



## Review

## Unravelling metabolic factors impacting iNKT cell biology in obesity

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## ABSTRACT

Obesity and related diseases have reached epidemic proportions and continue to rise. Beyond creating an economical burden, obesity and its co-morbidities are associated with shortened human life expectancy. Despite major advances, the underlying mechanisms of obesity remain not fully elucidated. Recently, several studies have highlighted that various immune cells are metabolically reprogrammed in obesity, thereby profoundly affecting the immune system. This sheds light on a new field of interest: the impact of obesity-related systemic metabolic changes affecting immune system that could lead to immunosurveillance loss. Among immune cells altered by obesity, invariant Natural Killer T (iNKT) cells have recently garnered intense focus due to their ability to recognize lipid antigen. While iNKT cells are well-described to be affected by obesity, how and to what extent immunometabolic factors (e.g., lipids, glucose, cytokines, adipokines, insulin and free fatty acids) can drive iNKT cells alterations remains unclear, but represent an emerging field of research. Here, we review the current knowledge on iNKT cells in obesity and discuss the immunometabolic factors that could modulate their phenotype and activity.

## 1. Introduction

Obesity has become a major public health problem as it reaches worldwide epidemic proportions. In 2022, over 2.5 billion adults were overweight, including 890 million obese [1]. Obesity is associated with life-threatening diseases such as type 2 diabetes, cardiovascular diseases and cancer [2]. Beyond creating an economical burden, obesity and its co-morbidities are associated with shortened human life expectancy [3]. Now more than ever, it is critical to understand the underlying mechanisms to prevent obesity-associated pathologies.

This disease is associated with insulin resistance, hyperglycemia, dyslipidemia and hypertension, which together are termed “metabolic syndrome”. A systemic chronic low-grade inflammation characterized by increased concentrations of pro-inflammatory cytokines such as tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and IL-6 is also observed in abdominal obesity and may have a role in the pathogenesis of obesity-related metabolic disorders [4]. This inflammation is caused,

among others, by an expansion and infiltration of pro-inflammatory macrophages in adipose tissue and a loss of regulatory immune cells, such as invariant Natural Killer T (iNKT) cells and Regulatory T cells (Treg) [4–10]. The immunometabolic impact of obesity on immune cells has been well illustrated by Michelet et al. (2012) [11]. They demonstrated that Natural Killer (NK) cells from obese patients are metabolically paralyzed and have an altered cytotoxic function. This alteration is due to a lipid accumulation in NK cells that interferes with their normal intrinsic metabolism, essential for their function. This results in immunosurveillance impairment and increases the risk of tumor growth in obesity [11]. More recently, many other studies have reported altered repartition and/or immune dysfunction of unconventional T-cells (e.g.,  $\gamma\delta$  T cells, iNKT cells and Mucosal-Associated Invariant T cells) in obesity [8,12]. These studies suggest a metabolic reprogramming of various immune cells in obesity that may profoundly affect immune system, shedding light on a new field of interest: the impact of obesity-related systemic metabolic changes affecting immune system that could

**Abbreviations:**  $\alpha$ GC,  $\alpha$ -galactosylceramide; AMPK, AMP-activated protein kinase; APCs, antigen presenting cells; ApoE, Apolipoprotein E; AT, adipose tissue; ATMs, AT-macrophages; BMI, body mass index; DCs, dendritic cells; E4BP4, E4-binding Protein 4; ER, endoplasmic reticulum; FA, fatty acid; FFAs, free fatty acids; HDL, high-density lipoprotein; HFD, high-fat diet; IFN- $\gamma$ , interferon gamma; IL, interleukin; iNKT, invariant Natural Killer T; IRE1 $\alpha$ , inositol-requiring enzyme 1 $\alpha$ ; LDL, low-density lipoprotein; LDLR, LDL receptor; MHC, Major histocompatibility complex class; NK, Natural Killer; PBMcs, peripheral blood mononuclear cells; PERK, protein kinase RNA-like ER kinase; PLZF, promyelocytic leukemia zinc finger; SFAs, Saturated fatty acid; TCR, T-cell receptor; TNF- $\alpha$ , tumor-necrosis factor- $\alpha$ ; UPR, unfolded protein response; VLDL, very-low-density lipoprotein.

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lead to immunosurveillance loss. However, the underlying mechanisms of immune dysregulation related to obesity are not fully elucidated. In this review, we decided to focus on the current knowledge on iNKT cells in obesity and discuss the immunometabolic factors that could modulate their phenotype and activity.

## 2. iNKT cells at the edge between innate and adaptive immunity

### 2.1. iNKT cells and their specific features

Discovered in the nineties, Natural Killer T (NKT) cells are nowadays considered as unconventional T cells, together with Mucosal-Associated Invariant and  $\gamma\delta$  T cells [13,14]. NKT cells display both T cells and NK cells features but the main characteristic of these cells is their ability to recognize (glyco-)lipid antigens, presented by CD1d molecule, via their specific T-cell receptor (TCR) [15,16]. NKT cells are divided into two main subsets based on their TCR diversity and antigen reactivity: (1) type I NKT cells, also known as iNKT cells and (2) type II NKT cells or variant NKT (vNKT) cells. While type I NKT cells have semi-invariant TCR, type II NKT cells have a more diverse TCR repertoire. Indeed, in mouse, the type I or invariant NKT TCR combines the canonical  $\alpha$ -chain V $\alpha$ 14-J $\alpha$ 18 paired with a limited repertoire of V $\beta$ -chains (V $\beta$ 8.1, V $\beta$ 8.2, V $\beta$ 8.3, V $\beta$ 7, or V $\beta$ 2). In human, this TCR has even more limited variability as it is composed by the  $\alpha$ -chain V $\alpha$ 24-J $\alpha$ 18 with the unique V $\beta$ 11 chain. However, the main difference between type I and type II NKT cells relies on the ability of type I, but not type II, to recognize  $\alpha$ -galactosylceramide ( $\alpha$ GC) [17]. These differences complicate the identification of type II NKT cells making their study more challenging and focusing efforts on the study of type I / invariant NKT cells.

Even if iNKT cells represent only a small proportion of T cells, they can regulate the function of multiple immune cells through cytokines and chemokines secretion. Therefore, iNKT cells play an important role in immune regulation and immunosurveillance, as they bridge innate and adaptive immunity [18,19]. Hence, iNKT cells have been implicated in several pathological conditions such as in cancer, autoimmune, metabolic and allergic diseases, and in various infectious conditions [16,20–23].

### 2.2. iNKT cells subsets

iNKT cells can be divided into 3 main subsets: NKT1, NKT2 or NKT17 in mice, also known respectively as Th1, Th2 and Th17 in humans (Fig. 1). This subdivision is based on cytokines production bias and transcription factors expression [15,24]. For example, T-bet<sup>+</sup> is exclusively expressed by NKT1/Th1 iNKT cells which also express low level of promyelocytic leukaemia zinc finger (PLZF). Upon activation, NKT1/Th1 iNKT cells mainly produce the Th1 cytokines: interferon gamma (IFN- $\gamma$ ) and TNF- $\alpha$ . However, iNKT cells have the uncommon ability to produce Th1 and Th2 cytokines at the same time [24]. Thereby, this subset is also able to produce low levels of the Th2 cytokine, such as IL-4. On the contrary, NKT2/Th2 iNKT cells are not characterized by a specific transcription factor but this subset expresses the highest levels of PLZF and GATA-binding protein 3 (GATA-3). Once activated, those cells secrete high levels of IL-4 and also low levels of IFN- $\gamma$ . Besides that, only the NKT17/Th17 iNKT cells express retinoic acid receptor-related orphan receptor- $\gamma$ t (ROR $\gamma$ t<sup>+</sup>) and mainly produce IL-17 upon stimulation [15,24–26]. Interestingly, other subsets can uniquely be found at the periphery, such as iNKT regulatory cell (iNKTreg), iNKT follicular helper cells (iNKT<sub>FH</sub>) and adipose tissue (AT)-resident iNKT cells. Here, we will only describe the latter but the other subsets have been reviewed elsewhere [27].

