이학박사학위논문

지방조직 염증반응 조절에 관여하는 지방세포 CD1d 유전자와 Invariant Natural Killer T 림프구의 역할 규명

Characterization of Adipocyte CD1d and Invariant Natural Killer T Cells in the Regulation of Adipose Tissue Inflammation

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ABSTRACT

Characterization of Adipocyte CD1d and Invariant Natural Killer T Cells in the Regulation of Adipose Tissue Inflammation

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Recent findings, notably on adipokines and adipose tissue inflammation, have revised the concept of adipose tissues being a mere energy storage depot. Instead, adipose tissues are emerging as endocrine and immunologically active organs with various effects on the regulation of systemic energy homeostasis. Compared with other metabolic organs such as liver and muscle, inflammatory response are dynamically regulated in adipose tissues and most of the immune cells in adipose tissues are involved in obesity-linked metabolic complications, including insulin resistance. Recently, most immune cells such as macrophages, granulocytes, helper T cells, cytotoxic T cells and B cells, have been implicated in the pathogenesis of adipose tissue inflammation. However, it has not been thoroughly understood yet how adipose tissue inflammation is initiated in obesity. To find out the causal factors for early response of adipose tissue inflammation, immune cell types and specific molecules have been investigated in high fat diet (HFD) fed obese mice. Notably, I found that there was significant changes of

adipose tissue iNKT cells, one of innate-like lymphocytes which recognize lipids as antigens. In this study, the roles of invariant natural killer T (iNKT) cells and the regulatory mechanisms of iNKT cell response in adipose tissue have been focused.

In chapter one, I have demonstrated that iNKT cells quantitatively decrease in adipose tissue of obese animals, and that activation-induced cell death (AICD) of iNKT cells would confer the decrement of iNKT cells in obesity. Moreover, iNKT cell-deficient Jα18 knockout (KO) mice became more obese and exhibited increased adipose tissue inflammation upon high fat diet (HFD). These findings suggest that iNKT cells would play a crucial role of anti-inflammatory response in adipose tissue inflammation in obesity.

In chapter two, I have examined the regulatory mechanisms of iNKT cell response in adipose tissues. It is of interest to observe that CD1d, a molecule involved in lipid antigen presentation to iNKT cells, was highly expressed in adipocytes and CD1d-expressing adipocytes stimulated iNKT cell activity through physical interaction. iNKT cell population and CD1d expression were reduced in adipose tissue of obese mice and humans compared to those of lean subjects. To investigate the role of adipocyte-expressing CD1d in the regulation of iNKT cell response in adipose tissue *in vivo*, I generated adipocyte specific CD1d KO mouse. In adipocyte CD1d KO mice, the absolute number of adipose iNKT cells decreased upon NCD and HFD. In addition, HFD-induced proliferation of iNKT cells was diminished in adipose tissue of adipocyte CD1d KO mice. These data suggest that adipocytes would regulate iNKT cell activity via CD1d molecules and the

interaction between adipocytes and iNKT cells may modulate adipose tissue inflammation in obesity.

Taken together, I suggest that excess energy intake would stimulate the activation of iNKT cells via adipocyte expressing CD1d molecules, which contributes to anti-inflammatory response in adipose tissue inflammation in obesity. However, reduction of CD1d expression in adipocytes would affect decrement of anti-inflammatory response mediated by iNKT cells, which results in accumulation of pro-inflammatory response in adipose tissues of late stage of obesity. Therefore, crosstalk between adipocytes and iNKT cells would be crucial to regulate adipose tissue inflammation in obesity.

Key words: obesity, adipose tissue inflammation, invariant natural killer T cell, adipocyte, CD1d, lipid antigen presentation

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BACKGROUNDS

1. Obesity and inflammation

1) Obesity-induced insulin resistance

The prevalence of obesity and overweight continues to rise, contributing increasingly to morbidity and mortality. In particular, obesity-induced insulin resistance is one of the key factors for the development of metabolic diseases such as hypertension, atherosclerosis, and type 2 diabetes (59). In obesity and type 2 diabetes, insulin resistance is manifested by decreased insulin-stimulated glucose disposal in skeletal muscle, enhanced lipolysis in adipose tissue, and impaired suppression of hepatic glucose output (42). These dysfunctions in metabolic tissues result in hyperglycemia and hyperlipidemia, which induces pancreatic β-cells to secret more insulin. However, with the aggravation of insulin resistance, β-cells are exhausted, and this leads to sustained hyperglycemia and type 2 diabetes (17). Therefore, insulin resistance has a crucial role in the pathogenesis of type 2 diabetes and metabolic complications. Subsequently, many groups have tried to elucidate the causal factors responsible for obesity-induced insulin resistance.

