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# The “Big Bang” in obese fat: Events initiating obesity-induced adipose tissue inflammation

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Obesity is associated with the accumulation of pro-inflammatory cells in visceral adipose tissue (VAT), which is an important underlying cause of insulin resistance and progression to diabetes mellitus type 2 (DM2). Although the role of pro-inflammatory cytokines in disease development is established, the initiating events leading to immune cell activation remain elusive. Lean adipose tissue is predominantly populated with regulatory cells, such as eosinophils and type 2 innate lymphocytes. These cells maintain tissue homeostasis through the excretion of type 2 cytokines, such as IL-4, IL-5, and IL-13, which keep adipose tissue macrophages (ATMs) in an anti-inflammatory, M2-like state. Diet-induced obesity is associated with the loss of tissue homeostasis and development of type 1 inflammatory responses in VAT, characterized by IFN- $\gamma$ . A key event is a shift of ATMs toward an M1 phenotype. Recent studies show that obesity-induced adipocyte hypertrophy results in upregulated surface expression of stress markers. Adipose stress is detected by local sentinels, such as NK cells and CD8<sup>+</sup> T cells, which produce IFN- $\gamma$ , driving M1 ATM polarization. A rapid accumulation of pro-inflammatory cells in VAT follows, leading to inflammation. In this review, we provide an overview of events leading to adipose tissue inflammation, with a special focus on adipose homeostasis and the obesity-induced loss of homeostasis which marks the initiation of VAT inflammation.

**Keywords:** Adiponectin · Adipose tissue · Diabetes mellitus type 2 · IFN- $\gamma$  · Inflammation · Insulin resistance · Macrophages · NK cells · Obesity · TNF

## Introduction

Over the past few decades, we have seen a dramatic worldwide increase in the incidence of obesity and its associated pathologies, such as insulin resistance, which contributes to the development of metabolic syndrome and diabetes mellitus type 2 (DM2) [1]. Metabolic syndrome is a cluster of conditions such as elevated blood glucose, elevated blood pressure, excess body fat, and

abnormal lipid (cholesterol) levels, which together increase the risk of diabetes mellitus and cardiovascular disease. DM2 is characterized by high blood glucose levels, which is a direct result of reduced systemic sensitivity to the anabolic hormone insulin. An important underlying cause of obesity-induced insulin resistance is chronic low-grade systemic inflammation (reviewed in [2]). The long-term presence of pro-inflammatory cytokines in the blood blunts the signal transduction capacity of the insulin receptor in

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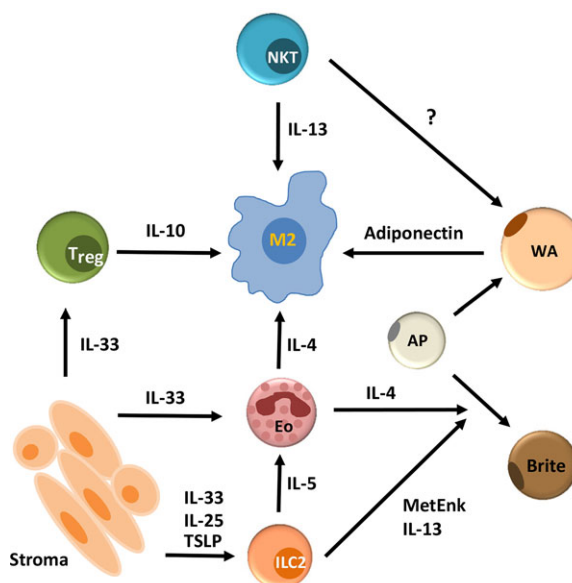
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insulin-sensitive tissues [3]. Obesity-induced systemic inflammation is thought to originate predominantly in adipose tissue. The human body contains various types of fat depots, generally divided in white and brown fat. The role of brown adipose tissue is to produce body heat and in humans is mostly found in newborns, even though adults do have small amounts of this type of fat [4]. The role of white adipose tissue (WAT) is to store nutrients in the form of a single large fat droplet. In addition, WAT is an important sensor of the metabolic state of an organism and is therefore one of the main endocrine organs in the body [5]. WAT can be found at multiple sites in the body and can be further subdivided based on location and on differences in precursor cells that give rise to these organs [6]. For example, retroperitoneal WAT is derived from a precursor that requires expression of the transcription factors Myf5 and Pax3, whereas these genes are not essential for the development of mesenteric WAT. In contrast, male perigonadal WAT does not require Myf5, but is partially dependent on Pax3 for its development in mice [6].

Of these various fat tissues, the intra-abdominal fat depots, collectively referred to as visceral adipose tissue (VAT), have been shown to be the predominant source of chronic systemic inflammation and most important for the development of DM2 [2]. When the perigonadal fat pads of mice were surgically removed 2 weeks before initiation of HFD feeding, a significant reduction of glucose intolerance and insulin resistance was observed in mouse models of obesity-induced DM2 [7]. VAT contains a relatively large population of immune cells, which changes dramatically in its composition during the development of obesity [2]. Recently, various studies have provided further insight in the earliest changes that occur within the immune cell composition of VAT in response to diet-induced obesity (DIO). In this review, we provide a brief overview of the events that ultimately results in adipose tissue inflammation and systemic insulin resistance. We focus on the initial stages of immune cell activation, which represent the equivalent of the astronomical ‘Big Bang’ for adipose tissue inflammation. Whereas many of these events will (partially) overlap, we present them as consecutive events to facilitate understanding.

