Activation of invariant natural killer T cells stimulates adipose tissue remodeling via adipocyte death and birth in obesity

Jeu Park,1 Jin Young Huh,1 Jiyoung Oh,2 Jong In Kim,1 Sang Mun Han,1 Kyung Cheul Shin,1 Yong Geun Jeon,1 Sung Sik Choe,1 Jiyoung Park,2 and Jae Bum Kim1

1National Creative Research Initiatives Center for Adipose Tissue Remodeling, Institute of Molecular Biology and Genetics, Department of Biological Sciences, Seoul National University, Seoul 08826, South Korea; 2Department of Biological Sciences, School of Life Sciences, Ulsan National Institute of Science and Technology, Ulsan 44919, South Korea

In obesity, adipose tissue undergoes dynamic remodeling processes such as adipocyte hypertrophy, hypoxia, immune responses, and adipocyte death. However, whether and how invariant natural killer T (iNKT) cells contribute to adipose tissue remodeling are elusive. In this study, we demonstrate that iNKT cells remove unhealthy adipocytes and stimulate the differentiation of healthy adipocytes. In obese adipose tissue, iNKT cells were abundantly found nearby dead adipocytes. FasL-positive adipose iNKT cells exerted cytotoxic effects to eliminate hypertrophic and pro-inflammatory Fas-positive adipocytes. Furthermore, in vivo adipocyte-lineage tracing mice model showed that activation of iNKT cells by alpha-galactosylceramide promoted adipocyte turnover, eventually leading to potentiation of the insulin-dependent glucose uptake ability in adipose tissue. Collectively, our data propose a novel role of adipose iNKT cells in the regulation of adipocyte turnover in obesity.

[Keywords: adipocyte death; adipocyte turnover; adipogenesis; adipose tissue remodeling; iNKT cell]

Supplemental material is available for this article.

Received June 7, 2019; revised version accepted October 18, 2019.
specific glycolipid antigen that potently activates iNKT cells and is an effective adjuvant against infections, tumors, and obesity-induced inflammation (Kawano et al. 1997). Numerical and functional reductions in iNKT cells in adipose tissue are closely associated with increased inflammatory responses, obesity, adipocyte hypertrophy, and insulin resistance in obesity (Lynch et al. 2009; Ji et al. 2012; Schipper et al. 2012; Huh et al. 2017).

Yearly, 10% of human adipocytes die and are replaced (Spalding et al. 2008). Patients with cachexia, human immunodeficiency virus, or lipodystrophy show loss of adipocytes (Prins et al. 1994; Domingo et al. 1999; Fischer-Posovszky et al. 2006; Hussain and Garg 2016). In mice, as the morbidity of obesity becomes worse, dead adipocytes are frequently found in epididymal adipose tissue (EAT). It has been suggested that several mechanisms, including apoptosis, necrosis, and pyroptosis, mediate adipocyte death in obesity (Cinti et al. 2005; Alkhouri et al. 2010; Giordano et al. 2013). It seems that different types of adipocyte death mediate different effects on inflammatory responses and metabolic diseases. For instance, targeted induction of apoptosis in adipocytes recruits anti-inflammatory macrophages into adipose tissue, leading to the alleviation of inflammatory responses (Pajvani et al. 2005; Fischer-Posovszky et al. 2011b), Fas (CD95), a member of the tumor necrosis factor (TNF) receptor family, plays a key role in programmed cell death (apoptosis) (Fischer-Posovszky et al. 2011a). When FasL binds to Fas, a death-inducing signaling complex is assembled and causes apoptosis in a caspase-8- and caspase-3-dependent manner, without significant induction of inflammatory responses (Rock and Kono 2008; Nagata and Tanaka 2017). However, necrosis has been characterized as accidental cell death with release of intracellular components, accompanied with pro-inflammatory responses and insulin resistance (Cinti et al. 2005; Fink and Cookson 2005; Alkhouri et al. 2010).

In obesity, adipose tissue expands through mainly two processes; enlargement of pre-existing adipocytes (adipocyte hypertrophy) and induction of new adipocyte differentiation from adipocyte progenitor cells (adipocyte hyperplasia) (Rosen and Spiegelman 2014). Adipocyte hypertrophy is closely associated with “unhealthy obesity” features, including fibrosis, inflammation, adipocyte death, and insulin resistance. In contrast, adipocyte hyperplasia is a relatively low-risk factor of metabolic diseases (Gustafson et al. 2009; Sun et al. 2011; Rydén et al. 2014). Genetic and pharmacologic stimulation of adipocyte hyperplasia could suppress unhealthy adipose tissue remodeling and improve metabolic phenotypes (Shepherd et al. 1993; Berg et al. 2001; Kim et al. 2007; Kusminski et al. 2012; Shao et al. 2018). Although surface markers of adipocyte progenitor cells have not been fully identified, platelet-derived growth factor receptor (PDGFR) α- or PDGFR β-expressing cells reportedly contribute to adipocyte hyperplasia upon high-fat diet (HFD), protecting against unhealthy adipose tissue expansion (Lee et al. 2012; Vishvanath et al. 2016; Shao et al. 2018). However, the regulatory mechanisms underlying the balance between adipocyte hypertrophy and hyperplasia in obese adipose tissue are largely unknown.

Previously, we have shown that HFD-fed iNKT cell-deficient mice (Ja18 KO) are prone to unhealthy adipose tissue expansion, including adipocyte hypertrophy and insulin resistance, as well as elevated pro-inflammatory responses in obesity (Huh et al. 2013). Although we and other groups have investigated the relationship between iNKT cells and adipose tissue inflammation in obesity (Ji et al. 2012; Lynch et al. 2012; Schipper et al. 2012; Huh et al. 2013; Satoh et al. 2016), it is not thoroughly understood how iNKT cells can prevent unhealthy adipose tissue expansion in obesity. In this study, we investigated the roles of adipose iNKT cells in the regulation of adipocyte death in obese adipose tissue. Moreover, by using specific lipid antigen and adipocyte lineage-tracing mouse model, we examined whether activated iNKT cells can modulate adipose tissue remodeling. Collectively, our findings suggest that adipose iNKT cell can drive healthy adipose tissue remodeling by modulating adipocyte death and birth in obesity.

Results

In obese adipose tissue, cytotoxic potential of iNKT cells is potentiated

Consistent with previous reports (Lynch et al. 2012; Huh et al. 2013), Ja18 KO mice gained more body weight and EAT mass, and increased adipocyte size than did wild-type (WT) mice upon HFD (Supplemental Fig. S1A–D). Although iNKT cells reportedly have cytotoxic ability, it is unclear whether adipose iNKT cells would kill adipocytes or remove damaged adipocytes. To address this, we investigated the survival rate of adipocytes in HFD-fed WT mice and Ja18 KO mice. To assess the frequency of dead adipocytes from WT and Ja18 KO mice, we used a BioSorter instrument (Supplemental Fig. S1E,F), which enables quantitative analysis of large adipocytes. As shown in Figure 1A, total dead adipocytes displayed a decreased trend in obese Ja18 KO mice than in obese WT control littersmates. When we examined the frequency of dead adipocytes in the small (≤60 µm) and large adipocyte (>60 µm) populations, the fraction of dead cells was significantly lower in the large adipocyte population isolated from HFD-fed Ja18 KO mice than in that of HFD-fed WT littersmates (Fig. 1B, Supplemental Fig. S1G), suggesting that iNKT cells probably participate in large adipocyte death in diet-induced obesity (DIO). In this study, large adipocytes were defined based on a diameter >60 µm, because the adipocyte population meeting this criterion was increased by HFD (Supplemental Fig. S1H). In addition, we found that iNKT cells were abundantly present nearby dead adipocytes that were identified as perilipin-negative cells (Cinti et al. 2005; Strissel et al. 2007) and surrounded by adipose tissue macrophages (Fig. 1C,D). Taken together, these results suggest that iNKT cells might be involved in the death of hypertrophic adipocytes in obesity.

