eWAT, leading to glucose and insulin intolerance[190, 192, 193]. In addition, it was observed that iNKTs in the eWAT express significant levels of IL-4 and IL-10. The activation of these cells by treatment with CD1d-α-GC tetramers augmented the expression of cytokines, leading to an increased *Arg1* expression and other factors related to the function of ATMs and ultimately improved insulin and glucose sensitivity[190, 192, 193]. These data indicate that iNKTs can act in synergy with eosinophils and ILC2s by recognizing the lipid content in eWAT to modulate the activity of resident Møs.

In the aftermath of these studies, Lynch and collaborators unveiled that iNKTs in the vWAT display a unique phenotype. They express low levels of IL7R, downregulate the expression of the transcription factor PLZF (thus far known as a marker of NKTs) after exiting the thymus and homing to the eWAT, and highly express IL-2 and IL-10[202]. These cytokines produced by iNKT are important for the maintenance of adTregs and to modulate ATMs, respectively[202, 203]. Deficiency in iNKT cells reduces the numbers of adTregs and the proportion of IL-10^{+} adTregs in the eWAT[202]. These data reinforce the idea that iNKTs act together with other regulatory cells in the vWAT to suppress inflammation and maintain its optimal metabolic activity by giving support to ATMs and consequently, to adipocytes. These data were recently reproduced using mice that lacked CD1d expression in Møs, neutrophils, DCs[204] and adipocytes[205], indicating a cross talk between NKTs, adipocytes and APCs to control the vWAT microenvironment[204, 205].

One important observation from these studies is that mice treated with CD1d-α-GC tetramers, which increases the number of iNKTs and improves insulin sensitivity, also induces weight loss. To understand the mechanism underlying this weight loss, a recent publication showed that activation of iNKTs promoted beiging of scWAT by the upregulation of UCP1 and induction of non-shivering thermogenesis. This increase in UCP1 was mediated by increased expression of Fibroblast Growth Factor (FGF) 21 directly induced by iNKT cells[206]. Interestingly, it was shown that treatment of mice and humans with liraglutide, an agonist of glucagon-like peptide 1 receptor (GLP1R), led to the proliferation of iNKTs, which consequently increased the levels of FGF21, UCP1, thermogenesis and promoted weight loss[206]. Liraglutide is a commercially available treatment for type 2 diabetes and obesity, which works, at least in part, by modulation of the iNKT-FGF21-UCP1 axis. Understanding how this pathway induces iNKT proliferation may help in the development of new approaches to avoid the side effects caused by GLP1R agonist treatment.

11. Type 2 innate lymphoid cells—The discovery of type 2 innate lymphoid cells (ILC2s) goes back to 2001 when the cytokine IL-25 (also known as IL-17E due to its homology to other members of the IL-17 family) was characterized by two different groups[207, 208]. Fort and colleagues observed that treating animals with IL-25 induced the expansion and expression of type 2 cytokines by a non-T/non-B cell that is CD11b^{-}NK1.1^{-}

Ly6C^{-}Ly6G^{-}[207]. Later, in 2002 and 2006 these cells were defined as IL-4–, IL-5–, IL-13–producing non–B/non–T (NBNT), c-kit^{+}, FcεR1^{-} cells and shown to be important for the clearance of helminthic infections[209, 210]. Thereafter several other groups studying these cells coined a diverse nomenclature, such as nuocytes[211], natural helper cells (NHCs)[212], innate helper type 2 cells (Ih2)[213] and multipotent progenitor type 2 (MPP type2)[214]. These studies found that ILC2s are fundamental at barrier surfaces to promote type 2 responses, important for immunity against helminths, in allergic inflammation and the resolution of pulmonary inflammation. It did not take long to for this population to be renamed type 2 innate lymphoid cells[215].

ILC2s are characterized by their lack of expression of CD3, CD4, CD8, CD19, B220, CD11b, FcεRI, TCRβ, TCRδ, CD5, NK1.1, Ter119, Ly6C, Ly6G and CD11c (lineage negative). These cells express CD90, IL-7Rα, c-Kit, Sca-1, IL-2Rβ, IL-2Rγ, CD25, IL-33R, KLRG1, IL-17RA, IL-17RB, CD45, CD38, CD44, GITR, CD69 and several cytokines, especially IL-4, IL-5 and IL-13[211–213, 216]. In addition, it was shown that ILC2s, similar to other innate lymphoid cells, rely on the transcription factor Inhibitor of DNA Binding (Id) 2 and the common γ chain receptor (IL-2Rγ) for their development, indicating the existence of a shared ILC progenitor[211–213]. Lastly, it was observed that ILC2 cells depend on the expression of the transcription factors TCF-1, RORα and GATA3 for theirs development and maintenance[216–218]. Interestingly, the expression of several surface markers and GATA3 is shared by ILC2s and adTregs, indicating that these cells may co-habit the same niche and work together to maintain vWAT homeostasis. Moreover, ILC2s and Tregs clustered together *in silico*, as demonstrated in scRNA-seq data of mouse eWAT, indicating a high degree of overlap in their transcriptomes[65].