## Adipose tissue in homeostasis

The immune system plays an important role in the control of adipose tissue structure and homeostasis. Two main functions of the immune system in fat can be distinguished: (i) inhibition of tissue inflammation and (ii) tissue (re)modeling. Much attention has been given to regulation of immune cell activation in VAT, since loss of immune cell inhibitory mechanisms drives the tissue inflammation that contributes to insulin resistance. However, various immunological deficiencies (described below) result either in reduced or increased adipose tissue mass, with obvious implications for the endocrine function of fat. Therefore, a better understanding of tissue remodeling by the immune system may have important implications for the current pandemic of obesity.



**Figure 1.** Control of adipose tissue homeostasis under lean conditions. Healthy adipose tissue is populated by a type 2-polarized immune system, which maintains ATMs in an M2-like state. Dominant immune cell subsets under these conditions are eosinophils, iNKT cells, and Treg cells, which produce IL-4, IL-13, and IL-10, respectively. Adipocytes also contribute to the type 2 immune response through the production of adiponectin. ILC2s regulate eosinophil numbers through the production of IL-5. Type 2 immune cells are supported by a stromal structure which promotes immune cell viability through the production of several cytokines, most importantly IL-33. In addition to sustaining a type 2 immune cell environment, adipose tissue cells engage in extensive cross-talk to (re)model adipose tissue structure and phenotype. One example is the formation of ‘brown in white’ adipocytes from adipocyte precursors ‘beiging’ through the production of IL-4, IL-13, and Met-Enkephalin by the ILC2/eosinophil axis. NKT: Invariant-Chain Natural Killer T cell; T<sub>Reg</sub>: regulatory T cell; Eo: Eosinophil; M2: M2 Macrophage; ILC2: Innate Lymphocyte 2; AP: adipocyte precursor; Brite: ‘Brown in white’ adipocyte; Stroma: adipose tissue stroma; MetEnk: Met-enkephalin.

## Inhibition of immune cell activation in adipose tissue

Under lean conditions, adipose tissue is populated by a number of immune cells (Fig. 1). These cells either inhibit immune cell activation or promote a Th2-type response, characterized by the production of cytokines such as IL-4, IL-5, and IL-13 [8]. In lean tissue, macrophages have been shown to be a dominant immune cell population, and the majority of these cells have an M2-like (or alternatively activated) phenotype (Fig. 1) [9]. Lean adipose tissue macrophages (ATMs) express arginase-1, which inhibits iNOS activity, and produce anti-inflammatory molecules, such as IL-10 and IL-1Ra [9]. Importantly, under nonobese conditions, macrophages have been shown to play a key role in inhibiting immune cell activation in murine fat. In mouse models where ATMs fail to respond to M2-polarizing stimuli, an increase of pro-inflammatory cytokines, such as TNF and IL-1 $\beta$ , has been observed. [10, 11]. The M2 phenotype of ATMs is maintained by a number of immune cells, as well as by adipocytes. IL-4 has been shown to be an important cytokine that

drives M2 polarization [10]. In adipose tissue, eosinophils are the dominant source of IL-4. Deficiency of these cells in  $\Delta$ dblGATA mice, or hyper-eosinophilia in IL-5 transgenic animals, has been shown to result in reduced or increased numbers of M2-like macrophages, respectively [11]. VAT-resident eosinophils depend on IL-5 for their survival, which is mainly produced by type 2 innate lymphoid cells (ILC2s) in this organ [12]. Elimination of any component of the ILC2/eosinophil/M2-ATM axis has been shown to result in an increase in pro-inflammatory cytokines in VAT and increased sensitivity to obesity-induced development of insulin resistance, making this particular axis one of the dominant regulatory mechanisms of adipose tissue homeostasis [11–13].

Invariant-chain natural killer T (iNKT) cells represent a second immune cell population which sustains M2 ATMs in VAT (Fig. 1). These cells are present at relatively high frequency in lean fat and recognize lipid antigens in the context of CD1d [14]. Lack of iNKT cells as a result of  $V\alpha$ 18 or CD1d deficiency in mice leads to reduced adipose tissue levels of IL-4 and IL-13, as well as to increased pro-inflammatory ATM numbers (reviewed in [15]). The role of iNKT cells may change in response to obesity, as it has been reported that these cells can also promote insulin resistance following DIO [15]. The exact mechanism via which iNKT cells inhibit adipose tissue inflammation under homeostasis, yet promote it in models of DIO, has yet to be resolved.