To evaluate the cytotoxic potential of adipose iNKT cells, the population of FasL-positive iNKT cells was quantified in EAT from normal chow diet (NCD)- and
HFD-fed mice. The fraction of FasL-positive iNKT cells (Fig. 1E,F) and absolute number of FasL-positive iNKT cells per fat pad were increased in obese mice [Fig. 1G]. Given that CD4 and CD8 T cells also exert cytotoxic effects by expressing FasL (Kagi et al. 1994; Kojima et al. 1994; Lowin et al. 1994), we investigated the populations of FasL-positive CD4 and CD8 T cells in EAT from DIO or genetically obese db/db mice compared with NCD-fed lean or db/+ heterozygote mice, respectively. Unlike adipose iNKT cells, the levels of FasL in CD4 and CD8 T cells did not change in DIO [Fig. 1H,I] nor db/db mice [Fig. 1J]. Furthermore, in hepatic and splenic iNKT cells, there were no significant differences in the fractions of FasL-positive iNKT cells [Fig. 1K,L]. Thus, these data imply that iNKT cells are a major cell type exhibiting increased FasL expression in obese adipose tissue.

Hypertrophic adipocytes express high level of Fas, accompanied with their mortality

Dead adipocytes were frequently observed in obese adipose tissue over HFD period [Fig. 2A; Supplemental Fig. S2A; Cinti et al. 2005; Strissel et al. 2007]. To investigate the characters of dead/dying adipocytes, the frequency of
dead adipocytes was analyzed at a single-cell level. As shown in Figure 2B, the fraction of dead adipocytes was ∼1.8% or 6.2% of total adipocytes upon NCD or HFD, respectively. The mRNA levels of the pro-apoptotic genes such as Caspase-3 and 8, and the Bax/Bcl2 ratio were also elevated in EAT (Fig. 2C; Supplemental Fig. S2B). Given that adipocytes have different characters depending on their sizes, we explored the relationship between adipocyte size and dead adipocyte frequency. The frequency of dead adipocytes was higher in the large adipocyte

Figure 2. Hypertrophic adipocytes strongly express Fas, accompanied with increased mortality. (A) Histological analysis of dead adipocytes in EAT from HFD-fed WT mice. (B) Dead adipocytes were analyzed using a BioSorter, after PI staining. (C) mRNA levels of apoptosis-related genes in adipocyte fractions. (D) Percentage of dead adipocytes per size category (small adipocytes, <60 μm; large adipocytes, >60 μm). (E) mRNA level of Fas in adipocyte fraction and stromal vascular cells (SVCs) from mice fed HFD for 3 d. (F) Percentage of total Fas-positive adipocytes from NCD- and HFD-fed mice. (G) Abundance of Fas-positive adipocytes in small and large adipocytes from NCD- and HFD-fed mice. (H) Correlation between adipocyte size and Fas protein expression. (I) Correlation between Fas protein expression and TUNEL intensity. Dotted lines are trend lines. r² is indicated on the graph. All data represent mean ± SEM. [*] P < 0.05 (t-test or two-way ANOVA with Bonferroni post-hoc test).
population (>60 µm) than in the small one (≤60 µm) in NCD- and HFD-fed WT and α18 KO mice [Fig. 2D; Supplemental Fig. S2C,D]. As shown in Figure 2D, the frequency of large adipocyte death was up-regulated in HFD-fed WT mice compared with that of large adipocyte death in NCD-fed WT mice. On the contrary, in HFD-fed α18 KO mice, the frequency of dead adipocytes in large adipocyte population was comparable to NCD-fed mice [Supplemental Fig. S2D], implying that the process of hypertrophic adipocyte death might be alleviated in HFD-fed α18 KO mice.

As adipose iNKT cells expressed the cytotoxicity mediator FasL, we examined the expression of Fas as a binding partner for FasL in iNKT cells. The mRNA level of Fas was tended to be up-regulated in adipocyte fractions after 3 d of HFD feeding, but not stromal vascular cells [Fig. 2E; Supplemental Fig. S2E]. The fraction of Fas-positive adipocytes was higher in HFD-fed than in NCD-fed mice [Fig. 2F; Supplemental Fig. S2F]. Next, we analyzed the relationship between adipocyte size and adipocyte Fas expression. The fraction of Fas-positive adipocytes was significantly increased in large adipocytes in both NCD and HFD [Fig. 2G]. Moreover, the intensity of adipocyte Fas in HFD-fed mice markedly increased with adipocyte size [Fig. 2H]. To determine the relevance of adipocyte Fas expression to the frequency of dead adipocytes, adipocytes isolated from HFD-fed mice were subjected to Fas and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stainings. As shown in Figure 2I, TUNEL-staining intensity was positively correlated with Fas expression in adipocytes, indicating that adipocyte Fas expression might be one of indicators of adipocyte death in obesity.

**Fas/FasL signaling is a key component in iNKT cell-induced adipocyte death**

To gain further insights into the mechanism by which iNKT cells induce adipocyte death, we conducted in vitro coculture experiments with differentiated 3T3-L1 adipocytes and DN32.D3 iNKT hybridoma cells. Differentiated adipocytes were directly cocultured with DN32.D3 cells in the absence or presence of a-GC, a potent iNKT cell lipid antigen [Fig. 3A]. Upon exposure to a-GC, the population of FasL-positive DN32.D3 cells was elevated [Fig. 3B], concurrently with an increase in the frequency of dead 3T3-L1 adipocytes and dead primary adipocytes [Fig. 3C,D; Supplemental Fig. S3A], indicating that FasL might be a potent mediator of iNKT cell-mediated adipocyte death. However, iNKT cells could also exert cytotoxic functions via secretion of cytotoxic molecules such as perforin/granzyme. To test the possibility that iNKT cells might induce adipocyte death through cytotoxic molecules secretion, condition media collected from coculture between DN32.D3 cells and 3T3-L1 adipocytes were incubated with differentiated 3T3-L1 adipocytes [Fig. 3E]. Treatment of condition media did not affect dead adipocyte frequency and mRNA expressions related with adipocyte function [Fig. 3F,G]. These data propose that physical contact between adipocytes and iNKT cells would be important to provoke iNKT cell-mediated adipocyte death.