2) Tissue inflammation and insulin resistance

Recent findings have demonstrated that obesity is tightly associated with systemic chronic inflammation. There are accumulating evidences that cytokine production by expanded adipose tissue results in elevated serum levels of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 in obese individuals (36, 94). Subsequently, elevated inflammatory stimuli activate inflammatory signaling including IKKβ/NFκB and JNK pathways, which negatively regulates insulin action in metabolic organs such as adipose tissue, liver and skeletal muscle (82).

Abdominal adiposity frequently accompanies with accumulation of hepatic lipid and inflammation in obesity. It has been reported that elevation of hepatic triglyceride (TG) content, serum free fatty acid (FFA) concentration, and NF-κB activity is induced upon high fat diet (HFD). The mRNA levels of NF-κB target genes such as IL-6, IL-1β, and TNF-α are also raised in liver by HFD (13). These pro-inflammatory cytokines participate in the development of insulin resistance and the activation of Kupffer cells, the resident hepatic macrophages.

Skeletal muscle is another major site of insulin resistance in obesity and type 2 diabetes. It has been reported that there is no significant change in macrophage infiltration into skeletal muscle in obese mice (90). Moreover, muscle-specific IKK2 knockout (KO) mice did not improve insulin sensitivity (72). Taken together,

skeletal muscle is a target of inflammation-induced insulin resistance rather than a site of initiation of inflammatory response.

In severe obesity, it is observed that increased inflammatory response in both of adipose tissue and liver. In mouse model, elevation of inflammatory response including inflammatory gene expression and macrophage infiltration are shown in adipose tissues less than 1 week of HFD while there was no significant degree of inflammation observed in liver and muscle (50). Therefore, it is very likely that adipose tissue would be an initiating tissue of HFD-induced inflammation in obesity.

2. Adipose tissue inflammation

1) Immune cells in adipose tissue inflammation

Adipose tissue is composed of various cell types including adipocytes, fibroblasts, endothelial cells, and various immune cells. Immune cells within adipose tissue are largely categorized into innate and adaptive immune cells. Innate immune cells include macrophages, neutrophils, eosinophils, and mast cells, whereas adaptive immune cells include various subtypes of T cells and B cells. Furthermore, natural killer T cells, one type of innate lymphocyte, were recently reported as regulators of adipose tissue inflammation in obesity (Table 1). In lean adipose tissue, several anti-inflammatory immune cells such as M2 type

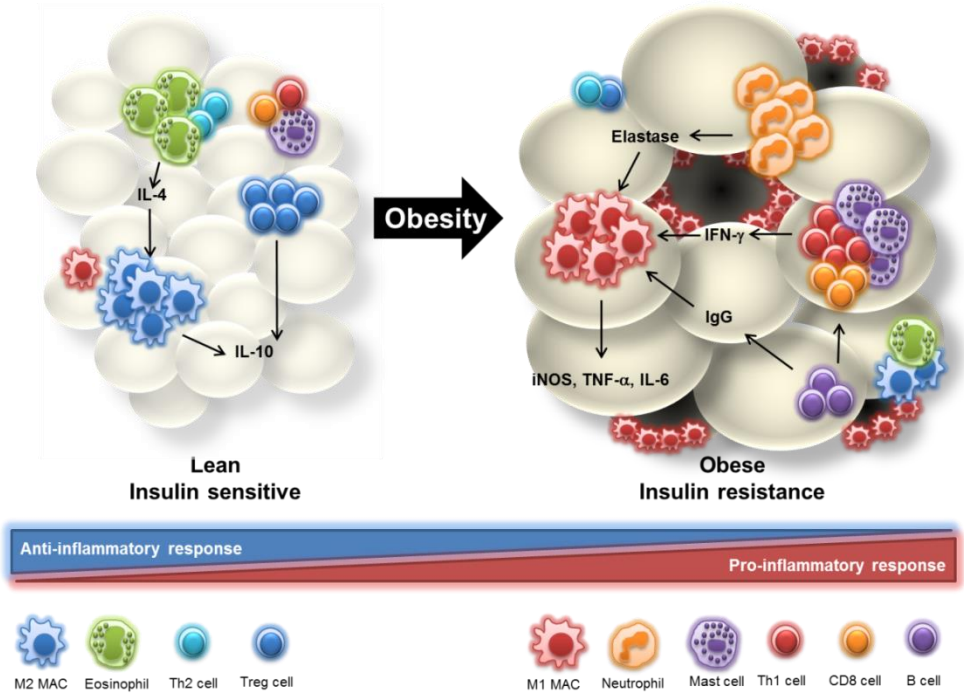
Table 1. Roles of immune cells in adipose tissue inflammation