Regulatory T (Treg) cells represent a second T-cell subset that is directly involved in the inhibition of adipose tissue inflammation. CD4<sup>+</sup> T cells are the most predominant T cells in adipose tissue and compared to other tissues, a very large fraction of these is Foxp3-positive, which was shown to depend on the cytokine IL-33 [16] and the nuclear receptor PPAR- $\gamma$  [17]. Regulatory T cells in VAT repress immune cell activation through production of the anti-inflammatory cytokine IL-10 [18]. Experimental ablation of Treg cells by injection of diphtheria toxin in mice expressing the diphtheria toxin receptor under the Foxp3 promoter was shown to result in an increase of pro-inflammatory cytokines such as TNF, IL-6, and RANTES in fat [18]. Importantly, elimination of Treg cells acutely reduced insulin sensitivity in these animals [18], whereas transfer of Treg cells into T-cell-deficient animals improved insulin sensitivity upon DIO [19].

In addition to immune cells, adipocytes and adipose stroma contribute to tissue homeostasis. Adipose tissue excretes a number of factors, generally referred to as adipokines, which play an important role in the regulation of systemic metabolism [20]. Many of these factors share homologies with cytokines and have profound impact on immune cell behavior. One of the most well-characterized adipokines is adiponectin (Fig. 1). This molecule shares functional homology with insulin, and has been shown to impair gluconeogenesis in the liver and promote glucose uptake [21]. In addition, adiponectin has a strong anti-inflammatory effect [22]. In vitro stimulation assays showed that the adiponectin receptor is expressed at relatively high levels on M2 macrophages, whereas M1 polarization results in its downregulation, explaining the stronger effect of adiponectin on the former ATM subset [22]. Adiponectin inhibits NF- $\kappa$ B activation and promotes IL-10 and IL-1Ra production by macrophages. Moreover, adiponectin

suppresses TLR4 signaling [22], which has been shown to be important for diet-induced insulin resistance [23].

## Immunological control of adipose tissue structure

Various immune cell subsets have been implicated in the control of adipose tissue remodeling. Kit<sup>W-sh</sup> mice, which lack mature mast cells, have been shown to have strikingly less adipose tissue mass than wild-type animals [24]. In addition, Kit<sup>W-sh</sup> mice show reduced adipose tissue expansion upon feeding with a high-fat diet, when compared with that in wild-type controls [24]. Mast cells were shown to promote microvessel growth by excreting IL-6 and IFN- $\gamma$ , which appear to be essential for healthy adipose tissue formation and expansion [24]. In contrast, mice deficient for IL-17 demonstrated increased adipose tissue mass gain in response to HFD [25]. A normal, low-fat diet did not result in altered adipose tissue mass in these animals, suggesting that adipogenesis induced by the abundant presence of nutrients differs from basic ontogenesis of adipose organs. Immune cell derived stimuli are also capable of inhibiting adipogenesis. IL-5 transgenic animals, which suffer from eosinophilia, have been shown to have reduced adipose tissue mass, whereas  $\Delta$ dblGATA mice, which lack eosinophils, have larger fat pads than wild-type animals, both under lean and obese conditions [11]. Deletion of iNKT cells, as a result of genetic deficiency for CD1d or  $J\alpha$ 18, results in an increase of adipose tissue mass only after high-fat feeding [26, 27]. The mechanisms via which these cells control white adipose tissue proliferation are currently unclear.

A cytokine of particular interest for adipose tissue homeostasis, because it affects both immune cell behavior and adipose tissue remodeling, is IL-33 [28]. IL-33 is a member of the IL-1 superfamily and binds to the receptor ST2, which is highly expressed on mast cells, Th2 CD4<sup>+</sup> T cells, and ILC2s [29]. Under homeostatic conditions IL-33 is mainly expressed by epithelial cells and tissue stroma [30]. Upon infection, IL-33 expression is highly induced in many tissues and it was therefore originally classified as a pro-inflammatory mediator which drives anti-helminth immune responses [29]. Under noninflammatory conditions, IL-33 sustains type 2 immune cells, including ILC2s and M2 macrophages, in order to maintain tissue homeostasis (reviewed in [29]). In VAT, IL-33 is abundantly expressed in adipose tissue stroma, predominantly by endothelial cells and fibroblast-like reticular cells [29, 30]. Adipose tissue Treg cells express high levels of ST2 and deficiency of this receptor results in a specific loss of Treg cells in fat, but not in other organs [16]. IL-33 also plays a role in the maintenance of adipose tissue eosinophils, as blocking of ST2 was shown to result in a significant reduction in the number of these cells, independently of ILC2s [31].