To investigate whether the FasL/Fas pathway is involved in adipocyte death, Fas and Fas downstream genes were suppressed in differentiated adipocytes using siRNAs [Supplemental Fig. S3B–D]. As shown in Figure 3H, knockdown of Fas in differentiated 3T3-L1 adipocytes significantly reduced adipocyte death induced by iNKT cells activated with α-GC, although there was no difference in the fractions of FasL-positive DN32.D3 cells between siNC- and siFas-transfected groups [Fig. 3I]. Similarly, when Caspase-3 or Caspase-8 was suppressed in differentiated adipocytes, activated iNKT cells failed to promote adipocyte death [Fig. 3J]. Given that CD1d is an antigen-presenting molecule that is essential for iNKT cell activation, the effect of adipocyte CD1d on iNKT cell-mediated adipocyte death was investigated. As indicated in Figure 3K and Supplemental Figure S3E, knockdown of CD1d in differentiated 3T3-L1 adipocytes blunted iNKT cell-induced adipocyte death, indicating that antigen presentation by adipocyte CD1d is an essential step in the mediation of adipocyte death by iNKT cells. Taken together, these data suggest that both the recognition of antigens loaded on adipocyte CD1d by iNKT cells and the interaction between FasL and Fas are crucial to provoke iNKT cell-mediated adipocyte death.

**Hypertrophic adipocytes with pro-inflammatory characters are vulnerable to iNKT cell-mediated adipocyte death**

In obesity, Fas expression in large adipocytes was elevated, which was positively correlated with the frequency of dead adipocytes [Fig. 2H,I]. To explore mechanistic understanding whether hypertrophic adipocytes would be vulnerable to iNKT cell-induced death, we decided to test lipid-overloaded large adipocytes treated with free fatty acids (FFAs). When differentiated adipocytes were challenged with saturated fatty acids (palmitic acid, PA) or mono-unsaturated fatty acids (oleic acid, OA) [Fig. 4A], adipocytes became enlarged with an unilocular lipid droplet [Fig. 4B]. Moreover, the mRNA levels of pro-inflammatory genes such as chemokine (C-C motif) ligand 2 (Ccl2), Il-6, and mRNAs and protein levels of Fas were markedly up-regulated in large adipocytes induced by PA, but not OA [Fig. 4C,D]. However, FFA treatment did not induce apoptosis-related genes such as Caspase-3, Caspase-8, and Bax/Bcl2 ratio [Fig. 4E]. After FFA treatment, DN32.D3 cells were directly cocultured with enlarged 3T3-L1 adipocytes to investigate whether enlarged adipocytes might be susceptible to death by activated iNKT cells. The fractions of FasL-positive DN32.D3 cells and dead 3T3-L1 adipocytes were increased in PA-induced, but not OA-induced hypertrophic adipocytes [Fig. 4F,G]. Given that saturated fatty acids such as PA potently stimulate pro-inflammatory responses in adipocytes, we asked whether suppression of pro-inflammatory responses in PA-induced hypertrophic adipocytes would attenuate the induction of adipocyte death by iNKT cells. To address this, lipid-overloaded adipocytes were treated with the anti-
inflammatory agent rosiglitazone. As shown in Figure 4C–G, rosiglitazone repressed pro-inflammatory responses and adipocyte death provoked by DN32.D3 cells, indicating that hypertrophic adipocytes with pro-inflammatory characters would be preferentially removed by activated iNKT cells. To acquire further insight into the relationship between inflammatory stimuli and adipocyte death, differentiated adipocytes were treated with TNFa. TNFa up-regulated the Fas mRNA level in differentiated 3T3-L1 adipocytes [Fig. 4H] and further promoted the cytotoxic effects of DN32.D3 cells against adipocytes [Fig. 4I]. Together, these data suggest that pro-inflammatory characters caused by excess nutrients and/or pro-inflammatory stimuli render unhealthy adipocytes susceptible to iNKT cell-mediated apoptosis.

In obesity, iNKT cell activation promotes adipocyte death

To explore physiological roles of iNKT cell-induced adipocyte death in vivo, WT and Jα18 KO mice fed NCD or HFD were administered α-GC and the frequency of adipocyte death was assessed. Administration of α-GC did not significantly affect total body weight and EAT mass upon NCD
and HFD [Supplemental Fig. S4A,B]. In adipose tissue, α-GC increased FasL expression in iNKT cells, but not CD8 T cells, which was persisted for 2 d [Fig. 5A]. Since the effect of α-GC on FasL expression in iNKT cells was remarkable at day 1, we decided to analyze adipocyte death 1 d after α-GC injection in following experiments. Upon NCD, the effect of α-GC on adipocyte death was not detected [Supplemental Fig. S4C], even though FasL expression was increased in iNKT cells [Supplemental Fig. S4D]. On the contrary, in HFD-fed mice, α-GC markedly increased the fractions of perilipin-negative dead adipocytes and TUNEL-positive adipocytes [Fig. 5B–D] as well as the up-regulated portion of FasL-positive iNKT cells and FasL mean fluorescence intensity [MFI] in adipose iNKT cells [Fig. 5E,F]. However, in HFD-fed Jα18 KO mice, α-GC did not affect adipocyte death [Fig. 5B,C] as well as total body weight and EAT mass [Supplemental Fig. S4E,F]. Moreover, the extent of adipocyte death induced by α-GC was diminished in FasL-neutralized WT mice [Fig. 5G], implying that α-GC-induced adipocyte death is mediated by FasL-positive iNKT cells. Concurrently, Caspase-3 and Caspase-8 mRNA levels were higher in adipocytes from α-GC-injected WT mice than in those from α-GC-injected Jα18 KO mice [Fig. 5H]. These data imply that adipocyte dysregulation by HFD might be required for iNKT cell-mediated adipocyte death.

We have previously demonstrated that adipocyte CD1d is crucial for the regulation of adipose iNKT cell activity [Huh et al. 2017]. To examine whether adipocyte CD1d is required for iNKT cell-mediated adipocyte death in obesity, HFD-fed adipocyte-specific CD1d KO (CD1dAKO) mice were treated with α-GC. Similar to HFD-fed Jα18 KO mice [Fig. 5B,C], there was no significant difference in the fraction of dead adipocytes in CD1dAKO mice with or without α-GC [Fig. 5I,J]. Moreover, the levels of FasL in adipose iNKT cells were not different between vehicle [Veh]- and α-GC-treated CD1dAKO mice [Fig. 5K,L]. These data suggest that antigen recognition of iNKT cells

Figure 4. Hypertrophic adipocytes with pro-inflammatory characters are preferentially removed by iNKT cells. (A) Schematic illustration of experimental setup. Differentiated 3T3-L1 adipocytes were challenged with FFAs (500 μM, palmitic acid; PA and oleic acid; OA) or TNFα (10 nM) for 2 wk or 1 d, respectively, and then cocultured with DN32.D3 cells for 48 h. Palmitic acid-treated 3T3-L1 adipocytes were challenged with rosiglitazone [Rosi, 10 nM]. (B) Microscopic images of 3T3-L1 adipocytes. (C) mRNA levels of Ccl2, Il-6, and Fas. (D) FasR mean fluorescence intensity [MFI]. (E) mRNA levels of apoptosis-related genes, including Casp3, Casp8, and Bax/Bcl2 ratio. (F,G) Percentages of FasL-positive DN32.D3 cells [F] and dead 3T3-L1 adipocytes [G]. (H) mRNA level of Fas in TNFα-treated 3T3-L1 adipocytes. (I) Percentage of dead 3T3-L1 adipocytes. ($^*$) P < 0.05 versus all other groups and ($^{\dagger}$) P < 0.05 [t-test or one-way ANOVA with Bonferroni post-hoc test]. All data represent mean ± SEM.
via adipocyte CD1d is an indispensable step in iNKT cell-mediated adipocyte death in DIO.