Interestingly, one of the first reports to characterize this immune population found their accumulation in the mesenteric adipose tissue (around the intestine)[212]. This was indicative that ILC2s could contribute to vWAT homeostasis. In 2013, Molofsky et al. revealed that ILC2 cells are resident at the vWAT, are expanded by IL-33, and are a major source of IL-5 and IL-13. This work demonstrated that the IL-5 produced by ILC2s was fundamental for maintenance of eosinophils within the vWAT. Mice deficient for IL-5 or lacking ILC2s have a dramatic reduction in the number of eosinophils[93]. As mentioned before in this review, eosinophils are an important source of IL-4 in the WAT, which support the maintenance of alternatively activated Møs. Diet-induced obese mice develop faster and more severe insulin resistance in the absence of eosinophils or IL-5[27, 93]. Additionally, the IL-13 produced by ILC2s was shown to contribute to the maintenance of AAMs expressing *Arg1* in the vWAT, which is associated with improved insulin sensitivity[219, 220].

As was observed for adTregs, IL-33 is fundamental for the maintenance of ILC2s in vWAT. Animals deficient for IL-33 have lower numbers of ILC2s, associated with increased fat content and body weight[181]. These phenomena were associated with the capacity of ILC2s to promote beiging of white adipocytes by the increase of the opioid peptide methionine-enkephalin, which upregulates UCP1[181]. Lee and colleagues have shown that the ILC2s promote beiging through the secretion of Type 2 cytokines that activate eosinophils and these 2 cell types can secrete IL-4 and IL-13 that bind to IL4Rα in adipocyte progenitors

(expressing PDGFRα), thus inducing their differentiation towards beige adipocytes instead of white adipocytes[221].

In addition to their effects on eosinophils and Møs, another study has shown that ILC2s contribute to the maintenance of adTregs in the eWAT through ICOS-ICOSL interactions in steady-state. During inflammation the production of IFNγ inhibits ILC2 cells and consequently the accumulation of adTregs in the eWAT[163]. This could be a possible mechanism to explain how adTreg numbers are drastically reduced in obesity.

Most recently it was shown that the high levels of TNFα in obese eWAT leads to the upregulation of PD-1 by ILC2. PD-1 can interact with PD-L1 expressed by the infiltrating M1-like Møs, subsequently reducing the expression of IL-5 and IL-13 by the ILC2, and jeopardizing the latter's homeostatic function[222]. Of note, this work did not use genetic models to remove the expression of PD-1 specifically in ILC2 to confirm this mechanism. Nonetheless it opens the possibility to utilize PD-1 blockade as a therapy for the metabolic syndrome/insulin resistance. Finally, it was observed also that ILC2s upregulate OX40L upon exposure to IL-33. The authors propose that OX40L can interact with OX40 expressed by adTregs, which expands the adTreg pool, contributing to the control of insulin sensitivity[223]. Altogether, it seems that the proposed therapeutic approach for adTregs through the administration of IL-33 can have beneficial synergistic effects through the expansion of ILC2 cells. Uncovering the potential side effects of IL-33 administration and if indeed ILC2s interact directly with adTregs can help in the development of approaches to treat type 2 diabetes.

12. B cells—B cells regulate immune responses mainly via the production of antibodies and cytokines. In mice, B cells reside in the lean eWAT and further accumulate in obese conditions[224, 225]. The absolute number of B cells is similar to that of T cells in both lean and obese eWAT[224].