Of particular interest is the effect of IL-33 on ILC2s. Exogenous administration of IL-33 increases the number of ILC2s in VAT, with a concomitant increase of IL-5 levels and eosinophil cell numbers [12]. Strikingly, in response to IL-33, ILC2s regulate adipocyte phenotype and function. For example, IL-33 deficiency

results in increased body mass and increased formation of glucose intolerance in response to high-fat feeding, due to a lack of Type 2 ILCs [16, 32]. Moreover, 'Brite' (Brown in white) cell numbers, which are UCP1-expressing adipocytes in white fat with a brown adipocyte phenotype, were shown to be decreased in these animals [32, 33]. Two recent studies have shown that IL-33 activates ILC2s to produce IL-13 and the endogenous opioid Met-enkephalin, which drives adipocyte precursors to differentiate into Brite cells, a process also known as 'beiging' [32, 33]. Future studies are required to show whether other cells that produce IL-13 in fat, such as iNKT cells, are also capable of skewing pre-adipocyte differentiation. Nevertheless, since the role of IL-33 in beiging is most prominent in subcutaneous fat, it comprises most likely a different biological mechanism than its anti-diabetic and anti-adipogenic effects on VAT. In addition, the biological significance of beiging requires further study, as it is currently unclear whether it protects against development of insulin resistance following DIO.

### Reaching critical mass: obesity-driven immune cell accumulation in VAT

The early phases of DIO in visceral adipose fat are characterized by an increase in the amount of fat per adipocyte and by an accumulation of immune cells which initially are of limited inflammatory capacity. The most commonly used animal model for the induction of obesity and obesity-induced insulin resistance is by feeding animals, usually mice, with a high-fat diet (HFD). Within the first weeks after start of HFD feeding, mice accumulate neutrophils, macrophages, and NK cells in VAT. Data showing the biological relevance of these cells in VAT will be discussed below.

#### Neutrophils

Already in the first days after initiation of HFD feeding in mice, neutrophil numbers rapidly increase in adipose tissue and produce the proteolytic enzyme elastase [34]. Even after long term (>3 months) HFD feeding, however, neutrophils remain a minor fraction of adipose tissue leukocytes. Nevertheless, elastase deficiency or chemical inhibition of elastase was shown to result in enhanced insulin sensitivity compared to wild-type mice upon 12 weeks of HFD feeding. In vivo administration of exogenous elastase resulted in acute reduction of insulin sensitivity in hepatocytes [34]. These experiments demonstrate the importance of elastase in metabolic disease, even though it is currently unclear how this enzyme contributes to adipose tissue inflammation. The immediate trigger for neutrophil infiltration into the VAT following DIO is also unknown. It has been shown in humans that acute lipid overload induces an inflammatory boost, as demonstrated by an increase of circulating MCP1 and C-reactive protein [35]. Alternatively, HFD is thought to induce acute changes in the adipose tissue microenvironment, such as alterations in oxygen consumption, following a brief surge in adipocyte precursor proliferation

[36]. This acute stress may be involved in recruiting neutrophils to this site in the first days following initiation of high-fat feeding.

#### Macrophages and NK cells

Compared with that of neutrophils, the increase in the numbers of adipose tissue macrophages and NK cells appears to be delayed, occurring in weeks, rather than days after the start of HFD feeding. Although neutrophils in VAT are thought to be primarily of peripheral origin [34], ATMs and NK cells appear to increase partially or completely through proliferation of tissue-resident populations [7, 37, 38]. When mice fed an HFD were intravenously injected with labeled NK cells, tracing of the labeled cells demonstrated that only a small number of NK cells reach the adipose tissues from the periphery. In contrast, BrdU labeling, as a marker of proliferation, was significantly increased in adipose-tissue resident NK cells, but not in splenic NK cells following HFD [7]. This suggests that the increase in NK cells in VAT is due to proliferation of the tissue-resident population rather than influx from the periphery. The origin of increased macrophage populations in the VAT in response to HFD appears to be more complex. One study demonstrates that local proliferation of these cells is the dominant source of cellular increase [38]. Other studies, in which the capacity of macrophages to respond to chemo-attractants was blocked, showed reduced macrophage cell numbers in VAT due to decreased peripheral influx [37, 39]. Possibly, both depots contribute to the ATM pool in the case of chronic inflammation in VAT.

#### Regulatory cells: ILC2s, Treg cells, and eosinophils

In addition to the accumulation of pro-inflammatory cells in VAT, DIO is also associated with a decrease in anti-inflammatory immune cells. In humans and mice, ILC2s were shown to be reduced in adipose tissue of obese individuals, both relative to the total number of leukocytes and per gram of adipose tissue [32]. This decrease appears to involve the inhibition of ILC2s to respond to IL-33 in the presence of the type 1 cytokine IFN- $\gamma$  [40]. Similarly, Treg cells decrease in number per gram of fat following DIO, and especially relative to the number of effector CD8<sup>+</sup> and CD4<sup>+</sup> T cells. The relative inhibitory capacity of this cell population therefore decreases in response to DIO, which was shown to be an important contributor to VAT inflammation [16, 18, 19]. Finally, eosinophils also decrease in number following DIO, possibly as a result of the decreased numbers of ILC2s, which produce the IL-5 required for the maintenance of this population [11, 12]. Thus, the regulatory capacity of inhibitory immune cell subsets in VAT decreases due to a relative decrease in the contribution of these cells per gram of fat and as a percentage of the overall immune cell pool.