**Activation of iNKT cells causes healthy adipose tissue remodeling by inducing adipogenesis**

In adulthood, the ratio of adipocyte birth to adipocyte death is quite constant, which contributes to the maintenance of adipose tissue homeostasis (Salans et al. 1973; Jo et al. 2009). To examine the dynamics of adipocyte turnover by iNKT cell activation, we evaluated adipose tissue 1 wk after α-GC treatment of HFD-fed mice. Histological analysis revealed that small adipocytes were increased in α-GC-treated obese adipose tissue (Fig. 6A,B). When adipocyte differentiation is initiated in adipocyte progenitor cells, it seems that they actively proliferate to secure the number of progenitor cells, which is required for both adipocyte differentiation and maintenance of progenitor cells (Rodeheffer et al. 2008; Lee et al. 2012; Jeffery et al. 2015). Further, adipocyte progenitor cells start to accumulate lipid metabolites during adipogenesis (Lee et al. 2004). The proliferation rate and granularity of adipocyte progenitor cells were analyzed by FACS from two populations such as CD31−CD45−PDGFRα+ cells and CD31−CD45−CD29−CD34+ cells (Jeffery et al. 2015). Upon α-GC treatment, the proliferation rate and granularity of adipocyte progenitor cells were analyzed by FACS from two populations such as CD31−CD45−PDGFRα+ cells and CD31−CD45−CD29−CD34+ cells.
progenitor cells (CD31−CD45−PDGFRα+Ki67+) was increased 4.2-fold (Fig. 6C) and 1.8-fold (Fig. 6D), respectively, in WT mice, but not in Jα18 KO mice (Supplemental Fig. S5A,B). Similarly, the proliferation rate and granularity of another type of adipocyte progenitor cells (CD31−CD45−CD29+CD34+) was increased upon α-GC treatment (Fig. 6E,F). Next, after adipocyte progenitor cells (CD31−CD45−PDGFRα+) were sorted by FACS,
mRNA expression was analyzed. As shown in Figure 6G, the mRNA level of Pparg2 and Cebpa were up-regulated 1.7-fold and 2.0-fold, implying that adipocyte differentiation might be potentiated after activation of iNKT cells to replace dead adipocytes in DIO.

To investigate the origin of small adipocytes induced by α-GC treatment, we decided to utilize an adipocyte lineage-tracing mouse model (AdiponectinrtTA; TRE-Cre; Rosa26-loxp-stop-loxp-YFP) (Wang et al. 2013) in which adiponectin-expressing adipocytes express YFP in a doxycycline (Dox)-inducible manner (Fig. 6H). The mice were fed HFD for 6 wk, followed by Dox-containing HFD for 2 wk to label mature adipocytes with YFP [Supplemental Fig. S5C-E]. After withdrawing Dox, α-GC was administered. One week after α-GC administration, newly differentiated (perilipin-positive and YFP-negative) adipocytes were observed by microscopy (Fig. 6I). As shown in Figure 6J,K, newly differentiated small adipocytes were evidently observed in α-GC-treated, but not Veh-injected mice. To determine whether newly differentiated small adipocytes induced by iNKT cell activation would contribute to insulin sensitivity in adipose tissue, we performed an insulin-dependent glucose uptake assay using a Cy3-tagged glucose bioprobe. In α-GC-treated obese mice, small adipocytes exhibited elevated insulin-dependent glucose uptake ability compared with large adipocytes (Fig. 6L, M), which might contribute to improved insulin resistance in EAT and whole body of DIO mice [Supplemental Fig. S5F,G]. Overall, these data suggest that iNKT cells can trigger healthy adipose tissue remodeling in obese mice by modulating adipogenesis.

Discussion

Adipocyte death is frequently observed in obesity (Cinti et al. 2005; Strissel et al. 2007; Spalding et al. 2008). However, the causes and physiological implications of adipocyte death have not been fully understood. There are significant technical limitations to in vivo analysis of adipocyte death, because adipocytes are floating, physically fragile, and too large to be analyzed by conventional microscopes and FACS. Here, we tried to overcome these issues by adopting new approaches to the analysis of large adipocytes and we investigated the physiological functions of adipocyte death in obesity. Our findings demonstrated that adipose iNKT cells stimulated death of hypertrophic adipocytes with pro-inflammatory characters. In obese adipose tissue, FasL-positive iNKT cells were increased and removed hypertrophic Fas-positive adipocytes. Interestingly, activation of iNKT cells not only induced adipocyte death, but also involved in stimulation of de novo adipogenesis in obesity, which eventually led to improved insulin-dependent glucose uptake ability in adipose tissue (Fig. 7). Taken together, these data suggest that adipose iNKT cells play an important role in the regulation of adipocyte turnover, contributing to maintenance of adipose tissue homeostasis in obesity.

iNKT cells exert effector functions through FasL expression. Also, iNKT cells mediate effector functions via activation of NK cells that secrete perforin/granzyme (Wilson and Delovitch 2003). However, given that perforin KO mice do not show any change in dead adipocytes in obesity (Revelo et al. 2015), it seems that the perforin/
granzyme pathway is not crucial in obesity-induced adipocyte death. Here, we found that iNKT cells were abundantly localized around dead adipocytes and FasL-positive iNKT cells augmented adipocyte death. Given that absolute number of cytotoxic iNKT cells was increased in obese adipose tissue, it is likely that adipose iNKT cells would elevate cytotoxic capacity. To entail iNKT cell-mediated adipocyte death, it seems that iNKT cells have to be activated via adipocyte CD1d and that FasL of activated iNKT cells has to bind to Fas in adipocytes. Accordingly, our data revealed that hypertrophic adipocytes with pro-inflammatory characters activated iNKT cells and up-regulated FasL in iNKT cells. Nonetheless, it remains elusive which kinds of endogenous lipid antigens might be presented by adipocyte CD1d in obesity. Identification of endogenous lipid antigens would improve our understanding of the roles of iNKT cells in the regulation of adipocyte turnover.

We found that death of hypertrophic Fas-positive adipocytes was induced by FasL-positive iNKT cells in obese adipose tissue. In iNKT cell-depleted Jα18 KO mice, hypertrophic adipocytes were retained in obese adipose tissue, resulting in unhealthy adipose tissue expansion upon obesity. Hypertrophic Fas-positive adipocytes induced by saturated fatty acids exhibited pro-inflammatory characters and they were removed by activated iNKT cells. It has been demonstrated that hypertrophic or Fas-positive adipocytes per se secrete pro-inflammatory cytokines, such as IL-1α, IL-1β, IL-6, and CCL2, aggravating adipose tissue inflammation, insulin resistance, and type 2 diabetes (Salans et al. 1968; Brook and Lloyd 1973; Schaub et al. 2003; Farley et al. 2008; Wueest et al. 2010; Blüher et al. 2014; Cotillard et al. 2014). Clearance of Fas-positive cells by FasL is one of the major mechanisms inducing apoptosis without eliciting improper immune responses [Nagata and Tanaka 2017]. It has been demonstrated that targeted apoptosis in adipocytes through activation of Caspase-8, a key downstream component of Fas signaling, attenuates adipose tissue inflammation by recruiting anti-inflammatory macrophages [Pajvani et al. 2005; Fischer-Posovszky et al. 2011b]. In addition, macrophages that phagocyte apoptotic cells are able to secrete anti-inflammatory cytokines, such as IL-10, transforming growth factor-β, platelet activating factor, and prostaglandin E₂, to down-regulate pro-inflammatory responses [Fadok et al. 1998; Gao et al. 1998; Xu et al. 2006; Szondy et al. 2017]. In EAT of HFD-fed mice, α-GC administration altered the abundance of pro-inflammatory macrophages (CD11b⁺F4/80⁺CD11c⁺) and anti-inflammatory macrophages (CD11b⁺F4/80⁺CD206⁺) [Supplemental Fig. S5H,I]. It seems that the relative ratio of anti-inflammatory to pro-inflammatoery macrophages might be increased from 4 d after iNKT cell activation. Thus, it is feasible to speculate that apoptotic removal of hypertrophic and pro-inflammatory Fas-positive adipocytes by FasL-positive iNKT cells would contribute to the suppression of pro-inflammatory responses in obese adipose tissue.