To directly investigate the contribution of B cells to obesity several groups used mouse models lacking B cells (B^{null}). On a HFD, B^{null} mice had improved glucose and insulin sensitivity compared with WT controls[226], albeit similar body weight[224]. Adoptive transfer of splenic B cells from obese, but not lean, donors to obese B^{null} mice worsened glucose tolerance and increased plasma insulin[224]. Moreover, eWAT $CD8^+$ T cell pro-inflammatory gene expression was attenuated in B^{null} mice. Depletion of B cells (using anti-CD20 antibody) in diet-induced obese mice markedly improved metabolic parameters and eWAT inflammation[224]. It was further shown that B cells from obese mice produce more pro-inflammatory cytokines and less IL-10 in response to toll-like receptor engagement, compared with lean controls[227]. With that said, B cell deficiency drastically alters the microbiome and impairs intestinal fat absorption, which may contribute to the phenotype in these mice[228].

The most abundant antibody isotype in the eWAT is IgM. Compared to lean mice, obesity increases the antibody isotype IgG2c in both the serum and eWAT, without significant changes in other isotypes[224, 229]. IgG antibodies produced in obesity were shown to be highly pathogenic, since administration of IgGs isolated from obese, but not lean mice,

dramatically worsened metabolic parameters of obese B^{null} mice. This observation was specific to IgG antibodies, as treatment with IgMs from obese mice did not alter metabolic or inflammatory parameters of B^{null} mice. Surprisingly, although IgG antibodies are upregulated in the obese eWAT, deficiency of all IgG receptors worsened insulin and glucose intolerance in obesity[230]. Taken together these data indicate that B cells in obesity produce diverse IgG antibodies that can be either deleterious or beneficial. It is reasonable that the expression and function of the different IgG receptors on phagocytes will determine the specific response to each antibody.

Similar to other leukocytes, B lymphocytes are stratified into sub-types according to their specific marker expression and function. The most significant difference between B1 and B2 cells is their origin and distribution. B1 cells mostly originate from embryonic tissues and B2 cells arise from adult bone marrow progenitors (reviewed elsewhere[231]). B1 cells, defined[232] as $CD19^{hi}B220^{lo}IgM^{hi}CD23^{-}$, normally reside in the pleural cavity[233] and are a major source of IgM antibodies[234]. In addition to their antimicrobial abilities, IgMs recognize epitopes on dying cells, promoting their uptake by phagocytes, thus helping maintain tissue integrity[234]. B1 cells are further stratified to 2 distinct sub-types, B1a and B1b, which differ based on their surface expression of CD5 (expressed by B1a, but not B1b)[232]. Functionally, B1a cells responses are broad, since they are not restricted to B cell receptor engagement, while B1b cells expand and produce IgM in response to specific antigens[232] and are thus the major source of memory B1 cells. Most B cells belong to the B2 type, which are the follicular (lymphoid) and marginal-zone (splenic) B cells[232]. B2 cells are activated following antigen recognition, with the help of T cells in lymphoid tissues. The eWAT was shown to contain predominantly B2 cells, with small amounts of B1a and B1b[224]. B cells further accumulated in the obese eWAT, with no change to the distribution of the B cell sub-types[224]. B cells that accumulated in the obese eWAT mainly increased the production of IgM and IgG[224]. Interestingly, recent scRNA-seq data showed that the frequency of B cells from all eWAT leukocytes did not differ between lean and obese mice, however there were ~2 fold more B cells in the obese eWAT following caloric restriction[65]. Very little is known about B cells in weight loss and it will be interesting to see whether they accumulate and have a substantial role in this process. Moreover, further investigations are needed to understand the contribution of each B cell sub-population in obesity.

III. Concluding remarks

The WAT microenvironment enables most types of immune cells to reside and cooperate within it, as well as influence systemic processes. Current investigations aim at functionally characterizing the immune populations in the WAT, due to their vital contribution to normal tissue homeostasis (Figure 2), as well as pathophysiology. In this review, we mainly focused on the vWAT, because of the wealth of knowledge in that tissue. However, it is critical to keep in mind that many other tissues (liver, muscle, bone marrow, heart and more) may accumulate excess amounts of lipids, especially in obesity. This fat accrual outside of the AT will, most likely alter the local milieu and possibly change the immune landscape of many tissues.

Recent technological advances allowing investigations on the single cell level drove the discovery of heterogeneous subpopulations and cell states, which were largely underappreciated. Flow-cytometry based studies were instrumental in finding and isolating cells expressing a distinct set of markers. However, these types of inquiries are biased, since pre-selection of marker genes is necessary. Revolutionizing biological studies was the RNA-seq, and more so scRNA-seq, which allows unbiased identification of sub-populations that may share many marker genes. Using these methods, a large number of immune populations and cell states were found in the vWAT under lean, obese and weight loss conditions. The newly identified sub-populations should be carefully examined and characterized, and to fully understand the processes underlying WAT function, the interactions of the different cell types, immune or others, should be investigated. Additionally, for a more comprehensive picture, such studies should be followed by examination of systemic changes occurring in obesity across multiple tissues. We look forward to such discoveries, which will surely revolutionize our understanding of obesity and its adverse consequences on many tissues and co-morbidities. Such efforts will hopefully enable the development of much needed therapies.