## Adipose tissue

The crucial trigger for the increase of immune cells in adipose tissue in response to DIO is still unclear, but is likely to be derived from adipocytes. Via lineage tracing of adipocyte precursors, it was shown that the increase in adipose tissue mass early after HFD feeding is predominantly the result of adipocyte hypertrophy [36, 41]. Despite an early surge in pre-adipocyte proliferation, only prolonged exposure to HFD (> 4 weeks) increases mature adipocyte cell numbers (hyperplasia) [36, 41]. With the increase of adipose tissue mass, several changes occur in adipokine production. Most notably, adiponectin production drops, which results in decreased glucose uptake and reduced anti-inflammatory regulation of the local tissue environment [22]. Instead, adipocytes start producing more leptin. Leptin affects the hypothalamus, where it triggers satiety signals, thereby directly inhibiting the effects of ghrelin, a hormone produced in the gut that stimulates hunger [22]. Leptin levels in the blood closely correlate with the amount of adipose tissue mass, both in humans and mice [42]. In addition to its central effects, leptin has a profound effect on the immune system. The leptin receptor, also known as LEP-R or CD295, is expressed on most immune cells including neutrophils, macrophages, and NK cells. LEP-R shares structural homology with the IL-6 receptor and also signals through Stat3 [43]. Mice that are deficient for leptin (*ob/ob*) or for LEP-R (*db/db*) are obese due to increased food intake as a result of never feeling sated. In addition, they have a strong reduction in functional immune cells, such as (regulatory) T cells [44], NK cells, and dendritic cells (reviewed in [45]). Importantly, T cells and B cells have been shown to increase expression of LEP-R in response to activation [46]. Moreover, survival of activated lymphocytes was enhanced when leptin was added to the cell culture [46]. Increased leptin production by adipose tissue may be an important initial trigger for immune cell increase in VAT in response to obesity. Indeed, in the first weeks after start of HFD feeding, macrophage and NK-cell numbers and functionality are specifically increased in VAT, whereas they are reduced in *ob/ob* mice [7, 47]. Moreover, leptin has been shown to promote NK-cell survival in the bone marrow [48] and intravenous administration of leptin resulted in a specific increase of granulocytes, monocytes, and NK cells in the circulation of rats [49].

In addition to leptin, adipose tissue produces other adipokines that affect immune cell numbers in VAT, most notably monocyte chemo-attractant protein-1 (MCP-1) and IL-6 (Fig. 2). MCP-1 is a potent chemokine that recruits monocytes and its levels are increased in the serum of DM2 patients [50]. Mice deficient for MCP-1 have a significant reduction in adipose tissue inflammation and a delayed onset of insulin resistance in response to DIO [37]. The role of IL-6 in obesity appears to be more complex and shares parallels with IL-33; IL-6 is generally assumed to be a pro-inflammatory mediator and its levels have been shown to be significantly increased in obese individuals [51]. The IL-6 receptor comprises the gp130 and IL-6R subunits. Although IL-6 can bind directly to this receptor complex, IL-6 can also be bound to a soluble form of gp130 and be transpresented to cells that express only

the IL-6R subunit [52]. Transpresentation of IL-6 bound to gp130 has been shown to be crucial for the accumulation of macrophages in adipose tissue [53]. Paradoxically, IL-6-deficient mice develop late-onset glucose tolerance [54]. IL-6 has been shown to stimulate IL-4R expression on macrophages, thereby promoting M2 polarization of these cells [10], and macrophages express high levels of the IL-6 receptor. Therefore, similarly to IL-33, under homeostatic conditions IL-6 appears to function as an anti-inflammatory mediator by preventing M1 macrophage formation. In response to DIO, IL-6 levels increase [55], allowing other, pro-inflammatory cells to be activated by this cytokine. Therefore, the effects of IL-6 on the development of DM2 appear to be time- and concentration dependent.

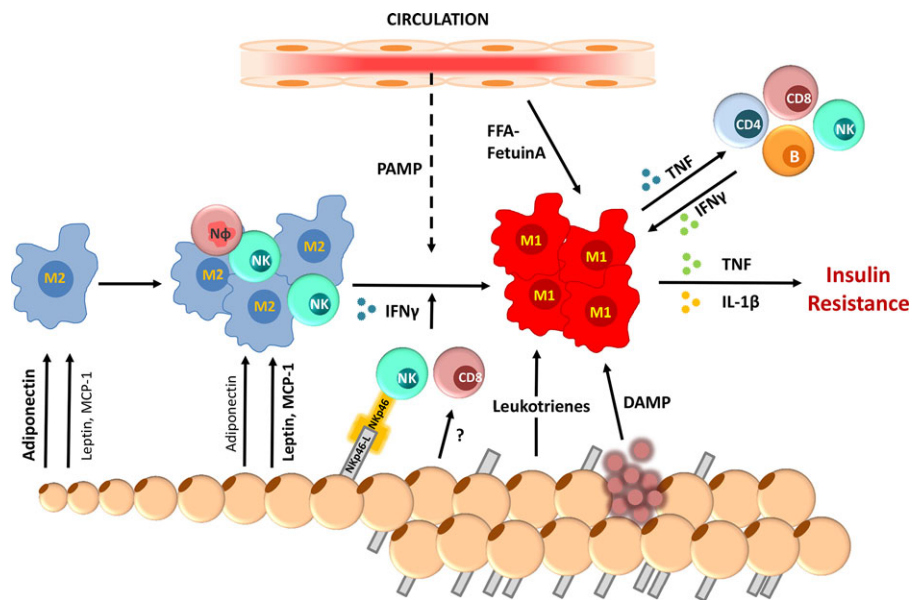
In addition to the factors mentioned above, there is an increasingly large number of adipokines that has been shown to mediate immune cell function in adipose tissue, such as resistin, visfatin, and apelin [56]. The exact role of these molecules in the promotion or inhibition of obesity-induced VAT inflammation and the development of insulin resistance is mostly unclear. However, the sheer number of immune-modulatory molecules derived from adipose tissue clearly illustrates the extensive communication of fat with its tissue-resident immune system.