Immune Cell	# changes in obese AT	Correlation with human	Fat mass of loss of function in each immune cell	Role of adipose tissue inflammation	Insulin sensitivity
Dendritic cell	↑	Yes	ND	ND	ND
Neutrophil	↑	ND	↑	Pro-inflammatory	↓
Mast Cell	↑	Yes	↓	Pro-inflammatory	↓
Eosinophil	↓	ND	↑	Anti-inflammatory	↑
B cell	↑	ND	↓ (VAT:SAT ratio)	Pro-inflammatory	↓
CD8+ T cell	↑	ND	↔	Pro-inflammatory	↓
CD4+ Th1	↑	ND	ND	Pro-inflammatory	↓
CD4+Treg	↓	Yes	ND	Anti-inflammatory	↑

↑, increased; ↓, decreased; ↔, not changed; ND, not determined.

Figure 1.

Role of immune cells in adipose tissue inflammation and insulin resistance. In lean adipose tissue, IL-4 secreted by eosinophils and Th2 cells activates M2 type macrophages, which express arginase and anti-inflammatory cytokines such as IL-10. Regulatory T (Treg) cells also play an important role in anti-inflammatory response via cell-cell contact or cytokine secretion involving IL-10. However, in obese adipose tissue the number of pro-inflammatory immune cells increases and that of anti-inflammatory immune cells decreases. Neutrophils, which are early responders to inflammatory response, infiltrate the adipose tissue where they secrete elastase and also stimulate M1 type macrophage infiltration and pro-inflammatory cytokine secretion. In addition, levels of IFN-γ–secreting cell types, such as Th1 cells, CD8 T cells, and mast cells, are elevated in obese adipose tissue. B cells also play a pro-inflammatory role through secretion of obesity-induced IgG.


IL-4
IL-10
Obesity
Elastase
IFN-γ
IgG
iNOS, TNF-α, IL-6
Lean
Insulin sensitive
Obese
Insulin resistance
Anti-inflammatory response
Pro-inflammatory response
M2 MAC
Eosinophil
Th2 cell
Treg cell
M1 MAC
Neutrophil
Mast cell
Th1 cell
CD8 cell
B cell

macrophages, regulatory T cells, and eosinophils are engaged in the maintenance of insulin sensitivity. However, during progression of obesity, changes in the number and activity of many immune cells contribute to prominent roles in the progression of insulin resistance via production of pro-inflammatory mediators (Figure 1).

2) Macrophages

Macrophages, tissue-resident phagocytes, perform various roles including scavenging cellular debris derived from apoptotic cells, regulating angiogenesis, and remodeling the extracellular matrix (17). Although macrophages comprise 10-15% of stromal vascular cells (SVCs) in visceral adipose tissues (VAT) of lean subjects, their numbers increased to 40-50% of the SVCs of VAT in obese humans and mouse models (90). Monocytes are differentiated into classically activated macrophages (M1) or alternatively activated macrophages (M2) upon stimulation. The major populations of adipose tissue macrophages (ATMs) that reside in lean adipose tissue are different from those residing in obese adipose tissues. For example, in the lean status, the predominant ATM population is M2 macrophage, which expresses high levels of arginase-1, the mannose receptor (CD206), and CD301 and secretes anti-inflammatory cytokines including IL-10 and IL-1 receptor antagonist (IL-1Ra). Th2 type cytokines such as IL-4, IL-10, and IL-13 stimulate the M2 polarization (17, 52). In contrast, in obesity, interferon (IFN)-γ and lipopolysaccharide (LPS) drive polarization of recruited monocytes toward

classically activated M1 type macrophages and promote the secretion of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, IL-12, and MCP-1 (33, 58). One of the key characteristics of M1 macrophages is the surface expression of CD11c proteins in addition to macrophage-specific markers such as F4/80 and CD11b. Previous studies have demonstrated that the major population of infiltrated M1 macrophages in adipose tissue originates from circulating monocytes in the blood (54). Interaction between MCP-1 and CCR2 appears to be crucial for obesity-induced macrophage infiltration into adipose tissue. Compared with M2 macrophages, accumulation of pro-inflammatory M1 macrophages in adipose tissue provokes whole body insulin resistance. For example, ablation of CD11c-positive cells leads to marked augmentation of insulin sensitivity, accompanied by diminished inflammatory response including macrophage infiltration and inflammatory cytokine gene expression in adipose tissue and lower levels of serum inflammatory cytokines (69).