### 2.3. iNKT cells distribution

After their maturation in the thymus involving a positive selection with lipid antigen, iNKT cells migrate to lymphoid or non-lymphoid tissues where only a small fraction recirculates. Thus, their frequency in tissues is clearly higher than in blood [15,28]. iNKT cells are therefore often considered as non-circulating tissue resident cells. In mouse, iNKT cells are mostly found in the liver, adipose tissue, lung, intestine, bone marrow, thymus, spleen and the peripheral blood. They represent approximately 0.5 % of the T cell population in peripheral blood, 2.5 % in the spleen, 15–20 % in the visceral adipose tissue and up to 40 % in the liver [15,18,28]. In human, most studies on iNKT cells are carried out on peripheral blood mononuclear cells (PBMCs), because PBMCs are easily available. The frequency of iNKT cells in human peripheral blood ranges from undetectable level to more than 1 % of total iNKT cells pool but accounts on average for 0.01 %-0.2 % of T-cells [15,29]. The huge

	Found in thymus and in peripheral tissues			Only found in peripheral tissues
	iNKT Th1/NKT1	iNKT Th2/NKT2	iNKT Th17/NKT17	AT-iNKT/NKT10
<b>Preferential location</b>	Liver Spleen	Lungs Intestines	Lymph nodes Lungs Skin	Only in adipose tissues
<b>Key cytokine</b>	INF- $\gamma$	IL-4	IL-17A	IL-10
<b>Other major cytokines produced</b>	TNF- $\alpha$ ; IL-4	IL-10; IL-13; (INF- $\gamma$ )	IL-21; IL-22; IL-4; IL-13	IL-4; IL-13; IL-17; IL-2; INF- $\gamma$ ; TNF- $\alpha$
<b>Transcription factors</b>	PLZF <sup>low</sup> GATA-3 <sup>low</sup> T-bet <sup>pos</sup>	PLZF <sup>high</sup> GATA-3 <sup>high</sup>	PLZF <sup>int</sup> GATA-3 <sup>low</sup> ROR $\gamma$ t <sup>pos</sup>	PLZF <sup>neg</sup> E4BP4 <sup>pos</sup>

**Fig. 1.** Characteristics of the major iNKT cells subsets. Created with BioRender.com. iNKT cells: invariant natural killer T cells; AT-iNKT: adipose tissue-resident iNKT cells; E4BP4: E4-binding Protein 4; GATA3: GATA-binding protein 3; IFN- $\gamma$ : interferon- $\gamma$ ; IL-: Interleukin-; PLZF: promyelocytic leukaemia zinc finger; T-bet: T-box transcription factor TBX21; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ .

inter-patient variation of their frequency results from different factors such as sex and age. Indeed, Singh et al. (2022) demonstrated that peripheral blood iNKT cells are less abundant in men than in women but also that their frequency in peripheral blood is negatively correlated with the age [30]. Apart from blood, human iNKT cells are predominantly found in the visceral adipose tissue, thymus, liver and spleen. However, their frequency in these human organs is ten-fold lower than in mice [15,28].

#### 2.4. Lipid antigens and CD1d restriction

The main feature of iNKT cells is to recognize lipid antigens, only if they are presented by the CD1d molecule. CD1d is a highly conserved non-polymorphic Major histocompatibility complex class (MHC)-I homolog [16,31]. It presents lipids as antigens, instead of classical peptides, to unconventional T-cells which are not restricted to classical MHC [35]. CD1d is constitutively expressed by antigen presenting cells (APCs) like monocytes/macrophages, B cells, dendritic cells (DCs) and granulocytes but also cortical thymocytes (essential for iNKT development) [15,16]. However, its expression appears to not be restricted to hematopoietic cells but is also observed on adipocytes, intestinal epithelial cells and hepatocytes [32–34].

##### 2.4.1. Lipid loading and recycling on CD1d molecule

CD1d molecule is a heterodimer composed of two  $\alpha$ -helices that binds non-covalently to  $\beta$ 2-microglobulin. This transmembrane protein contains two highly hydrophobic pockets (named A' and F') which allow the anchor of lipid antigen hydrophobic chains.

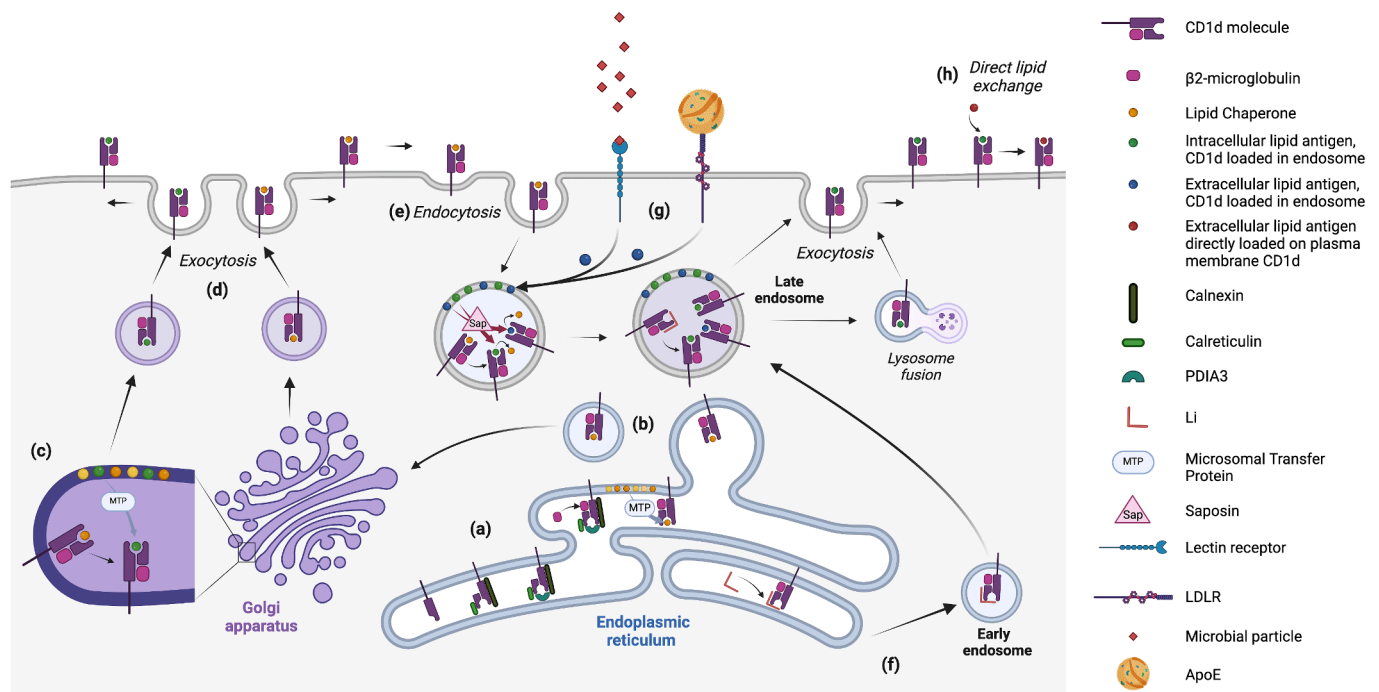
Similar to MHC-I, the synthesis, folding of CD1d molecules and

assembly with  $\beta$ 2-microglobulin occurs in the lumen of endoplasmic reticulum (ER) and is facilitated by ER chaperones, (i.e., calnexin, calreticulin and protein disulfide-isomerase A3 (PDIA3)) (Fig. 2). CD1d chains are also stabilized by phospholipids derived from the ER or Golgi membrane, considered as lipid chaperones. Loading of lipid chaperone onto CD1d is mediated by the microsomal transfer protein (MTP). After this step, properly folded CD1d containing lipid is ready to traffic through endosomal compartments to reach the plasma membrane. Importantly, CD1d is highly recycled by internalization followed by endosome and lysosome fusion mediated by adaptor protein complex [34–38]. Interestingly, CD1d can directly reach endo-lysosomal compartment from ER, possibly via an alternative pathway involving MHC class II-associated invariant chain (Ii) (Fig. 2) [39].

Exogenous lipids are endocytosed after binding to extracellular lipid carrier proteins such as Apolipoprotein E (ApoE) or lectin receptors (e.g., mannose receptor) (Fig. 2) [34,37,40,41]. Of note, before loading onto CD1d, some lipids have to be processed into lipid antigens by enzymes, like lipid hydrolases. Then, endogenous and/or exogenous lipid antigens are loaded onto CD1d in endosomes where this process is facilitated by low pH and the presence of lipid transfer proteins, such as GM2 activator (GM2A), saposins A-D (Sap) and Niemann-Pick type C protein (Fig. 2) [42]. Once loaded with new endogenous/exogenous lipid antigen, CD1d travels back to the cell surface. Of note, although most of iNKT cells-reactive lipid antigens are loaded onto CD1d in the endocytic compartment, some lipid antigens can be directly loaded at the cell surface (Fig. 2) [34,36].

##### 2.4.2. Lipid antigens diversity

While a controversy arose about endogenous lipid antigen able to



**Fig. 2.** CD1d trafficking and lipid loading. (a) CD1d heavy chains are folded in the lumen of endoplasmic reticulum (ER) via ER chaperones: calnexin, calreticulin and protein disulfide-isomerase A3 (PDIA3). Then, folded CD1d chains are assembled with  $\beta$ 2-microglobulin. Thanks to microsomal transfer protein (MTP), CD1d chains can also be stabilized by lipid chaperones derived from the membrane of the (b) ER or (c) Golgi. (d) Properly folded CD1d containing lipid traffics through endosomal compartments to reach the plasma membrane. (e) CD1d is recycled by internalization followed by endosomal and lysosome fusion. (f) CD1d can directly reach endo-lysosomal compartment from ER, possibly via an alternative pathway involving MHC class II-associated invariant chain (Ii). (g) Lipids are transported from extracellular environment to endocytic compartments via lipid carrier protein and lipoprotein receptors or lectin receptors. These extracellular lipids contained in endocytic compartments are able to supersede ER-derived lipids already loaded on CD1d. The lipid antigen exchange on CD1d is facilitated by ER-resident lipid transfer proteins, such as saposins (SAP). Once loaded with new endogenous/exogenous lipid antigen, CD1d travels back to the cell surface. (h) Although majority of iNKT cells-reactive lipid antigens are loaded onto CD1d in endocytic compartment, some lipid antigens can be directly loaded at the cell surface. Created with [BioRender.com](https://BioRender.com). ApoE: Apolipoprotein E; LDLR: LDL receptor.

activate iNKT cells, the first unquestioned exogenous lipid antigen described in the literature was the  $\alpha$ GC. Discovered in a marine sponge,  $\alpha$ GC is actually derived from *Sphingomonas* bacteria that colonize those marine sponges. Since that discovery, several exogenous and endogenous lipid antigens have been found but  $\alpha$ GC remains one of the most used antigens to activate iNKT cells due to its potency [15,19].