## The “Big Bang”: Initiation of inflammation in VAT

Whereas the accumulation of immune cells predisposes an organ to tissue inflammation, in itself this local accumulation is insufficient for the activation of the immune system. A key event in the induction of obesity-induced VAT inflammation and development of insulin resistance appears to be the polarization of macrophages from an M2 to a pro-inflammatory M1-like state [9, 13]. In evidence of this, depletion of M1 macrophages in both humans and mice has been shown to cause significant improvement of systemic insulin resistance [13, 57]. Pro-inflammatory licensing of macrophages is therefore crucial for the development of VAT inflammation, as they appear to be the primary source of pro-inflammatory cytokines that are found in the circulation of subjects with diabetes [55]. In addition, macrophages potently recruit T cells via the production of chemokines such as CXCL16 [58], a molecule of which the expression is greatly increased in adipose tissue in response to obesity [59]. Thus, macrophages contribute both directly and indirectly to the excretion of pro-inflammatory cytokines.

## Systemic sources of immune activation

Various stimuli for M1 macrophage priming have been proposed, including free fatty acids (FFAs) and microbial components [23, 60]. FFAs are normal components of our food, but they are also the vehicle by which triacylglycerol stored in adipose tissue is transported through the bloodstream [61]. FFAs are used as a source of fuel by many tissues, including skeletal muscle and



**Figure 2.** Model for development of obesity-induced adipose tissue inflammation. In response to HFD, adipocytes initially become hypertrophic and later hyperplastic (indicated by increase in size and number of adipocytes, respectively). This is associated with a shift in adipokine production from adiponectin to leptin/MCP-1 and an increase in the number of ATMs, neutrophils, and NK cells in visceral fat. As obesity persists, adipocyte stress drives CD8<sup>+</sup> T-cell and NK-cell activation through NKp46, resulting in local production of IFN- $\gamma$ . Together with PAMPs coming from the periphery, this locally produced IFN- $\gamma$  licenses ATMs toward a pro-inflammatory M1 state. This makes these cells sensitive to a range of pro-inflammatory stimuli, such as leukotrienes, FFA-Fetuin-A complexes, and DAMPs from necrotic adipocytes. As a result, ATMs produce pro-inflammatory cytokines, such as TNF and IL-1 $\beta$ , and recruit more pro-inflammatory cells, including CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells, into the VAT to amplify the immune response. The chronic systemic presence of pro-inflammatory cytokines derived from this response ultimately contributes to the development of insulin resistance. M1: M1 Macrophage; M2: M2 Macrophage; N $\Phi$ : Neutrophil; NK: natural killer cell; IFN- $\gamma$ : interferon gamma; PAMP: pathogen-associated molecular pattern; FFA: free fatty acid; DAMP: danger-associated molecular pattern; TNF: tumor necrosis factor.

the brain [61]. Uncontrolled (i.e., nontreated) diabetes patients have increased amounts of FFAs in circulation [62]. Importantly, FFAs were shown to be able to stimulate TLR4 on macrophages, but only when bound to the protein carrier Fetuin-A [23, 63]. Palmitate, an FFA found in many food sources, including palm oil, cheese, and meat, has been shown to drive activation of the inflammasome, the protein complex responsible for the formation of pro-inflammatory cytokines IL-1 $\beta$  and IL-18 [64]. Recently, the role of FFAs as initiators of inflammation is being questioned. In large cohort studies of obese nondiabetic individuals, the levels of FFAs in the serum did not correlate with BMI [65, 66]. Importantly, meta-analysis shows that serum FFA levels are not predictive for the formation of DM2 [61]. FFA uptake from the circulation is controlled by insulin and the increase of FFA levels in the blood of uncontrolled DM2 patients is therefore thought to be a result of insulin resistance rather than its cause [61]. Indeed, well-controlled DM2 patients do not have higher serum levels of FFA than healthy subjects [61, 67]. Notably, *in vitro* studies demonstrate that FFAs can only induce pro-inflammatory cytokine excretion in macrophages when they are first primed with LPS or IFN- $\gamma$  [64]. Thus, before a free fatty acid can function as a pro-inflammatory mediator, polarization of ATMs is first required. This observation in turn triggered the hypothesis that obesity-induced gut dysbiosis drives adipose tissue inflammation, through the leakage of microbial components (or pathogen-associated molecular patterns (PAMPs)) into the blood stream (Fig. 2). Although large-