3) T cells

T cells develop and mature in the thymus, and are then repopulated into peripheral tissues. T cells have various repertoires of T cell receptors and are able to discriminate self from non-self after negative and positive selection during their development in the thymus. Upon antigenic stimulation, T cells play key roles in the control of immune response for defense against foreign antigens. There are

various subpopulations of T cells, including CD4, CD8, and natural killer T (NKT) cells (38). Most subtypes of T cells are involved in the regulation of adipose tissue inflammation in obesity.

The number of total T cells increased in obese VAT in parallel with an increase in their proliferation and infiltration in response to adipose tissue-specific factors (8). Moreover, it has been shown that one of the T cell chemo-attractant factors, RANTES, is induced in both SVCs and adipocytes after activation by IFNγ and TNFα. Therefore, obesity-induced factors would contribute to quantitative and qualitative changes in T cell populations, leading to the accumulation of pro-inflammatory response in obese adipose tissues.

A. CD4 T cells

CD4 T lymphocytes recognize peptide antigens loaded on MHC class II molecules of antigen presenting cells. Naïve CD4 T cells differentiate into various subtypes of CD4 T cells such as Th1, Th2, Th17, and regulatory T (Treg) cells. In general, T cell differentiation is regulated by a variety of cytokines, including IFN-γ for Th1, IL-4 for Th2, IL-6 and TGF-β for Th17, and TGF-β for regulatory T cells (96). Th1 and Th17 cells mediate pro-inflammatory response whereas Th2 and Treg cells contribute to anti-inflammatory response.

Winer et al. have shown that adoptive transfer of wild-type (WT) CD4 T

cells into recombination activating gene (RAG)-null mice that lack lymphocytes results in decreased body weight gain and fat mass with improved glucose tolerance upon HFD (91). They suggested that Th2 cells, but not Treg cells, contribute to these beneficial effects on energy metabolism.

Regulatory T cells, characterized as CD4+CD25+Foxp3+, are a well-known anti-inflammatory T cell subtype. The proportion of Treg cells among CD4 T cells is relatively high in adipose tissue compared with spleen, lymph nodes, and lung. In addition, there is a positive correlation between the proportion of Treg cells and aged adipose tissue (24). The number of Treg cells decreases in adipose tissues of obese mice models such as *ob/ob, db/db,* and DIO relative to lean mice. Depletion of Treg cells in mice by diphtheria toxin (DT) aggravates adipose tissue inflammation and insulin resistance. On the other hand, expansion of Treg cells in mice by IL-2 injection attenuates adipose tissue inflammation and improves insulin sensitivity, in part through IL-10–mediated suppression of the proliferation of conventional T cells. Notably, VAT Treg cells have adipose tissue-specific T cell receptor (TCR) repertoires compared with splenic Treg cells but the identities of the antigens specific to VAT Treg cells remain to be explored.

Th1 cells and Th17 cells are immune cell types that play critical roles in the onset of autoimmune diseases and tissue inflammatory response. Th1 cells primarily secret IFN-γ, which stimulates monocyte differentiation into M1 type macrophages. IFN-γ treatment of adipose tissues ex-vivo results in an increase in IP-10, MIG, and TNFα, implying that IFN-γ exacerbates adipose tissue

inflammation in obesity (71). Consistent with the above observations, IFN-γ KO mice display improved insulin sensitivity, accompanied by a decrease in HFD-induced adipose tissue inflammation. In summary, it has been proposed that a relative decrease in anti-inflammatory cell types such as Th2 and Treg cells compared with pro-inflammatory cells such as Th1 is associated with the induction of infiltration of circulating monocytes and subsequent M1 polarization in obese adipose tissue.