Most iNKT cells-reactive lipid antigens show a similar structure characterized by a polar head and two alkyl chains which both insert into one of the two hydrophobic pockets of CD1d inducing a conformational change. Most of them belong to three categories: glycosphingolipids, diacylglycerols and phospholipids [16,19,34]. A large part of known lipid antigens is derived from bacteria wall, as  $\alpha$ -galacturonosylceramide,  $\alpha$ -glycuronylceramides and diacylglycerol. At the opposite, endogenous lipid antigens and mechanisms leading to their generation are not fully understood. However, some candidates have already been identified and include (i) glycosphingolipids like isoglobotrihexosylceramide (iGb3) and ganglioside D3 (GD3), and (ii) glycerophospholipids such as phosphatidylinositol (PI), phosphatidylethanolamine (PE) and lyso-phosphatidylcholine (lyso-PC) [31,34,43].

The chemical structure of lipid antigens (acyl chains, head groups) and their physical properties (i.e., hydrophobicity, polarity) influence the response of iNKT cells both at qualitative and quantitative levels. By designing  $\alpha$ GC analogs to elicit different iNKT cells responses, several research groups demonstrated that the loading mechanism of lipid antigens onto CD1d depends on their structure and can predict cytokine output [44,45]. Glycosphingolipids such as  $\alpha$ GC are endocytosed after binding to extracellular lipid carrier proteins such as ApoE and loaded onto CD1d in endosomes. These intracellularly produced CD1d–lipid complexes are translocated to lipid raft microdomains on the plasma membrane and activate a strong and prolonged iNKT cell responses characterized by a mixture of Th1- and Th2-type cytokines. However, certain  $\alpha$ GC analogs featuring more polar or less hydrophobic N-acyl substitutions can be directly loaded onto CD1d from the extracellular compartment and therefore do not require loading into the endosome. Such complexes are rapidly dissociated in the endosome and do not reach lipid rafts, leading to Th2-type transient activation.

Beyond lipid loading mechanism, the chemical structure of the lipid antigen also influences their nesting in CD1d and the subsequent iNKT cells response. Indeed, the lipid tail of lipid antigens is enclosed inside CD1d molecule since CD1d inner surface is covered by hydrophobic residues [34]. At the other extremity, the polar moiety (i.e., sugar head) of the lipid protrude out to be available for interactions with iNKT cells TCR. Thus, the length of the sugar head could modulate affinity of iNKT cells for lipid antigen and the subsequent response [18,34]. Moreover, all CD1d-loaded lipid antigens do not perfectly fit with optimal stimulating capacity. The strength of lipid antigenicity depends on the stability of its interaction with CD1d. The composition of the lipid antigen tail (length and saturation) determines the kind of interaction, with a longer tail resulting in a more sustained and stable interaction. Transient CD1d–TCR interactions can lead to the secretion of IL-4 (Th2), while a sustained interaction will likely induce both IL-4 and IFN- $\gamma$  production (Th1 and Th2) [46].

## 2.5. iNKT cells activation modes

One key concept to understand the iNKT cells plasticity is that these cells are context-dependent responder. Obviously, TCR engagement can activate these cells but they can also react to an inflammatory context with a weak or even without TCR signal. Therefore, four context-dependent activation pathways have been described: an activation via (1) a strong TCR signal or (2) through cytokine(s). A mixed activation mode involving a weak TCR signal through a poorly antigenic endogenous lipid combined with (3) cytokine-mediated activation, or (4) NK cell receptor(s) activation (Fig. 3) [16,47].

As iNKT cells can display either pro- or anti-inflammatory phenotype, their effector functions is widely influenced by the context. It

means that the kind of APCs and lipid antigens, the strength of the subsequent TCR signaling, the mode of activation (TCR-dependent or -independent activation), the presence of cytokines but also the tissue-specific environment can shape the behavior of iNKT cells [19,31]. For example, during strong TCR-driven activation, iNKT cells are able to produce a mixture of pro- and anti-inflammatory cytokines, while without TCR engagement, they are only able to produce IFN- $\gamma$  [16]. In conclusion, these different activation modes highlight the large plasticity of iNKT cells to deal with pathogens, inflammation and sterile stresses, revealing the plethora of contexts in which these cells can be involved.

## 2.6. iNKT cells are key players in immune response

Like innate immune cells, iNKT cells rapidly trigger their effector functions following stimulation. Shortly after activation, iNKT cells have the characteristics to copiously secrete cytokines and chemokines within minutes up to several hours [18,19,31]. These cells can produce a large range of cytokines that include IL-4 and IFN- $\gamma$  but also IL-2, IL-6, IL-10, IL-13, IL-17, IL-21 and TNF- $\alpha$  [19,48,49]. The iNKT cells are also able to exert cytolytic activity through production of cytolytic enzymes (i.e., perforin and granzyme B). Thereby, iNKT cells are able to kill cells presenting lipid antigen in a CD1d-dependent manner [18].

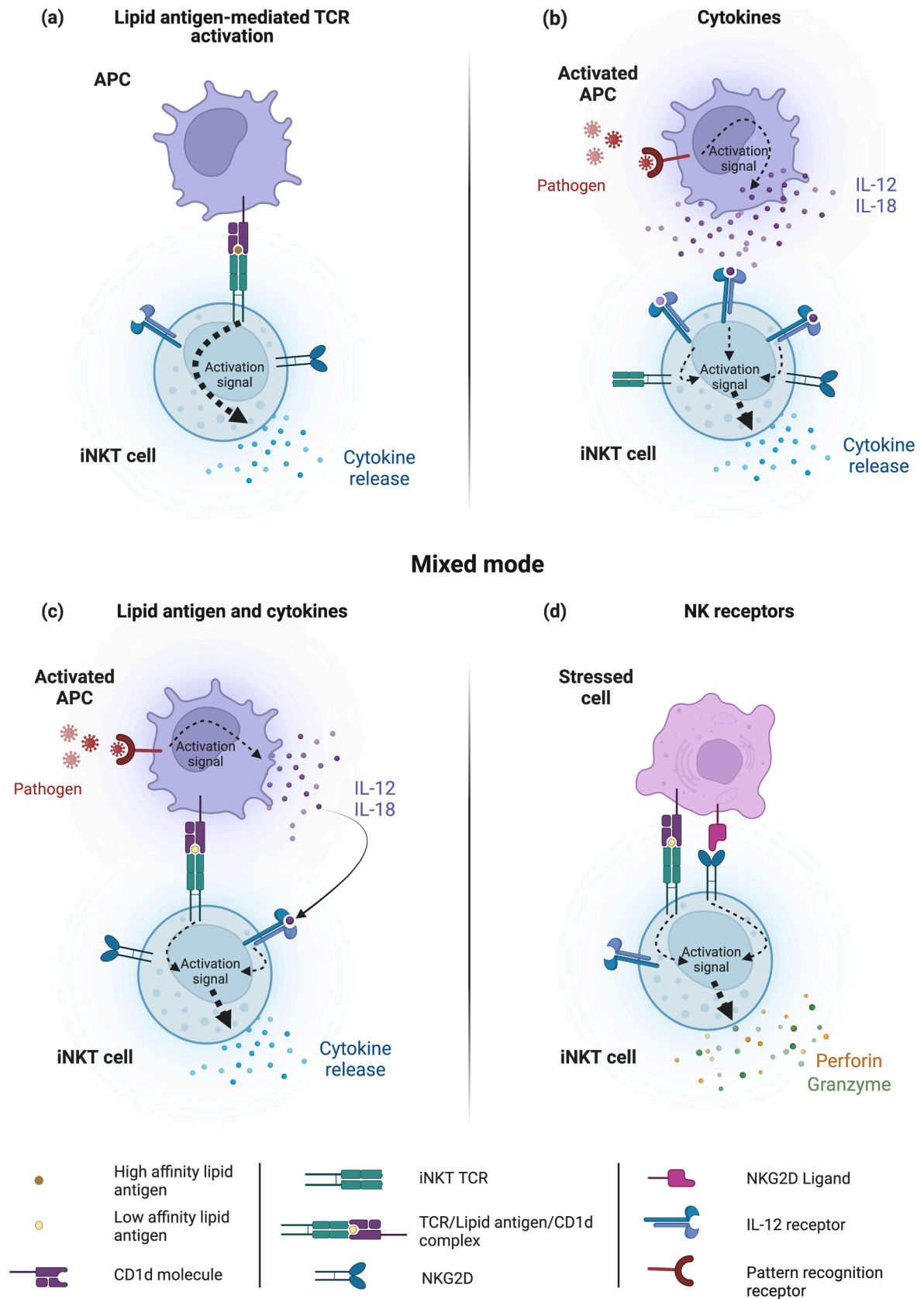
Cytokine production by iNKT cells is required to activate and regulate a large array of immune cells (from innate and adaptive immunity), such as monocytes/macrophages, DCs, B cells, T cells, NK cells and neutrophils. By this way, iNKT cells are involved in protective immunity against pathogens and tumor cells. For example, iNKT cells play a critical role in immunosurveillance against tumors in both mouse and human due to their capacity to elicit a cytotoxic response. They can induce direct or indirect tumor lysis [29,50]. Some tumor cells (e.g., B lymphoma cells) express high levels of CD1d loaded with endogenous tumor-associated lipid. After TCR engagement through CD1d-lipid antigen recognition, iNKT cells can induce direct cytolysis of tumor cells [50]. Moreover, once activated iNKT cells have the capacity to recruit and activate other immune cells, such as NK, B cells and T cells at tumor site. Indeed, IFN- $\gamma$  produced by iNKT cells could subsequently activate NK cells and mature cytotoxic CD8<sup>+</sup> T cells well known for their activity in immunity against tumor [19,29]. For more information about iNKT cells interactions with other immune cells, we refer readers to this excellent review [16]. Given their ability to interact with other immune cells and their rapid effector function, iNKT cells are emerging as key orchestrators of various immune responses.