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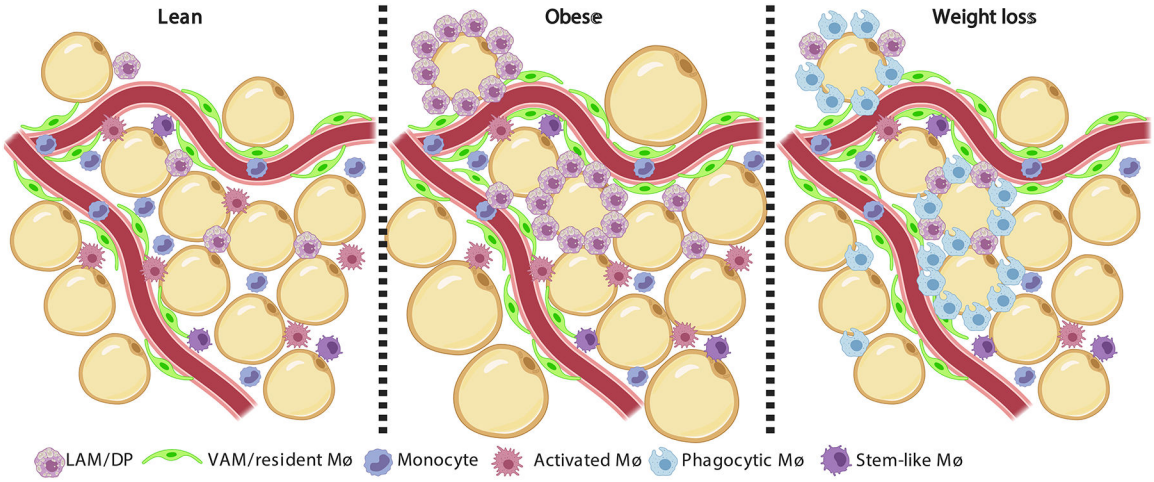

Figure 1. Adipose tissue macrophage landscape in lean, obese and following caloric restriction-induced weight loss.

The major leukocyte type in the white adipose tissue (WAT) is the macrophage. Increasing evidence show that adipose tissue macrophages (ATMs) are highly heterogeneous, and their landscape drastically changes in obesity and following weight loss. The lean epididymal WAT (eWAT) is dominated by tissue resident vascular associated macrophages (VAMs)[51], which are tightly associated with blood vessels and responsible for the endocytosis of macro-molecules from the circulation. Monocytes and monocyte-derived macrophages also reside in the lean eWAT, namely lipid-associated macrophages (LAMs[52], also known as double-positive, DP[51]). LAMs are thought to assist with lipid handling. Another ATM sub-population was found to express many chemokines, cytokines and their receptors and was named activated macrophage[65, 66]. Lastly, a macrophage sub-population that expresses many cell cycle genes and resembles a population found in atherosclerotic lesions (termed stem-like)[235] was also found in the lean eWAT[65]. Obesity increases the abundance of macrophages in the eWAT, with specific enrichment of LAMs/DPs and VAMs. While absolute VAM numbers increase by ~2 fold, there is a 10 fold growth in the LAMs/DPs sub-population. LAMs/DPs accumulate especially around adipocytes and form crown-like structures (CLS) and are thought to protect against adipocyte hypertrophy and worsening of the metabolic syndrome[52]. Weight loss subsequent to obesity causes the accumulation of a novel macrophage sub-population that is rare in lean and obese eWAT[57, 65]. This ATM sub-population was termed Phagocytic macrophage, due to their expression of genes related with phagocytosis and endocytosis[65], and was proposed to participate in clearing dying adipocytes and leukocytes from the shrinking eWAT. Following weight loss, the LAMs/DPs return to their proportions in lean eWAT.