Emerging evidence suggests that adipocyte hypertrophy is an indicator of adipose tissue dysfunction and leads to insulin resistance and dyslipidemia in obesity, whereas adipocyte hyperplasia plays relatively beneficial roles in the relief of metabolic complications (Salans et al. 1968; Stern et al. 1972; Cotillard et al. 2014; An et al. 2017). However, the factors that modulate the fate of adipose tissue expansion into hypertrophy or hyperplasia have not been fully elucidated. Our data suggest that adipose iNKT cells would suppress unhealthy adipose tissue expansion by removing hypertrophic adipocytes and promoting the generation of small adipocytes. The new and small adipocytes exhibited a stronger glucose uptake capacity than old and hypertrophic adipocytes, probably leading to improvement of insulin resistance. Although it remains to be clarified how adipogenesis is promoted by activated iNKT cells in obesity, macrophages reportedly can cause proliferation and differentiation of PDGFRα-positive adipocyte progenitor cells [Lee et al. 2013; Hill et al. 2018], and iNKT cells interact with macrophages around dead adipocytes [Lynch et al. 2015]. Moreover, it has been suggested that FasL might promote proliferation and differentiation of adipocyte progenitor cells [Solodov et al. 2018]. Thus, elucidation of ternary relations between adipocytes, iNKT cells, and macrophages in adipocyte turnover will be helpful to decipher the dynamic regulation of adipose tissue remodeling.

Numerous studies have highlighted T cell cytotoxicity as an immunotherapeutic approach to remove harmful or dysregulated cells [Fesnak et al. 2016; June et al. 2018]. However, the application of such immunotherapy for the treatment of metabolic disorders such as obesity and type 2 diabetes has not been explored. It is feasible to speculate that iNKT cells would be able to sense and respond to imbalanced lipid metabolites in obese adipose tissue because they are activated by recognizing lipid antigens loaded onto CD1d. Among several tissues, such as adipose tissue, liver, and spleen, where iNKT cells are abundant, adipose iNKT cells prominently expressed FasL in DIO and genetically obese mice. Both Fas and FasL are up-regulated in obese and diabetic patients [Fischer-Posovszky et al. 2004; Blüher et al. 2014] and Fas-mediated apoptosis provokes adipocyte loss in patients with acquired lipodystrophy disorders [Hussain and Garg 2016]. Nevertheless, the sources of FasL have not been yet elucidated. Further, even though iNKT cells are abundant in human adipose tissue [Lynch et al. 2012], their roles in adipocyte pathophysiology have not been fully defined. Based on our findings, we believe that the cytotoxic roles of iNKT cells in human adipose tissue are worth being considered in the fight against metabolic dysfunction.

In conclusion, we have elucidated a novel role of adipose iNKT cells in the regulation of adipocyte turnover, potentially leading to healthy adipose tissue expansion. In obesity, activated adipose iNKT cells participate in adipose tissue remodeling through the stimulation of damaged adipocyte death as well as the induction of adipogenesis [Fig. 7]. Collectively, our data suggest that adipocyte turnover by iNKT cells is a key process to maintain adipose tissue homeostasis.
Materials and methods

Animals and treatments

C57BL6/J mice were obtained from Central Lab Animal Incorporation. After 1 wk of acclimatization, 8-wk-old mice were fed a NCD or a 60% HFD purchased from Research Diets Incorporation for 8 wk. db/db mice were purchased from Central Lab Animal Incorporation and sacrificed at 12 wk of age. Ja18 heterozygous mice were bred to generate WT mice and knock-out (KO) littermates. Adipocyte-specific CD1d KO (CD1d<sup>AKO</sup>) mice were generated by crossing adiponectin Cre mice with CD1d<sup>flow</sup> mice (C57BL/6-C1d<sup>tm1<sub>Abken</sub></sup>/J, The Jackson Laboratory). AdipoChaser mice [Adiponectin<sup>−<sub>TA</sub></sup>, TRE-Cre, Rosa26-loxp-stop-loxp-YFP] were kindly provided by Dr. Philipp Scherer (UT Southwestern). AdipoChaser mice were kept on a HFD for 6 wk and were then fed a Dox (600 mg/kg)-containing HFD for 2 wk. To activate iNKT cells, α-GC (1 μg/mouse; Adipogen) was intraperitoneally injected. Glucose tolerance test was performed using intraperitoneal glucose injection (1 g/kg body weight) after overnight fasting. All experiments with mice were approved by the Seoul National University Institutional Animal Care and Use Committee (SNUIACUC). Neutralizing anti-FasL monoclonal IgG (BioLegend) was previously described (Hattori et al. 1997). HFD-fed WT mice were incubated intraperitoneally with 300 μg of anti-FasL antibody, followed by α-GC injection. Age-matched control mice were treated similarly with control IgG. Mice were monitored 1 d after α-GC injection to investigate whether neutralization of FasL affects iNKT cell-induced adipocyte death.

Primary adipocyte generation and analysis

Adipocyte fractionation from whole epididymal adipose tissue was conducted as previously described (Lee et al. 2011). Briefly, adipose tissues were digested with type I collagenase for 30 min in a water bath at 37°C with shaking. After centrifugation at 1000 rpm and at room temperature (RT) for 5 min, floating adipocytes were collected. Primary adipocytes were washed several times with phosphate-buffered saline (PBS). Primary adipocytes were incubated with fluorescence-labeled Fas mAb (eBioscience) at RT for 1 h to detect Fas-positive adipocytes. To distinguish dead/dying adipocytes from live adipocytes, primary adipocytes were stained with propidium iodide (PI, eBioscience) or TUNEL assay (Roche) at RT for 1 h. After staining, the primary adipocytes were analyzed using a BioSorter instrument (Union Biometrica) following the manufacturer’s instruction.

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described (Ham et al. 2016). Whole epididymal adipose tissues were removed and fixed with 4% paraformaldehyde for 1 h. The samples were blocked with 1% horse serum for 1 h and then stained with primary antibody at 4°C overnight. Monoclonal antibodies (mAbs) against PERILIPIN-1 (Fitzgerald), CD11b (eBioscience), phycocrythrin-conjugated PBS57-loaded CD1d-tetramer (National Institutes of Health Tetramer Core Facility), and GFP (Santa Cruz Biotechnology) were used to detect adipocytes, macrophages, iNKT cells, and YFP-positive adipocytes, respectively. After a 1-h wash, the samples were incubated with fluorescence-labeled secondary antibody at RT for 4 h. After incubation with mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Lab Incorporation), and the samples were observed using a Zeiss LSM 700 confocal microscope (Carl Zeiss).