## 3. iNKT cells in obesity

Obesity creates an environment that often disfavors proper functioning of immune cells. [7,11,51–53]. Among immune cells affected by obesity, iNKT cells which recognize lipid antigens are also targeted through direct or indirect mechanisms. However, it is necessary to distinguish AT-resident from peripheral blood iNKT cells. Due to the difficulty to obtain human subcutaneous or omental adipose tissue, but also because the number of iNKT cells drops in obese adipose tissue, iNKT cells of human patient are largely observed in peripheral blood. Moreover, circulating immune cells, like iNKT cells, are considered to partially reflect the systemic conditions [54]. At the opposite, AT-resident iNKT cells are widely studied in mice as their frequency is 10-fold more than in human [15,28].

### 3.1. Peripheral blood iNKT cells in obesity

Several studies have reported that iNKT cells frequency is decreased in PBMCs from obese patients compared to age-matched lean individuals [54–56]. However, other teams failed to show any difference [57–60]. These discrepancies can be explained in two ways. First, a very significant variability in the frequency of iNKT cells among PBMCs is



**Fig. 3.** iNKT cells activation modes. iNKT cells can be activated by four context-dependent pathways: (a) via a strong TCR signal induced by a potent lipid antigen presented through CD1d molecule to iNKT cells; (b) via cytokines; (c) via a mixed activation mode involving a weak TCR signal through a poorly antigenic endogenous lipid combined with cytokine-mediated activation; (d) iNKT cells cytolytic activity could also be activated by NK cells receptors (e.g., NKG2D), in absence of strong TCR engagement. Created with [BioRender.com](https://BioRender.com). APC: antigen-presenting cell; IL-: Interleukin-; TCR: T-cell receptor.

already observed between healthy individuals. Secondly, in our opinion, this controversy is mainly related to difference in body mass index (BMI) criteria. Indeed, obesity is most often only defined on BMI > 30 kg/m<sup>2</sup>. Beyond this value, only gradation exists (Grade III obesity: BMI > 40 kg/m<sup>2</sup> vs. Grade II obesity: BMI 35–40 kg/m<sup>2</sup> vs. Grade I obesity: BMI 30–35 kg/m<sup>2</sup>). Thus, studies which succeeded to show an obesity-related decrease in iNKT cells number were most of the time carried out on Grade III obese patients. Moreover, this way of sorting patients does not consider the visceral fat accumulation. Wang et al. (2016) showed that proportion of iNKT cells is lower in patient with excessive fat accumulation, irrespectively of their BMI [54]. Interestingly, the reduction in peripheral blood iNKT cells number was correlated with several autoimmune diseases and even some cancers [16,61], reviving interest of immunometabolic impact of obesity on auto-immune diseases and cancer genesis. Importantly, gender is an important factor to consider in obesity. For example, it can influence prevalence and body fat distribution [62]. On the other side, studies have also highlighted that iNKT cells frequency is influenced by gender [30]. However, to our knowledge, no studies focused on the impact of gender on iNKT cells in obesity context. Hence, future studies should consider both obesity and gender aspects either by matching patients or by sorting patients according to their BMI and gender.

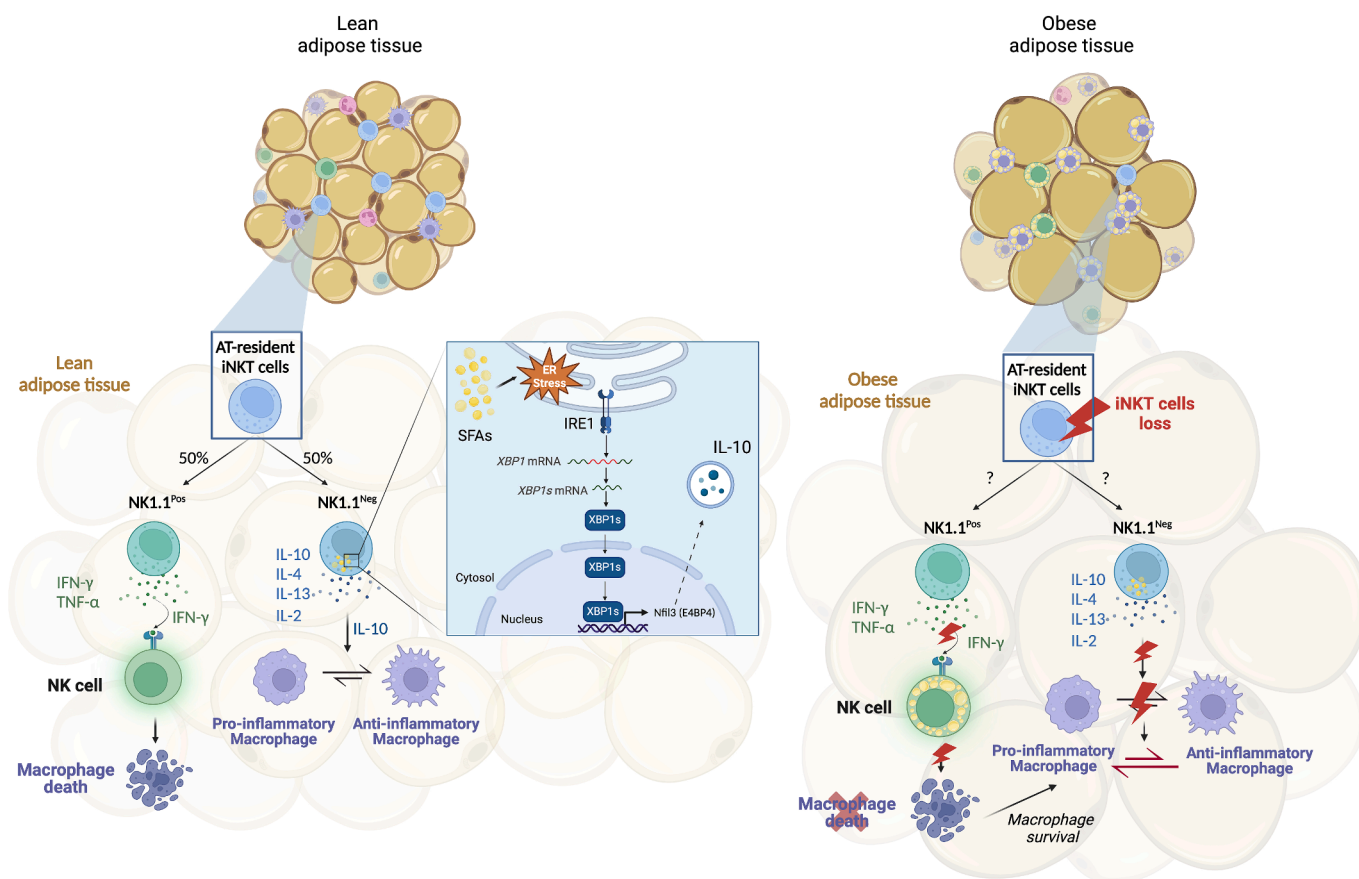
Obesity has been shown to induce upregulation of CD25 and/or CD69 on peripheral iNKT cells, suggesting a chronic activation of these cells [57,59,60]. Beside these phenotypical alterations, the activity of peripheral iNKT cells from obese patients has also been shown to be impaired. However, some controversy exists regarding these alterations. Ververs et al. (2021) measured iNKT cell cytokine production in supernatant using multiplex immunoassay following  $\alpha$ GC stimulation of PBMCs from obese adolescents [57]. While they failed to demonstrate a direct impact of obesity on IFN- $\gamma$  or IL-4 production, they observed an increased IFN- $\gamma$ /IL-4 ratio in obese adolescents compared to healthy controls. These results suggest a Th1-skewing of peripheral blood iNKT cells toward a pro-inflammatory phenotype [57]. At the opposite, Li et al. (2020) determined by flow cytometry the frequency of cytokine-producing iNKT cells in PBMCs stimulated with PMA/ionomycin. They observed an impairment of the production of both IFN- $\gamma$  and IL-2 but not of IL-17, granzyme and IL-4 by iNKT cells from obese patients compared to healthy controls [60]. These contradictory results can be explained by several differences in the experimental design. First, the stimulation methods are different. Indeed, Ververs et al. (2021) used the canonical lipid antigen  $\alpha$ GC which specifically activates the TCR of iNKT cells after CD1d loading, whereas Li et al. (2020) stimulated iNKT cells with the non-specific combination PMA/ionomycin. Second, the methods to measure the cytokines production are different. The first team measured the secretion of cytokines in the supernatant, while the second monitored the percentage of iNKT cells expressing intracellular cytokines. Third, the average age of patients in the cohorts is very different, from 14 to 16 years in the first case, from 45 (obese) to 49 (healthy control) years in the second case. Even if the PMA/ionomycin stimulation does not really mimic TCR activation through CD1d-loaded lipid antigen, the results from Li et al. (2020) have the advantage to underscore significative differences for both IFN- $\gamma$  and IL-2, highlighting iNKT cells dysfunction in obese patients compared to healthy controls. Li et al. (2020) also assessed by flow cytometry the production of intracellular cytokines by iNKT cells after stimulation with  $\alpha$ GC. They did not see any significant increase of cytokines production in response to  $\alpha$ GC stimulation and consequently any difference between obese and healthy control. However, as iNKT cells internalize their TCR upon stimulation, it becomes unsuitable to analyze these cells by flow cytometry after stimulation involving the TCR [61,63–65]. Altogether, these studies on peripheral blood iNKT cells from obese patients suggest a decrease of their number accompanied by an alteration of cytokine production. Nevertheless, these studies were conducted on small patient cohort and need to be confirmed. Moreover, the mechanisms involved in iNKT cells impairment are not understood and need more investigations.