scale, metagenome-wide association studies on microbial gut DNA revealed only mild dysbiosis in the intestines of DM2 patients [68], several micro-organisms could indeed be associated with markers of metabolic syndrome [69]. Animal studies indicate that an HFD increases endotoxin levels in the circulation [60, 70]. However, this could only be achieved when food with a very high fat content was used, whereas more commonly used high-fat diets failed to do so [60]. Nevertheless, studies in humans indicate that HFDs acutely increase systemic endotoxin levels [71]. More importantly, a large prospective cohort study showed that systemic endotoxin levels are a risk factor for the development of DM2 [72]. Thus, there is clear evidence that systemic factors, especially diet-induced endotoxemia, play a role in the development of insulin resistance. Whether and how these factors affect chronic systemic inflammation has yet to be elucidated.

### Local sources of VAT inflammation

Systemic endotoxemia does not explain, however, why VAT macrophages specifically would be primed toward an M1 phenotype. Rather, a local trigger is required to initiate macrophage polarization in particularly this tissue. IFN- $\gamma$  is a potent inducer of M1 polarization [73]. Mice deficient for this cytokine have reduced M1 macrophage accumulation in the VAT and improved insulin sensitivity in response to HFD feeding [74–76]. Therefore,

obesity-induced IFN- $\gamma$  production has been suggested to be an important initiating event in VAT inflammation.

NK cells are the body's sentinels and browse tissues looking for signs of cellular stress, activating the immune system in response to viral infection or oncogenic transformation. NK cells are armed with a broad repertoire of activating and inhibitory receptors that recognize self-ligands on target cells. In the absence of inhibitory ligands ('missing self') and/or in the presence of activating ligands ('induced self'), NK cells are triggered to induce a cytolytic response, or produce cytokines such as IFN- $\gamma$  [77]. In several models, NK cells have been shown to drive M1 macrophage polarization through the production of IFN- $\gamma$  [73, 78].

Hypertrophy is a well-known stress factor for adipocytes, leading to micro-hypoxia, ER stress, and extracellular matrix confinement [79, 80]. Indeed, we have observed that in response to HFD feeding, murine adipocytes of the visceral fat induce expression of stress ligands which bind to the activating NK-cell receptor Nkp46 (NCR1 in mice) (Fig. 2) [7]. Early after initiation of HFD feeding, the number of IFN- $\gamma$ -producing cells increases, and in particular the number of NK cells [7]. Antibody-mediated depletion of NK cells or deficiency of NCR1 in mice was shown to result in a significant reduction of M1-polarized ATMs upon HFD feeding, whereas the total number of ATMs was not greatly affected [7, 81]. Furthermore, the development of diet-induced insulin resistance could be delayed by preventing NK-cell activation using soluble NCR1 [7]. These findings are in line with several recent studies in humans and mice. VAT from obese humans shows a significant increase in the number of NK cells compared with that in subcutaneous fat, and these cells produce higher levels of IFN- $\gamma$  [76]. In addition, human adipocytes can stimulate NK cells to produce IFN- $\gamma$  *in vitro* [82].

In addition to NK cells, T cells are an important source of IFN- $\gamma$  in VAT [7, 19]. Both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell numbers strongly increase in response to DIO and ultimately become the most prevalent lymphocytes in VAT, albeit with delayed kinetics compared to NK cells [7, 83]. In the first weeks after DIO, CD4<sup>+</sup> T cells retain a T<sub>H</sub>2 phenotype and contribute to the inhibition of VAT inflammation [19]. CD8<sup>+</sup> T cells on the other hand, have been shown to recruit macrophages through the production of MCP-1 and their deletion in mice results in a significant decrease in systemic inflammation and insulin resistance upon DIO [84]. It is currently unclear whether CD4<sup>+</sup> and CD8<sup>+</sup> T cells are activated in VAT through engagement of their T-cell receptor (TCR), or whether they are recruited in a chemokine-dependent fashion, independent of TCR engagement. Obese, but not lean adipose tissue was shown to be able to induce proliferation of CD8<sup>+</sup> T cells *in vitro* [84]. However, whether this is also the way via which CD8<sup>+</sup> T-cell numbers increase in obese VAT *in vivo* remains to be investigated.

Finally, NKT cells can be a source of IFN- $\gamma$  in VAT. Whereas iNKT cells initially have an anti-inflammatory role in VAT through the production of IL-4 and IL-13, in response to HFD the number of TNF and IFN- $\gamma$ -producing NKT cells strongly increases [7, 83]. Whether and how these cells contribute to initiation of VAT inflammation is still a matter of debate, especially since people using the

same mouse models deficient for iNKT cells found opposing effects on the development of insulin resistance following DIO [14, 83].