Flow cytometry and fluorescence-assisted cell sorting (FACS)

Flow-cytometric iNKT cell analysis was carried out as previously described [Shin et al. 2017]. The levels of FasL in iNKT cells and DN3.2.D3 cells, an iNKT cell hybridoma cell line, were assessed using FasL mAb (eBioscience). Fas expression and the frequency of dead 3T3-L1 adipocytes were investigated using Fas mAb (eBioscience) and PI, respectively. The proliferation rate was analyzed using Ki67 mAb (eBioscience). Macrophages were analyzed using CD11b, F4/80, CD11c, and CD206 mAbs (eBioscience). Cells were analyzed using a FACS Canto II instrument (BD Biosciences). To isolate adipocyte progenitor cells, epididymal adipose tissues were fractionated into adipocytes and stromal vascular cells (SVCs) and SVCs were stained using CD29 (BioLegend), CD34, CD31, CD45, and PDGFRα (BD Biosciences) mAbs. After SVCs were stained with mAbs against, CD31 “CD45−PDGFRα+”, adipocyte progenitor cells were sorted using a FACS Aria II (BD Biosciences) at the National Center for Inter-university Research Facilities (NCIRF) at Seoul National University.

Coculture

3T3-L1 pre-adipocytes were grown to confluence and then differentiated in 12-well culture plates. Differentiated 3T3-L1 adipocytes were pretreated with α-GC (100 ng/mL) for 4 h and then washed with PBS. DN3.2.D3 cells (1 × 10<sup>5</sup>) were incubated with differentiated 3T3-L1 adipocytes for 48 h. FFA treatment (500 μM) was conducted as previously described [Kim et al. 2015]. Briefly, FFAs were dissolved in ethanol and diluted in DMEM containing 1% fetal bovine serum and 2% bovine serum albumin (BSA) at 55°C for 10 min. BSA-conjugated FFA-containing media were used for challenging differentiated 3T3-L1 adipocytes for 2 wk at 2-d intervals. Then, the cells were challenged with TNFs [10 nM] for 1 d. Dead adipocytes and FasL-positive DN3.2.D3 cells were analyzed by FACS.

Apoptosis assay

The frequency of apoptotic adipocytes was determined using an in situ cell death detection kit (Roche) according to the manufacturer’s protocol. TUNEL-positive cells were analyzed under a microscope, and statistical analysis was performed using LSM510 software (Carl Zeiss). For the detection of annexin V-positive cells, we used the annexin V staining kit purchased from BD Biosciences. According to the manufacturer’s protocol, the frequency of annexin V-positive adipocytes was analyzed using FACS.

Ex vivo glucose bioprobe uptake assay

Ex vivo glucose bioprobe uptake was assayed as previously described [Kim et al. 2015].

Western blot analysis

Western blotting was performed as previously described [Sohn et al. 2018].

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was carried out as previously described [Lee et al. 2017]. The mRNA levels of cyclophilin were used for normalization. Sequence information for the primers used is provided in Supplemental Table S1.
siRNA duplexes for Cd1d1, caspase-3, caspase-8, and Fas were synthesized at Bioneer Incorporation. siRNAs were delivered into 3T3-L1 cells by electroporation. As a negative control, a scrambled siRNA (siNC) was used.

**Statistical analysis**

Data are reported as the mean ± standard error of the mean (SEM). Means were compared by two-tailed Student’s t-tests or two-way ANOVA with a Bonferroni post-hoc test. Statistical analyses were considered significant at $P < 0.05$.

**Acknowledgments**

We would like to thank Dr. Philipp Scherer for providing the adipocyte lineage-tracing mouse model. The authors thank the National Institutes of Health Tetramer Core Facility for the generous gift of CD1d tetramers. This study was supported by the National Research Foundation (NRF) grant funded by the Korean government (MIST) (2011-0018312, J.B.K., 2017M3A9C4068956, J.P.). J.P. (Seoul National University) J.K., S.M.H., K.C.S., and Y.G.J. were supported by the BK21 plus program.

**Author contributions** J.P. (Seoul National University) designed and conducted the study and wrote the paper. J.Y.H., J.O., J.I.K., Brennan MJ, Brenner MB. 2013. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat Rev Immunol 13: 101–117. doi: 10.1038/nri3369

Brook CG, Lloyd JK. 1973. Adipose cell size and glucose tolerance in obese children and effects of diet. Arch Dis Child 48: 301–304. doi: 10.1136/adc.48.4.301

Choe SS, Shin KC, Ka S, Lee YK, Chun JS, Kim JB. 2014. Macrophage HIF-2α ameliorates adipose tissue inflammation and insulin resistance in obesity. Diabetes 63: 3359–3371. doi: 10.2373/db13-1965

Choe SS, Huh JY, Hwang II, Kim JJ, Kim JB. 2016. Adipose tissue remodeling: its role in energy metabolism and metabolic disorders. Front Endocrinol (Lausanne) 7: 30.

Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Ohin MS. 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 46: 2347–2355. doi: 10.1194/jlr.M50294-3LR200

Cottillard A, Portou C, Torcivia A, Bouillot JL, Dietrich A, Klöting N, Grégoire C, Lolmede K, Blüher M, Clément K. 2014. Adipocyte size threshold matters: link with risk of type 2 diabetes and improved insulin resistance after gastric bypass. J Clin Endocrinol Metab 99: E1466–E1470. doi: 10.1210/jc.2014-1074

DiSpigno JR, Mathis D. 2015. Immunological contributions to adipose tissue homeostasis. Semin Immunol 27: 315–321. doi: 10.1016/j.smim.2015.10.005

Domingo P, Matias-Guiu X, Pujol RM, Francia E, Lagarda E, Sambeat MA, Vázquez G. 1999. Subcutaneous adipocyte apoptosis in HIV-1 protease inhibitor-associated lipodystrophy. AIDS 13: 2261–2267. doi: 10.1097/00002030-199911120-00008

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest 101: 890–898. doi: 10.1172/JCI1112

Farley SM, Purdy DE, Ryabinina OP, Schneider P, Magun BE, Iordanov MS. 2008. Fas ligand-induced proinflammatory transcriptional responses in reconstructed human epidermis. Recruitment of the epidermal growth factor receptor and activation of MAP kinases. J Biol Chem 283: 919–928. doi: 10.1074/jbc.M705852200

Fesnak AD, June CH, Levine BL. 2016. Engineered T cells: the promise and challenges of cancer immunotherapy. Nat Rev Cancer 16: 566–581. doi: 10.1038/nrc.2016.97

Fink SL, Cookson BT. 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun 73: 1907–1916. doi: 10.1128/IAI.73.4.1907-1916.2005

Fischer-Posovszky P, Tornqvist H, Debatin KM, Wahракти M. 2004. Inhibition of death-receptor mediated apoptosis in human adipocytes by the insulin-like growth factor I (IGF-I)/IGF-I receptor autocrine circuit. Endocrinology 145: 1849–1859. doi: 10.1210/en.2003-0985
Fischer-Posovszky P, Hebestreit H, Hofmann AK, Strauss G, Möller P, Debatin KM, Wabitsch M. 2006. Role of CD95-mediated adipocyte loss in autoimmune lipodystrophy. *J Clin Endocrinol Metab* 91: 1129–1135. doi:10.1210/jc.2005-0737