### 3.2. Adipose tissue-resident iNKT cells in lean condition

In adipose tissue, a large diversity of cells is able to present lipid antigens to iNKT cells through CD1d. It includes macrophages, DCs but also adipocytes. By acting as non-professional APCs, adipocytes can regulate the activation of AT-resident iNKT cells and maintain their number in AT [16,28]. Compared with other tissues, iNKT cells are highly enriched in adipose tissue from mouse but also in human in a lean state, representing up to 10–30 % of T cells [16,66–68].

Recent findings have pointed out that iNKT cells undergo tissue-specific metabolic adaptation and regulation [69,70]. First, they do not express PLZF unlike other tissue-resident iNKT cells known so far (Fig. 1) [71]. Moreover, AT-resident iNKT cells do not rely on glycolytic metabolism to support their activation unlike splenic and hepatic iNKT cells [69]. Aguiar et al. (2023) highlighted distinctive transcriptional and metabolic signatures in AT-resident iNKT cells involving both AMP-activated protein kinase (AMPK) pathway and fatty acid (FA) oxidation [69]. AMPK is a nutrient and energy-sensor which controls energetic homeostasis [72]. AMPK maintains the energy balance by decreasing the ATP-consuming processes while the metabolic pathways such as FA transport and oxidation are increased to preserve ATP during energy deficiency [72]. Aguiar et al. (2023) demonstrated that FA oxidation inhibition reduces the activation of AT-resident iNKT cells but not splenic iNKT cells [69]. AMPK deletion impairs IFN- $\gamma$  and IL-4 production by AT-resident iNKT cells without affecting the response of splenic and hepatic iNKT cells to  $\alpha$ GC. Of note, the loss of AMPK expression in AT-resident iNKT also abrogates the ability of AT-resident iNKT cells to produce IL-10, which is critical to maintain AT homeostasis [69].

In addition to impact intrinsic metabolism of iNKT cells, adipose microenvironment also drives the expression of the E4-binding Protein 4 (E4BP4) transcription factor, mediating IL-10 production, in AT-resident iNKT cells (Fig. 1) [55,68,71]. Interestingly, E4BP4 appears to not be expressed by other types of iNKT cells at steady state. LaMarche et al. (2020) sought to determine what factors in the adipose microenvironment induce E4BP4 in iNKT cells and endow them with regulatory capacity [71]. They demonstrated that E4BP4 upregulation does not require TCR-CD1d interaction, nor any other cell–cell contact, but depends on lipid soluble factors present in adipose microenvironment. Accordingly, nonantigenic lipids seem to be involved in driving E4BP4 expression in AT-resident iNKT cells. Adipose tissue is characterized by high concentrations of extracellular lipids, 85 % of which are the free fatty acids (FFAs) palmitate (C16:0), oleate (C18:1), and linoleate (C18:2) [73]. LaMarche et al. (2020) showed that the amount of lipids inside AT-resident iNKT cells is approximately twice as high as in the splenic iNKT cells, as measured by LipidTox staining, consistent with increased FA uptake [71]. Interestingly, they could recapitulate this lipid accumulation and the rapid upregulation of E4BP4 and *Il10* expression in palmitate-treated splenic iNKT cells. This was specific to iNKT cells since conventional T cells did not upregulate IL-10 production in response to palmitate. The next step was to decipher the molecular mechanisms underlying E4BP4 upregulation in palmitate-treated iNKT cells. Interestingly, their analysis of single cell RNA-seq data revealed an upregulation of several transcripts associated with apoptosis and cellular stress in AT-resident iNKT cells compared to splenic iNKT cells. Furthermore, an average of 10 % of AT-resident iNKT cells are in apoptosis, compared with only 1 % in the spleen. Altogether, these observations suggest that iNKT cells are undergoing a fat-mediated stress. Saturated fatty acids (SFAs), like palmitate, are known to induce the ER stress via inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ )-XBP1s axis, one of three pathways engaged by the unfolded protein response (UPR) in response to ER stress in several kinds of immune cells, notably in iNKT cells [74]. Accordingly, LaMarche et al. (2020) investigated this IRE1 $\alpha$ -XBP1s axis and demonstrated that the transcription factor XBP1s, that is derived from the splicing of the *XBP1* mRNA mediated by IRE1 $\alpha$ 's RNase activity, drives the upregulation of both E4BP4 and IL-10 in palmitate-treated splenic iNKT cells (Fig. 4) [71]. Interestingly, LaMarche et al. (2020)



**Fig. 4.** Adipose tissue-resident iNKT cells in lean and obese conditions. Created with [BioRender.com](https://www.biorender.com/). AT: adipose tissue; ATMs: AT-macrophages; E4BP4: E4-binding Protein 4; ER: endoplasmic reticulum; IFN- $\gamma$ : interferon- $\gamma$ ; IL-: Interleukin-; IRE1 $\alpha$ : inositol-requiring enzyme 1 $\alpha$ ; Nfil3: Nuclear Factor, Interleukin 3 Regulated; NK: Natural Killer; SFAs: saturated fatty acids; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ .

showed that when donor splenic iNKT cells, which do not express E4BP4, are adoptively transferred and recovered a week later, their expression profile can be modified [71]. While donor iNKT cells recovered from spleen show low or no expression of E4BP4, the ones recovered from adipose tissue upregulate E4BP4. These “newly” AT-resident iNKT cells are completely functional as demonstrated by IL-10 production following  $\alpha$ GC stimulation [71]. Altogether, these data highlight a key role of FFAs in shaping the regulatory phenotype of AT-resident iNKT cells, both as metabolites to fuel FA oxidation and ER stress inducers to drive IL-10 production. However, these observations have still to be confirmed in humans, which remains challenging due to the smaller number of iNKT cells.

These characteristics define a new subset of AT-specific iNKT cells, also named NKT10. As this particular NKT10 phenotype seems to arise outside thymic development, adipose tissue micro-environment appears to shape both metabolic profile and function of resident iNKT cells.

Even if these cells predominantly produce potent anti-inflammatory cytokines (i.e., IL-10, IL-4, IL-13 and IL-2) following  $\alpha$ GC activation, they are also able to produce, to a lesser extent, IFN- $\gamma$  [55,67,68,71,75,76]. This can be explained by the fact that NKT10 cells can still be equally divided into two functionally distinct sub-populations based on NK1.1 expression (Fig. 4): (1) NK1.1<sup>NEG</sup> iNKT cells which mostly produce anti-inflammatory cytokines (especially IL-10) and (2) NK1.1<sup>POS</sup> iNKT cells characterized by the release of large amounts of IFN- $\gamma$  and TNF- $\alpha$  in response to adipose tissue lipid antigens [71]. By producing anti-inflammatory cytokines, NK1.1<sup>NEG</sup> iNKT cells are able to influence macrophages polarization.