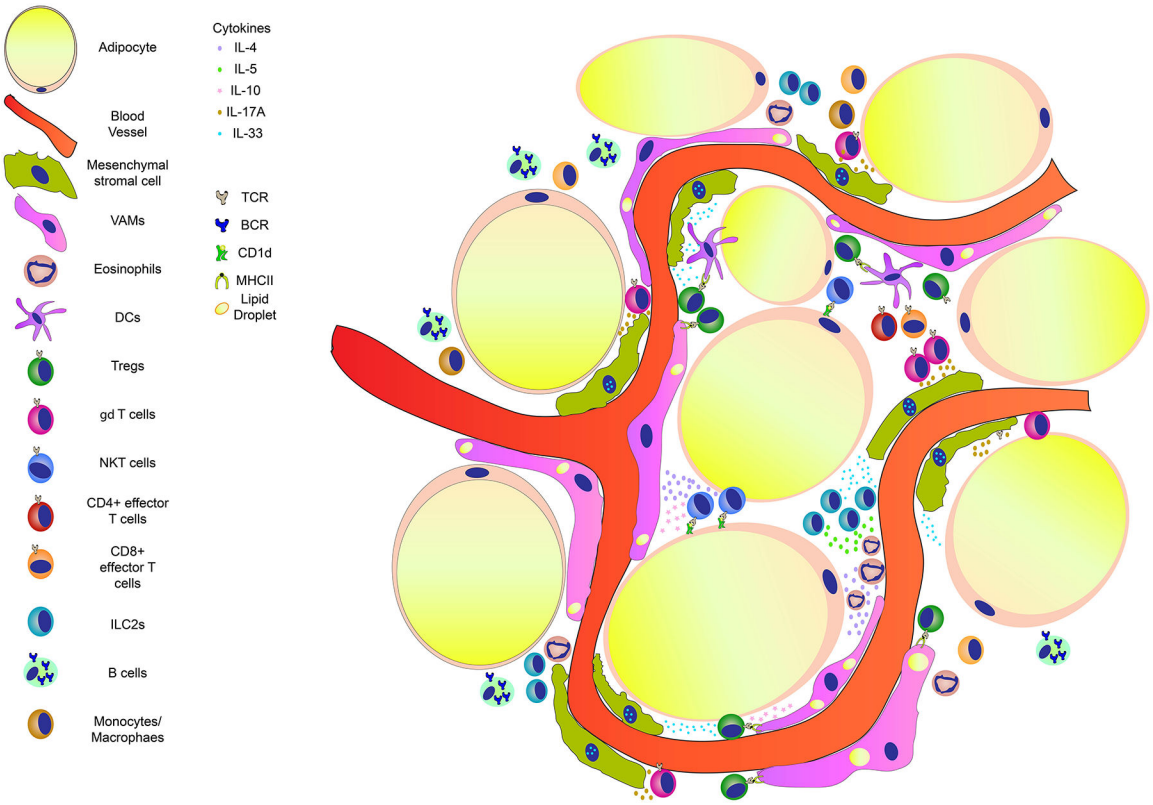

Figure 2. Major players of immune homeostasis in the lean eWAT.

The eWAT is an organ highly vascularized and replete of leukocytes. Vasculature associated adipose tissue macrophages (VAMs) are normally in intimate contact with the blood vessels. They display a rapid endocytic capacity for blood borne particles and also contain lipid droplets in their cytoplasm[51]. These cells are regulated by IL-4 and IL-10 produced by iNKT cells that are activated by CD1d expressing cells in the eWAT[193]. These cytokines contribute to an anti-inflammatory/ homeostatic environment shaping the activity of VAMs. γδ T cells positive for the transcription factor RORγT express high levels of IL-17A. This cytokine regulates the numbers of mesenchymal stromal cells (MSCs or Fibroblastic reticular cells)[179]. MSCs play an important role by producing IL-33, which is fundamental for the expansion of regulatory T cells (Tregs) within the adipose tissue. eWAT Tregs have a peculiar phenotype, displaying high expression of the receptor for IL-33[157] and the transcription factor PPARγ[154]. Tregs can recognize antigens and be activated by VAMs or Dendritic cells (DCs). Upon activation Tregs release IL-10, which in turn contribute to the physiological eWAT environment. Another important cell population is the innate lymphoid cells type 2 (ILC2s)[93]. These cells are resident in a healthy eWAT producing high amounts of IL-5, which, in turn attracts and expands eosinophils. Production of IL-4 by eosinophils help to maintain the phenotype and activity of VAMs[27]. Other leukocytes reside in the eWAT (such as T cells, B cells, monocytes, macrophage and dendritic cells), however, their function in homeostasis remains to be investigated. TCR: T cell receptor; BCR: B cell receptor; MHCII- major histocompatibility complex 2.