In summary, the increase in macrophage cell numbers in VAT in response to HFD feeding is promoted by various factors, both derived from local and systemic sources. However, in order to achieve their full pro-inflammatory potential, licensing of ATMs by NK cells and CD8<sup>+</sup> T cells through immune cell derived cytokines such as IFN- $\gamma$  is required (Fig. 2). The observation that IFN- $\gamma$  deficient mice still develop insulin resistance in response to HFD feeding, though delayed compared to wild-type animals, also indicates that this cytokine is not the only factor that drives VAT inflammation [75].

An additional layer of complexity has recently been added with the surprising observation that recruitment of pro-inflammatory macrophages in VAT is not absolutely required for the development of obesity-induced insulin resistance. Mice in which IL-6 *trans* signaling was impaired showed reduced recruitment of macrophages into the VAT, but still developed glucose intolerance [53]. Possibly, redundancy between pro-inflammatory mediators ensures recruitment of pro-inflammatory cells to the VAT even in the absence of macrophages.

## Inflation of inflammation in the VAT

The later stages of adipose tissue inflammation are well studied and have been elaborately described in excellent reviews [2, 15, 85]. Therefore, we will describe this phase only briefly here. Priming of macrophages in adipose tissue makes them susceptible to a range of pro-inflammatory stimuli that are associated with obesity, described below. In response to obesity, adipocytes induce expression of enzymes that convert arachidonic acid into pro-inflammatory mediators, including leukotrienes [39, 86]. Deficiency in key components of leukotriene biology has been shown to prevent cytokine secretion by macrophages and obesity-induced insulin resistance [39, 86]. In addition, obesity has been shown to induce necrotic cell death of adipocytes as a result of micro-hypoxia [79]. Necrosis results in the release of danger-associated molecular patterns (DAMPs), such as double-stranded DNA and heat shock proteins, which drives macrophages to produce pro-inflammatory cytokines [87]. Advanced obesity is therefore characterized by adipocytes in VAT that are surrounded by a ring of pro-inflammatory macrophages, known as crown-like structures [88]. In addition, priming makes murine macrophages susceptible to activation by FFAs [63]. These pro-inflammatory stimuli drive the activation of the JNK and NF- $\kappa$ B signaling cascades and of the inflammasome in macrophages [87, 89, 90]. The inflammasome is a protein complex, which converts pro-IL-1 and pro-IL-18 into IL-1 $\beta$  and IL-18, both potent activators of the immune system [91]. Either genetic deficiency or chemical inhibition for components of the JNK, NF- $\kappa$ B, or inflammasome pathways has been shown to strongly reduce obesity-induced insulin resistance in humans and mice [87, 89, 90].

The activation of macrophages in VAT and the generation of an inflammatory environment in this tissue subsequently drives the



recruitment of a plethora of pro-inflammatory cells, mostly with a  $T_H1$  signature.  $T_H1$  effector CD4 T cells, CD8 T cells, and B cells most strongly increase during the later stages of adipose tissue inflammation [2, 15, 19, 85]. Not only are these cells important sources of IFN- $\gamma$  that further drive ATM polarization, they also contribute to chronic systemic inflammation by producing pro-inflammatory cytokines such as TNF (Fig. 2). Ultimately, it is the chronic leakage of these pro-inflammatory cytokines into the blood stream that forms a strong risk factor for the development of systemic insulin resistance by dampening the signaling capacity of the insulin receptor in local tissues [3].

## Conclusions

Although the immunological complexity of VAT inflammation is becoming increasingly clear, many questions remain unanswered. Subcutaneous adipose tissue is comparable in many aspects to its visceral counterpart, including the capacity to produce leptin and adiponectin [92], yet it accumulates much less pro-inflammatory macrophages in response to DIO [93]. Adipose tissue stroma is an important source of adipokines and has been shown to activate CD8<sup>+</sup> T cells in HFD-fed animals [84, 92]. Therefore, differences between the connective tissues that support visceral and subcutaneous adipocytes are likely to be important. In addition, the chronic systemic presence of pro-inflammatory mediators cannot in itself explain the development of insulin resistance. Although some inflammatory diseases, such as psoriasis, ulcerative colitis, and systemic vasculitis are associated with an increased risk for developing DM2, others, such as Crohn's disease, inflammatory arthritis, and systemic autoimmune disorders (e.g., SLE, scleroderma) do not [94]. Clearly, communication between metabolic and immunological regulators is required for the development of DM2.

Finally, a better understanding of the underlying mechanisms behind adipose tissue inflammation holds great promise as a target for future therapeutic intervention. Current clinical practice mainly focuses on the reduction of blood glucose levels in DM2 patients [95], which is a consequence of insulin resistance, whereas obesity-induced VAT inflammation is crucial for the development of this disease. Several phase II and III clinical trials have been initiated to inhibit key immunological processes of VAT inflammation in DM2 patients, such as NF- $\kappa$ B signaling, IL-1 $\beta$  function, or arachidonic acid metabolism, with promising results [85]. Thus, targeting of inflammatory processes in VAT of obese patients appears to be a promising future strategy for prophylaxis against diabetes development.

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