Of note, these cells are major IL-10 producers, likely due to their higher lipid accumulation and sensitivity to ER stress, which in turn,

switches on IRE1 $\alpha$ -XBP1s axis and drives the upregulation of both E4BP4 and IL-10 [71]. At the opposite, NK1.1<sup>POS</sup> cells reveal their function in lean adipose tissue: through IFN- $\gamma$  secretion, these cells promote AT-macrophages (ATMs) killing by NK cells [71]. Indeed, iNKT-deficient or IFN- $\gamma$  KO lean mice exhibit significant ATMs accumulation and metabolic alteration without any significant body weight modulation in comparison to WT mice [55,71]. Mechanistically, in lean adipose tissue, IFN- $\gamma$  produced by NK1.1<sup>POS</sup> cells transactivates AT-NK cells which, in turn, promote ATMs death thanks to perforin-granzyme B pathway (Fig. 4) [71,77]. Hence, NK1.1<sup>POS</sup> cells play an unexpected “anti-inflammatory role” in lean adipose tissue by restraining the ATMs expansion. Thus, AT-resident iNKT cells sub-populations have complementary functions to maintain metabolic integrity in AT. For this reason, AT-resident iNKT cells have to be considered as key players in maintaining adipose tissue homeostasis and overall metabolic balance under normal conditions by (1) promoting anti-inflammatory macrophages polarization and (2) limiting pro-inflammatory ATMs population accumulation through regulatory cytokines secretion.

### 3.3. Adipose tissue-resident iNKT cells in obesity

During obesity, adipocytes hyperplasia and hypertrophy result in adipose tissue dysfunction, metabolic disorders such as dyslipidemia, insulin resistance and glucose intolerance which could lead to obesity-related diseases [78]. This adipose tissue expansion is accompanied by AT-resident immune cells (i.e., CD4<sup>+</sup>, CD8<sup>+</sup>, regulatory T cells, B cells and macrophages) redistribution but also pro-inflammatory immune cells infiltration [10,51,53]. For example, pro-inflammatory macrophages and CD8<sup>+</sup> T cells are increased. This evolution of AT landscape

gradually leads to a pro-inflammatory environment which in turn contributes to the development of a chronic low-grade inflammation promoting the metabolic dysregulation observed in obese patients [51,52]. Therefore, immune cells represent potent mediators of obesity-induced inflammation and associated diseases. Among AT-immune cells affected by obesity, iNKT cells are not left out: a reduction of iNKT cells number was observed in obese adipose tissue in mice and humans [66,75,79]. This depletion was correlated to BMI increase and glucose intolerance in human [75]. However, the origin of this iNKT cells loss during adipose-tissue expansion is still misunderstood. One hypothesis is that obesity reduces CD1d expression on adipocytes surface, which are no longer able to maintain iNKT cells physiological activation [28,80]. Of note, important weight loss (i.e., bariatric surgery for human or a return to a standard fat diet for mouse) remarkably restores the iNKT cells number in AT [66,75,79].

Interestingly, reduction of iNKT cells number is strongly correlated with macrophage infiltration in adipose tissue [55], raising the new question: is it a simple correlation or a consequence? Mouse models of obesity have provided many clues to this question. First, Schipper et al. (2012) demonstrated that AT-resident iNKT cells of lean mice (i.e., under low-fat diet) present an activated profile along with basal secretion of anti-inflammatory cytokines, while *Cd1d1*<sup>-/-</sup> mice, under the same diet, develop both adipose tissue inflammation and insulin resistance [67]. Second, under high-fat diet (HFD), mice lacking iNKT cells (i.e.,  $\alpha$ 18-deficient or *Cd1d1*<sup>-/-</sup>) are predisposed to weight gain, hyperplasia of adipocytes, insulin resistance and show higher levels of ATMs. Interestingly, adoptive transfer of iNKT cells in those obese mice reverses HFD-induced phenotype by driving weight loss and improving both adipocyte hyperplasia and insulin sensitivity [55]. The same findings were observed in WT obese mice following residual iNKT cells stimulation by  $\alpha$ GC [55,76]. This obesity-associated metabolic disorder improvement by  $\alpha$ GC administration seems to rely on expansion of iNKT cells and subsequent production of IL-4 and IL-10, which can promote ATMs polarization toward anti-inflammatory phenotype [55,75,76,79]. Thus, iNKT cells can shape adipose tissue environment and metabolic balance by influencing macrophages polarization. These findings point to a protective role for AT-resident iNKT cells in adipose tissue. When each NK1.1 subset was separately adoptively transferred in  $\alpha$ 18-deficient mice under HFD, NK1.1<sup>NEG</sup> iNKT cells were able to restore metabolic function by improving glucose tolerance and reversing adipocytes hypertrophy unlike NK1.1<sup>POS</sup> iNKT cells [71]. These results are consistent with the recently described function of NK1.1<sup>POS</sup> iNKT cells in promoting ATMs killing through NK cells transactivation. In obese state, since NK cells are in a “paralyzed state” due to lipid accumulation, they are no longer able to exert their cytotoxic activity and to prevent ATMs accumulation [11]. Altogether, these studies demonstrate that both subsets of AT-resident iNKT cells play an important role in the control of AT homeostasis. Together with macrophage infiltration, their depletion participates to adipose tissue low-grade inflammation but also insulin resistance.

While many reports have described AT-resident iNKT cells as anti-inflammatory and protective against metabolic disease, several groups have reported the opposite finding that AT-resident iNKT cells contribute to obesity-related metabolic disease. They suggest that lipid excess induces chronic stimulation of AT-resident iNKT cells, driving a pro-inflammatory response via TNF- $\alpha$  and IFN- $\gamma$  production [81–83]. The factors that could influence these discrepancies include both the genetic and age of the mice, the duration and composition of the diet but also the gut microbiome [84].

What would be the origin of AT-resident iNKT cells depletion in obesity? This question has not been discussed again since the identification and characterization of the two subpopulations of iNKT cells (NK1.1<sup>POS</sup> and NK1.1<sup>NEG</sup>) in adipose tissue. These two subpopulations are not similarly influenced by the adipose environment. Indeed, NK1.1<sup>NEG</sup> iNKT cells, unlike NK1.1<sup>POS</sup>, accumulate intracellular lipids which are proposed to be responsible for the activation of ER stress and

induction of the IRE1 $\alpha$ -XBP1s branch. These observations refer us to the recent notion that different tissue-resident cell subpopulations can preferentially reside within different, but conserved, subtissular niches. For example, recently published single-cell RNA sequencing data allowed to highlight that obesity-linked AT remodeling induces a unique ATM subpopulation characterized by lipid droplets and termed lipid-associated macrophages (LAMs) [85]. LAMs are enriched around adipocytes in crown-like structures where they appear to play an important role in AT remodeling. Given these new data, it would be interesting to analyze the spatial distribution of both NK1.1<sup>POS</sup> and NK1.1<sup>NEG</sup> iNKT cells subpopulations within lean and obese adipose tissue. One hypothesis would be that NK1.1<sup>NEG</sup> iNKT cells are concentrated in crown-like structures, accumulating more lipids released by adipocytes and dying from sustained ER stress leading to apoptosis.

#### 4. Metabolic factors that could affect iNKT cells in obesity

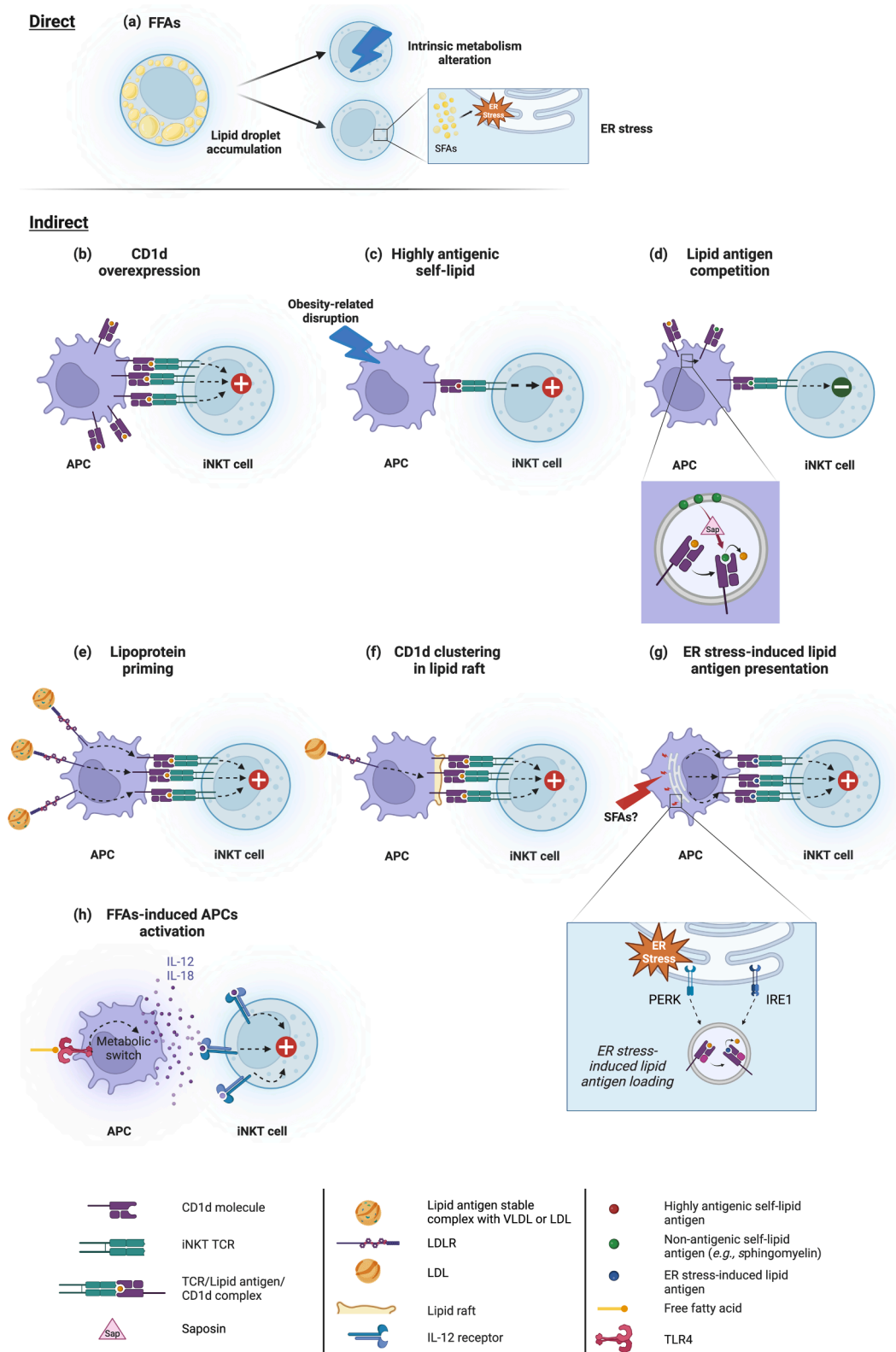
While there is clear evidence that iNKT cells are affected by obesity, how and to what extent metabolic factors like lipids, glucose and FFAs can drive iNKT cells alterations remains unclear, even if this research field shows growing interest. Do obesity-linked metabolites such as FFAs have a direct impact on these cells? Are new or more lipid antigens presented to iNKT cells? We will summarize last findings in this field.