Fischer-Posovszky P, Keuper M, Nagel S, Hesse D, Schürmann A, Debatin KM, Strauss G, Wabitsch M. 2011a. Downregulation of FLIP by cycloheximide sensitizes human fat cells to CD95-induced apoptosis. *Exp Cell Res* 317: 2200–2209. doi:10.1016/j.yexcr.2011.06.016

Fischer-Posovszky P, Wang QA, Asterholm IW, Rutkowski JM, Scherer PE. 2011b. Targeted deletion of adipocytes by apoptosis leads to adipose tissue recruitment of alternatively activated M2 macrophages. *Endocrinology* 152: 3074–3081. doi:10.1210/en.2011-1031

Gao Y, Herndon JM, Zhang H, Griffith TS, Ferguson TA. 1998. Antiinflammatory effects of CD95 ligand (FasL)-induced apoptosis. *J Exp Med* 188: 887–896. doi:10.1084/jem.188.5.887

Gaya M, Barral P, Burbage M, Aggarwal S, Montaner B, Warren Navia A, Aid M, Tsui C, Maldonado P, Nair U, et al. 2018. Initiation of antiviral B cell immunity relies on innate signals from spatially positioned NKT cells. *Immunity* 49: 1361–1365. doi:10.1016/j.immuni.2018.07.006

Giordano A, Murano I, Mondini E, Perugini J, Smorlesi J, Severi I, Barazzoni R, Scherer PE, Cinti S. 2013. Obese adipocytes show ultrastructural features of stressed cells and die of pyroptosis. *J Lipid Res* 54: 2423–2436. doi:10.1194/jlr.M038638

Gustafson B, Gogg S, Hedjadijar S, Jendahl L, Hammarstedt A, Smith U. 2009. Inflammation and impaired adipogenesis in hypertrophic obesity in man. *Am J Physiol Endocrinol Metab* 297: E999–E1003. doi:10.1152/ajpendo.00377.2009

Ham M, Lee JW, Choi AH, Jang H, Choi G, Park J, Kozuka C, Sears DD, Masuakzi K, Kim JB. 2013. Macrophage glucose-6-phosphate dehydrogenase stimulates proinflammatory responses with oxidative stress. *Mol Cell Biol* 33: 2425–2435. doi:10.1128/MCB.01260-12

Ham M, Choe SS, Shin KC, Choi G, Kim JW, NoH JR, Kim YH, Ryu JW, Yoon KH, Lee CH, et al. 2016. Glucose-6-Phosphate dehydrogenase deficiency improves insulin resistance with reduced adipose tissue inflammation in obesity. *Diabetes* 65: 2624–2638. doi:10.2337/db16-0060

Hattori K, Hirano T, Ushiyama C, Miyajima H, Yamakawa N, Ebata T, Wada Y, Ikeda S, Yoshino K, Tateno M, et al. 1997. A metalloproteinase inhibitor prevents lethal acute graft-versus-host disease in mice. *Blood* 90: 348–358. doi:10.1182/blood.V90.2.348

Hill DA, Lim HW, Kim YH, Ho WY, Foong YH, Nelson VL, Nguyen HCB, Chegreddy K, Kim J, Habertheruer A, et al. 2018. Distinct macrophage populations direct inflammatory versus physiological changes in adipose tissue. *Proc Natl Acad Sci* 115: E5096–E5105. doi:10.1073/pnas.1802611115

Huh JY, Kim JJ, Park YJ, Hwang IJ, Lee YS, Sohn JH, Lee SK, Alfaada AA, Kim SS, Choi SH, et al. 2013. A novel function of adipocytes in lipid antigen presentation to iNKT cells. *Mol Cell Biol* 33: 328–339. doi:10.1128/MCB.00552-12

Huh JY, Park YJ, Ham M, Kim JB. 2014. Crosstalk between adipocytes and immune cells in adipose tissue inflammation and metabolic dysregulation in obesity. *Mol Cells* 37: 365–371. doi:10.1007/s10059-014-0074

Huh JY, Park J, Kim JJ, Park YJ, Lee YK, Kim JB. 2017. Deletion of CD1d in adipocytes aggravates adipose tissue inflammation and insulin resistance in obesity. *Diabetes* 66: 835–847. doi:10.2337/db16-1122

Huh JY, Park YJ, Kim JB. 2018. Adipocyte CD1d determines adipose inflammation and insulin resistance in obesity. *Adipocyte* 7: 129–136.
tumorigenesis by inhibiting the SREBP1c-PTTG1 axis in kidney cancer. Mol Cell Biol 37: e00265-17. doi:10.1128/MCB .00265-17

Li C, DiSpirito JR, Zemmour D, Spallanzani RG, Kuswanto W, Benoist C, Mathis D. 2018. TCR transgenic mice reveal stepwise, multi-site acquisition of the distinctive fat-Treg phenotype. Cell 174: 285–299.e12. doi:10.1016/j.cell.2018.05.004

Lowin B, Hahne M, Mattmann C, Tschopp J. 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature 370: 650–652. doi:10.1038/370650a0

Lynch L, O’Shea D, Winter DC, Geoghegan J, Doherty DG, O’Farrell C. 2009. Invariant NKT cells and CD1d+ cells amass in human omentum and are depleted in patients with cancer and obesity. Eur J Immunol 39: 1893–1901. doi:10.1002/eji .200939349

Lynch L, Nowak M, Varghese B, Clark J, Hogan AE, Toxavidis V, park YJ, Park J, Huh JY, Hwang I, Choe SS, Kim JB. 2018. Regulation of the inflammatory response of natural killer T cells to a glycolipid antigen using IL-10-producing NKT10 cells are a distinct regulatory invariable NKT cell subset. Front Immunol 9: 1311. doi:10.3389/fimmu .2018.01311

Prins JB, Walker NI, Winterford CM, Cameron DP. 1994. Human adipocyte apoptosis occurs in malignancy. Biochem Biophys Res Commun 205: 625–630. doi:10.1016/hbr.1994.2711

Revelo XS, Tsai S, Lei H, Luck H, Ghazarian M, Tsui H, Shi SY, Schroer S, Luk CT, Lin GH, et al. 2015. Perforin is a novel immunoregulatory molecule in immune cells and macrophages in adipose tissue. Nature 514: 85–95. doi:10.1038/nature13780

Farrell PLZF and control the homeostasis of Treg cells and macrophages in adipose tissue. Nat Immunol 16: 37: 2918. doi:10.2337/db07-0767

Shao M, Vishvanath L, Busbuso NC, Hepler C, Shan B, Sharma AX, Chen S, Yu X, An YA, Zhu Y, et al. 2018. De novo adipocyte differentiation from Pdgfrα-preadipocytes protects against pathologic visceral adipose expansion in obesity. Nat Commun 9: 890. doi:10.1038/s41467-018-03196-x

Shepherd PR, Gnudi L, Tozzo E, Yang H, Leach F, Kahn BB. 1993. Adipocyte cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. J Biol Chem 268: 22243–22246.