##### 4.1. Direct impact on iNKT cells

Immune cells reprogram their metabolism in response to stimuli such as antigens, cytokines or pathogen-associated molecular patterns (PAMPs) to properly carry out their effector functions [86]. It has becoming increasingly clear that systemic metabolism modulations can participate to the development of immune dysfunction in obesity by impacting intrinsic metabolism and functions of immune cells [87,88]. This was very well highlighted by the team of L. Lynch [11]. They demonstrated that obesity in mice and human induces an accumulation of lipids in peripheral NK cells, leading to the “paralysis” of their glucose metabolism and cytotoxic functions. Interestingly, *ex vivo* treatment of healthy human peripheral NK cells with FFAs, either saturated or monounsaturated, was able to recapitulate the phenotype of peripheral NK cells in obesity. As reminder, larger amounts of FFAs are released in the blood of obese patients due to increased lipolysis of triglycerides from insulin-resistant visceral adipocytes [89–91]. FFA-*ex vivo* treated splenic murine NK cells lost their antitumoral activity when injected peritumorally into B16-tumor bearing mice.

Interestingly, LaMarche et al. (2020) recently demonstrated that SFAs such as palmitate can induce the expression of E4BP4 and IL-10 in murine splenic iNKT cells (Fig. 4) [71]. The underlying molecular mechanisms involve ER stress and activation of the IRE1 $\alpha$ -XBP1s pathway. The authors propose that the regulatory phenotype of AT-resident iNKT cells is shaped by the FFAs-rich AT environment which directly impacts iNKT cells via ER stress-mediated pathways. Surprisingly, LaMarche et al. (2020) did not observe any effect of palmitate on the production of IL-10 by conventional T cells. FFAs can affect immune cells by completely different mechanisms depending on the cell type; fatty acids, saturated or not, separately or mixed, paralyze the intrinsic glycolytic metabolism of NK cells and disrupt their effector functions [11]. However, only saturated fatty acids, known to saturate membranes and induce ER stress, induce the expression of the regulatory cytokine IL-10 by iNKT cells.

Because the FFAs concentration further increases in adipose tissue during obesity and since this tissue undergoes a profound remodeling with the appearance of new niches [85], we can suspect the appearance of new FFAs effects on AT-resident iNKT cells (Fig. 5a). Moreover, FFAs concentration also increases in peripheral blood as well as in other non-adipose tissues, such as liver. Those effects might not be limited to AT but might affect peripheral or non-AT-resident iNKT cells.



**Fig. 5.** Mechanisms that could affect iNKT cells in obesity through direct or indirect way. (a) Elevation of FFAs levels. (b) Greater presentation of self-lipid antigens or (c) presentation of a more antigenic self-lipid by peripheral APCs expressing CD1d. (d) Competition of non-antigenic lipid antigen with other antigenic lipids. (e) Stabilization of lipid antigen with lipoproteins and internalization through LDLR. (f) Clustering of CD1d in lipid raft. (g) ER stress in APCs. (h) FFAs-induced cytokines production. Created with [BioRender.com](https://www.biorender.com). APC: antigen-presenting cell; ER: endoplasmic reticulum; FFAs: free fatty acids; IRE1 $\alpha$ : inositol-requiring enzyme 1 $\alpha$ ; LDL: low density lipoprotein; LDLR: LDL receptor; PERK: protein kinase RNA-like ER kinase; SFAs: saturated fatty acids; TCR: T-cell receptor; TLR-4: Toll-like receptor-4; VLDL: very low-density lipoprotein.

## 4.2. Indirect impact via lipid antigen presentation

The obesity could also modify CD1d-mediated lipid antigens presentation and subsequently leads to a modulation of the iNKT response in terms of amplitude and/or cytokine output. The alteration of the extracellular lipid environment, the reprogramming of intrinsic lipid synthesis pathways within APCs, as well as the modulation of the different steps leading to the presentation of lipid antigens on CD1d (uptake, loading, membrane CD1d distribution) are all potential events that could indirectly impact iNKT cells in obesity. Various metabolic factors like FFAs, lipoproteins or stress conditions are likely to be at the origin of such modulations.

### 4.2.1. Lipid environment

New advances support the existence of an ever-increasing repertoire of lipid antigens that is available for CD1d presentation [92]. Since each tissue is characterized by its own lipidome, itself modulated under pathological conditions [93,94], we can predict that the current list of potential lipid antigens will continue to expand. In the same vein, several teams including ours demonstrated modulations of the plasma lipidome of obese patients in comparison with the one of lean individuals [95,96]. We also observed modifications of the PBMCs phospholipidome from obese patients in comparison to lean patients [96]. This disruption of plasma and PBMCs (phospho)lipidome in obesity could lead to greater presentation of self-lipid antigens (Fig. 5b) or the presentation of a more antigenic self-lipid (Fig. 5c) by peripheral APCs expressing CD1d such as monocytes, dendritic cells or B lymphocytes, which could drive the activated profile of peripheral iNKT cells in obese patients [57,59,60]. This hypothesis is reinforced by the demonstration that CD1d is upregulated on the surface of peripheral monocytes in obese compared to lean patients (personal communication – unpublished data). While CD1d has already been shown to be transcriptionally upregulated in a pro-inflammatory context [97], the content of CD1d is also probably dictated by the lipid antigen cargo of the cell (Fig. 5b).

In AT, iNKT cells can be directly activated by adipocytes, as they express CD1d. Moreover, adipocytes possess a functional lipid antigen presentation pathway, as well as a biosynthetic pathway for the production of lipid self-antigens [98,99]. Interestingly, their cytokine output was skewed toward a low IL-4 and high IFN- $\gamma$  profile when iNKT cells were co-cultured with *ex vivo* FFAs-treated adipocytes [70].

During lipid loading onto CD1d in the lysosome, small lipids with a short tail or even without a tail could enable previously non-antigenic lipids to become antigenic by filling one of the hydrophobic pockets, and thereby promoting the essential conformational change upon binding [100,101]. Such lipids called spacer lipids are FFAs, like palmitate, and would have the property of expanding the repertoire of lipid antigens. This kind of scaffold effect could be promoted in obesity where FFA concentrations are increased in plasma and tissues.

Since CD1d can load lipids even if they do not exhibit antigenic properties, such lipids in the microenvironment can enter into competition with other antigenic lipids and exert a competitive inhibition on TCR-mediated iNKT cell stimulation. Using *in vivo* experiments, elevated levels of specific sphingomyelin species in lysosomes resulted in defective iNKT cell development [43]. The same sphingomyelin species were able to compete  $\alpha$ GC, reducing the IL-2 secretion from iNKT cells in a dose dependent manner [102,103]. Interestingly, such attenuation mechanism could occur in obesity as the same sphingomyelin species are significantly elevated and associated with many of obesity-linked comorbidities (Fig. 5d) [104].

### 4.2.2. Lipoproteins

Recent work demonstrated an opposing and indirect modulatory role of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) lipoproteins on the activation of iNKT cells [57]. In APCs-iNKT cells co-culture assays, the priming of APCs with both  $\alpha$ GC and LDL induced robust and higher production of IFN- $\gamma$  in comparison to standard  $\alpha$ GC-

induced response. At the opposite, IL-4 production was enhanced following APCs-priming with both LDL or HDL with or without  $\alpha$ GC stimulation [57]. In contrast to very-low-density lipoprotein (VLDL) and HDL, LDL does not bind ApoE, which suggests an ApoE-independent role for LDL in iNKT cell activation. Since biochemical studies demonstrated that glycosylceramides can insert into phospholipid rafts with a similar composition as the phospholipid shell of lipoproteins [105], Engelen et al. (2023) investigated whether lipoproteins can form a complex with  $\alpha$ GC. They demonstrated that all the lipoproteins (LDL, VLDL), except HDL, are able to form a stable complex with  $\alpha$ GC [106]. These complexes are efficiently taken by APCs through LDL receptor (LDLR) and lead to robust iNKT cells activation *in vitro* and *in vivo* (Fig. 5e). This study reveals a potential novel mechanism of lipid antigen delivery to APCs and provides further insight into the immunological capacities of circulating lipoproteins.