Shin KC, Hwang I, Choe SS, Park J, Ji Y, Kim J, Lee GY, Choi SH, Ching J, Kovalik JP, et al. 2017. Macrophage VLDLR mediates obesity-induced insulin resistance with adipose tissue inflammation. Nature 548: 818–823. doi:10.1038/nature23080

Sohn JH, Lee YK, Han JS, Jeon YG, Kim JI, Choe SS, Kim JI, Lee GY, Choi SH, Ching J, Kovalik JP, et al. 2017. Macrophage VLDLR mediates obesity-induced insulin resistance with adipose tissue inflammation. Nature 548: 818–823. doi:10.1038/nature23080

Sato M, Hoshino M, Fujita K, Iizuka M, Fujii S, Clingan CS, Van Kaer L, Iwabuchi K. 2016. Adipocyte-specific CD1d-deficiency mitigates diet-induced obesity and insulin resistance in mice. Sci Rep 6: 28473. doi:10.1038/srep28473

Schaub FJ, Liles WC, Ferri N, Sayson K, Seifert RA, Bowen-Pope DF. 2003. Fas and Fas-associated death domain protein regulate monocyte chemoattractant protein-1 expression by human smooth muscle cells through caspase- and calpain-dependent release of interleukin-1α. Circ Res 93: 515–522. doi:10.1161/01.RES.0000092305.42313.7C

Schipper HS, Rakhshandehroo M, van de Graaf SF, Venken K, Schaub FJ, Liles WC, Ferri N, Sayson K, Seifert RA, Bowen-Pope DF. 2003. Fas and Fas-associated death domain protein regulate monocyte chemoattractant protein-1 expression by human smooth muscle cells through caspase- and calpain-dependent release of interleukin-1α. Circ Res 93: 515–522. doi:10.1161/01.RES.0000092305.42313.7C

Shin KC, Hwang I, Choe SS, Park J, Ji Y, Kim J, Lee GY, Choi SH, Ching J, Kovalik JP, et al. 2017. Macrophage VLDLR mediates obesity-induced insulin resistance with adipose tissue inflammation. Nature 548: 818–823. doi:10.1038/nature23080

Sohn JH, Lee YK, Han JS, Jeon YG, Kim JI, Choe SS, Kim JI, Lee GY, Choi SH, Ching J, Kovalik JP, et al. 2017. Macrophage VLDLR mediates obesity-induced insulin resistance with adipose tissue inflammation. Nature 548: 818–823. doi:10.1038/nature23080

Sato M, Hoshino M, Fujita K, Iizuka M, Fujii S, Clingan CS, Van Kaer L, Iwabuchi K. 2016. Adipocyte-specific CD1d-deficiency mitigates diet-induced obesity and insulin resistance in mice. Sci Rep 6: 28473. doi:10.1038/srep28473

Schaub FJ, Liles WC, Ferri N, Sayson K, Seifert RA, Bowen-Pope DF. 2003. Fas and Fas-associated death domain protein regulate monocyte chemoattractant protein-1 expression by human smooth muscle cells through caspase- and calpain-dependent release of interleukin-1α. Circ Res 93: 515–522. doi:10.1161/01.RES.0000092305.42313.7C

Schipper HS, Rakhshandehroo M, van de Graaf SF, Venken K, Schaub FJ, Liles WC, Ferri N, Sayson K, Seifert RA, Bowen-Pope DF. 2003. Fas and Fas-associated death domain protein regulate monocyte chemoattractant protein-1 expression by human smooth muscle cells through caspase- and calpain-dependent release of interleukin-1α. Circ Res 93: 515–522. doi:10.1161/01.RES.0000092305.42313.7C

Shin KC, Hwang I, Choe SS, Park J, Ji Y, Kim J, Lee GY, Choi SH, Ching J, Kovalik JP, et al. 2017. Macrophage VLDLR mediates obesity-induced insulin resistance with adipose tissue inflammation. Nature 548: 818–823. doi:10.1038/nature23080

Sohn JH, Lee YK, Han JS, Jeon YG, Kim JI, Choe SS, Kim JI, Yoo HJ, Kim JB. 2018. Perilipin 1 [Pli1] deficiency promotes inflammatory responses in lean adipose tissue through lipid dysregulation. J Biol Chem 293: 13974–13988. doi:10.1074/jbc .RA118.003541

Solodove I, Meilik B, Volovitz I, Sela M, Manheim S, Yarkoni S, Zipori D, Gur E, Shani N. 2018. Fas-L promotes the stem cell potency of adipose-derived mesenchymal cells. Cell Death Dis 9: 695. doi:10.1038/s41419-018-0702-y

Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hofstedt J, Näslund E, Brönn T, et al. 2008. Dynamics of fat cell turnover in humans. Nature 453: 783–787. doi:10.1038/nature06902

Stern JS, Hollander N, Batchelor BR, Cohn CK, Hirsch J. 1972. Adipocyte cell size and immunoreactive insulin levels in obese and normal-weight adults. Lancet 300: 948–951. doi:10.1016/S0140-6736(72)92474-9

Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW 2nd, DeFuria J, Sick Z, Greenberg AS, Ohlin MS. 2007. Adipocyte death, adipose tissue remodeling, and obesity complications. Diabetes 56: 2910–2918. doi:10.2337/db07-0767

iNKT cells are required for adipocyte turnover
Sun K, Kusminski CM, Scherer PE. 2011. Adipose tissue remodelling and obesity. *J Clin Invest* **121**: 2094–2101. doi:10.1172/JCI45887

Szondy Z, Sarang Z, Kiss B, Garabuczi E, Köröskényi K. 2017. Anti-inflammatory mechanisms triggered by apoptotic cells during their clearance. *Front Immunol* **8**: 909. doi:10.3389/fimmu.2017.00909

Vishvanath L, MacPherson KA, Hepler C, Wang QA, Shao M, Spurgin SB, Wang MY, Kusminski CM, Morley TS, Gupta RK. 2016. Pdgfrβ+ mural preadipocytes contribute to adipocyte hyperplasia induced by high-fat-diet feeding and prolonged cold exposure in adult mice. *Cell Metab* **23**: 350–359. doi:10.1016/j.cmet.2015.10.018

Wang QA, Tao C, Gupta RK, Scherer PE. 2013. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. *Nat Med* **19**: 1338–1344. doi:10.1038/nm.3324

Wilson SB, Delovitch TL. 2003. Janus-like role of regulatory iNKT cells in autoimmune disease and tumour immunity. *Nat Rev Immunol* **3**: 211–222. doi:10.1038/nri1028

Wu D, Molofsky AB, Liang HE, Ricardo-Gonzalez RR, Jouihan HA, Bando JK, Chawla A, Locksley RM. 2011. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science* **332**: 243–247. doi:10.1126/science.1201475

Wueest S, Rapold RA, Schumann DM, Rytka JM, Schildknecht A, Nov O, Chervonsky AV, Rudich A, Schoenle EJ, Donath MY, et al. 2010. Deletion of Fas in adipocytes relieves adipose tissue inflammation and hepatic manifestations of obesity in mice. *J Clin Invest* **120**: 191–202. doi:10.1172/JCI38388

Xu W, Roos A, Schlagwein N, Woltman AM, Daha MR, van Kooten C. 2006. IL-10-producing macrophages preferentially clear early apoptotic cells. *Blood* **107**: 4930–4937. doi:10.1182/blood-2005-10-1444