On another side, lipoproteins uptake can be at the origin of membrane fluidity fluctuations through cholesterol accumulation which, in turn, can dysregulate lipid raft turnover [57,107]. Ververs et al. (2021) suggest that LDL could increase stabilization and the clustering of CD1d in lipid rafts of APCs, thereby promoting lipid antigen presentation and Th1-type iNKT cells response (Fig. 5f) [33]. However, this study has limitations: even if lipoproteins were washed before APCs-iNKT cells co-culture, a direct impact of lipoproteins on iNKT cells cannot be ruled out. Thus, further studies are required to ensure lipoprotein-mediated indirect effect on iNKT cells activation and underlying mechanisms.

Together, these data demonstrate a priming effect of LDL on the ability of APCs to take and present lipid antigens in a proper way to activate iNKT cells. These findings are very relevant in the context of obesity where HDL cholesterol/LDL cholesterol blood ratios are generally reduced. They are also in agreement with the activated profile of peripheral iNKT cells in obesity previously described by our group and several others (personal communication – unpublished data), [57,59,108].

### 4.2.3. ER stressed-APCs

ER stress is frequently observed in multiple tissues and cell types from dietary and genetic obesity mouse models [109,110] and from obese humans [111–113]. This state of so-called metabolic ER stress is provoked by a multitude of stimuli, mainly nutrients like glucose and lipids, arising from both systemic and tissue microenvironmental changes to face energy surplus [109].

Under cellular conditions disrupting ER function, an increase of unfolded/misfolded proteins is sensed by ER membrane effectors that trigger UPR to restore ER homeostasis. UPR is divided into three arms based on three ER transmembrane receptors: (1) IRE1 $\alpha$ ; (2) protein kinase RNA-like ER kinase (PERK); (3) activating transcription factor 6 (ATF6). These stress transducers reduce ER stress and re-establish cellular homeostasis by expanding ER size (via regulation of membrane lipid and protein biosynthesis), promoting degradation of unfolded/misfolded protein via ER-associated protein degradation (ERAD), or downregulating transcription of secretory proteins while upregulating transcription of chaperones to increase protein folding [114,115].

Recently, ER-stressed myeloid APCs have been shown to activate iNKT cells in a CD1d-dependent manner (Fig. 5g). These data are even more convincing as two teams showed similar results a few months apart [38,116]. Briefly, APCs were pre-stimulated with classical ER-stress inducers (i.e., Thapsigargin and Tunicamycin) and then co-cultivated with iNKT cells. The latter produced in response copious amounts of cytokines (i.e., IL-4, IL-2 and IFN- $\gamma$ ) and presented activated phenotype (i.e., CD25 upregulation). Of course, no activation of iNKT cells was observed when they were directly cultivated in CD1d protein-bound plate loaded with ER-stress inducers [38]. This indirect iNKT cells activation was strictly dependent on CD1d, even if CD1d surface expression remains unchanged after ER-stress induction. Both studies incriminate the PERK-EIF2 $\alpha$  branch of UPR as the pathway playing a key role in CD1d-

dependent iNKT cells activation [38,116]. Of note, one point of divergence lies between the two studies: Govindarajan et al. (2020) showed that the RNase IRE1 $\alpha$  was also involved in this phenomenon [38]. They identified neutral lipids (i.e., ceramides and diacylglycerols) as lipid antigens produced by ER-stressed APCs mediating iNKT cells activation. Intriguingly, the neutral lipids fraction but not the polar lipids fraction from ER-stressed APCs was able to recapitulate previous observations [38]. Interestingly, this pathway was observed *in vitro* in murine and human iNKT cells and was confirmed *in vivo* in mice after intravenous injection of ER-stressed APCs leading to splenic iNKT cells activation. These studies therefore shed light on ER-stress in APCs as potent trigger of iNKT cells activation in a CD1d-dependent manner via upregulation of endogenous lipid antigens. However, some questions remain unanswered. Does ER stress induce synthesis of new lipid antigens or does it promote the loading of pre-existing lipid antigens onto CD1d?

We demonstrated that increased levels of SFAs like palmitate and stearate can efficiently induce ER stress and UPR in macrophages [117–119]. Indeed, SFAs induce a saturation of membrane phospholipids, which impairs its integrity and fluidity [119]. IRE1 RNase and PERK can sense the loss of ER membrane fluidity mediated by SFAs and trigger UPR to restore homeostasis [120]. ER stress has been described in adipose tissue macrophages from HFD-fed mice and obese patients [111–113,121]. In obese individuals with type 2 diabetes, circulating immune cells display UPR markers [122]. Based on these recent findings, new hypothesis emerges: SFAs, whose levels are increased in obesity and which are known to trigger ER stress in myeloid cells, could promote the presentation of endogenous lipid antigens to iNKT cells via CD1d. Hence, iNKT cells will be chronically activated, which could lead to their exhaustion/anergy and eventually loss of iNKT cells observed in obese individuals (Fig. 5g). However, this hypothesis has to be challenged but cannot alone summarize the impact of obesity on iNKT cells.

#### 4.3. Indirect impact through TLR-mediated activation of APCs

Beyond modulation of lipid antigen presentation, other mechanisms could affect iNKT cells activation through an indirect way. Indeed, in addition to induce an ER stress, SFAs are well-known to stimulate the pro-inflammatory cascades in macrophages leading to the secretion of pro-inflammatory cytokines [123]. This pro-inflammatory effect of SFAs was initially shown to be mediated by Toll Like Receptor (TLR)-4, although this result was later controversial [124]. Higher lipopolysaccharides (LPS) levels have been detected in obese patients as a result of increased intestinal permeability and have also been proposed as inflammation triggers in obesity [125]. Accordingly, in obesity, higher levels of LPS and SFAs could cooperate to activate APCs to produce pro-inflammatory cytokines such as IL-12, well-known to promote iNKT cells activation and IFN- $\gamma$  secretion (Fig. 5h) [126,127]. As serum level of IL-12 is increased in obesity [128], IL-12-mediated chronic activation of iNKT cells cannot be ruled out.

## 5. Conclusion

Obesity and related metabolic diseases have reached epidemic proportions that continues to rise [1]. Paradoxically, in addition to increased inflammation, obesity also causes immune dysregulation as evidenced by an increased risk of cancer and infections [2]. Recent studies have demonstrated that obesity can negatively affect the activity of several kinds of immune cells like NK cells, T cells, and DCs [4–9].

Since the identification of iNKT cells as unconventional T cells capable of lipid antigens recognition, numerous studies have focused on the behaviour of these cells in conditions characterized by dyslipidemia. Several teams reported a decrease in their number in both adipose tissue and blood of obese mice and patients [66,75,79,54–56]. Peripheral iNKT cells appear to be chronically activated in obese patients. *Ex vivo* stimulation experiments with  $\alpha$ GC or PMA/Ionomycin cocktail demonstrate an alteration of their activity in obese patients [60]. However,

controversies have been raised. Analysis of iNKT cells in larger patient cohorts will be necessary to clarify this point. Why iNKT cells are reduced in obese, why residual iNKT cells show an activated profile and are they still able to carry out their function correctly remain unanswered questions. Chronic activation of iNKT cells in obesity could lead to their exhaustion or anergy or paralysis (as previously described for peripheral NK cells) and ultimately to their apoptosis. In this review, we report the conditions and mechanisms that could affect iNKT cells in obesity. The highlighted mechanisms involve the impact of metabolic factors either directly on iNKT cells, or indirectly via the presentation of lipid antigens or cytokines by APCs. The alteration of the extracellular lipid environment, the reprogramming of intrinsic lipid synthesis pathways within APCs, as well as the modulation of the different steps leading to the presentation of lipid antigens on CD1d (uptake, loading, membrane CD1d distribution) are all potential events that could indirectly impact iNKT cells in obesity. We also highlight a central role for FFAs which could directly impact iNKT cells by modifying their intrinsic metabolism or by inducing ER stress-associated gene expression. Moreover, FFAs could also act indirectly on the presentation of lipid antigens by playing the role of spacer lipids, by inducing ER stress-mediated lipid antigens presentation in APCs or by activating the production of cytokines by APCs. The impact of FFAs will depend on their saturation status and concentration.

We propose various mechanisms supported by literature review, but in some cases without explicit supportive evidence. Hence, all of these proposals have to be confirmed *in vitro* and/or *in vivo* to better elucidate the immunometabolic impact of obesity on iNKT cells.

## CRedit authorship contribution statement

**Chloé Wilkin:** Writing – original draft. **Jacques Piette:** Writing – review & editing. **Sylvie Legrand-Poels:** Writing – review & editing, Funding acquisition.

## Declaration of competing interest

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

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