B. CD8 T cells

CD8 T cells recognize peptide antigens loaded by MHC class I molecules on antigen presenting cells and participate in pro-inflammatory cytokine secretion and cytolysis of target cells. It has been reported that the number of CD8 T cells is elevated in obese adipose tissue. Nishimura et al. have shown that the percentage of CD8 T cells in SVCs increases upon 2 weeks of HFD feeding whereas macrophage infiltration is induced after 6 weeks of HFD feeding (64). Depletion of CD8 T cells by injection of anti-CD8 antibody into DIO mice results in a decrease in the levels of pro-inflammatory cytokines such as IL-6 and TNFα with augmented glucose tolerance and insulin sensitivity independent of obesity. Moreover, in co-culture experiments of CD8 T cells with macrophages, CD8 T cells induce macrophage differentiation from monocytes and cytokine secretion, confirming the critical role of CD8 T cells in the control of macrophage

polarization and activation.

3. Invariant Natural Killer T Cells

1) Classification of Natural Killer T (NKT) cells

Natural killer T (NKT) cells are simply defined as T cells expressing NK lineage receptors such as NK1.1. NKT cell is one of innate lymphocytes that bridge innate and adaptive immune response. There are three types of NKT cells, invariant NKT (iNKT, type I), non-invariant NKT (type II), and NKT-like cells (32). Invariant NKT and non-invariant NKT cells are CD1d-dependent, whereas NKT-like cells are CD1d-independent (Table 2). iNKT cells specifically recognize a variety of lipid antigens loaded on CD1d molecules and do not recognize peptide antigens on MHC molecules. Among lipid antigens, α-galactosylceramide (α-GC) is the most potent CD1d-binding lipid antigen for iNKT cell activation. α-GC loaded CD1d is recognized by iNKT cells that express invariant TCR chains such as Vα14Jα18 in mouse and Vα24Jα18 in human. Generally, type II NKT cells are difficult to identify, although some study has been used particular antigens including sulfatide and sulfatide-loaded CD1d tetramers to which selectively respond. To date, the best way to investigate the role of type II NKT cells *in vivo* is to compare phenotype of CD1d deficient mice (which lack both type I and type II NKT cells) with Jα18-deficient mice (which lack only type I NKT cells) (39).

Table 2. Classification of NKT cells

	Type I	Type II	Type III
Other name	Invariant NKT cells	Non-invariant NKT cells	CD1d independent NK1.1+ cells
CD1d dependence	Yes	Yes	No
Known antigens	α-galactosylceramide	Sulfatide	ND
TCR α-chain	Vα14Jα18 (mice) Vα24Jα18 (humans)	Diverse	Diverse
NK1.1(CD161)	+/-	+/-	+

NKT-like cells are complicated and not well studied populations as subpopulation of NKT cells which can develop without CD1d expression in the thymus.

2) Thymic development of invariant Natural Killer T (iNKT) cells

iNKT cells develop in the thymus from the CD4+CD8+ double positive (DP) thymocyte which is the same precursor population as MHC-restricted T cells (30). iNKT cell development depends on the stochastic generation of the canonical iNKT cell T cell receptor (TCR) such as Vα14Jα18 in mice and Vα24Jα18 in human. DP thymocytes, rather than thymic epithelial cells that are responsible for selection of conventional MHC-restricted T cells, are the major cell types that mediate the positive selection of iNKT cells. Displaying self-lipid antigens on CD1d molecules is necessary for this positive selection of iNKT cells. In spite of the fact that the identities of these self-lipid antigens remain unclear, plasmalogen lysophosphatidylethanolamine (pLPE) was recently identified as a candidate (23). Moreover, promyelocytic leukaemia zinc finger protein (PLZF) is known as ‘master regulatory transcription factor’ for development and effector phenotype of iNKT cells (49, 76). There are four stages in the development of iNKT cells in the thymus of mouse: stage 0 (CD24+CD44-NK1.1-), stage 1 (CD24-CD44-NK1.1-), stage 2 (CD24-CD44+NK1.1-) and stage 3 (CD24-CD44+NK1.1+). iNKT cells

leave the thymus mainly at stage 2, after which they can complete their maturation in the peripheral tissues.

3) iNKT cell subsets

Upon activation, iNKT cells have capability of rapid production of cytokines such as IFN-γ, TNF, IL-2, IL-13, IL-4, IL-5, IL-9, IL-10, IL-17, IL-21 and GM-CSF. Depending on their cytokine profiles, iNKT cells could be divided into 3 kinds of subsets; Th1-like iNKT cell, Th2-like iNKT cell, and Th17-like iNKT cell (9). Th1-like cells secrete IFN-γ and enhance cell-mediated immunity, which is associated with elevated microbial immunity, tumor rejection, and autoimmunity. IL-12 secreted by antigen presenting cells is known as one of the cytokines related to increment of Th1-type response of iNKT cells. On the other hand, Th2-like iNKT cells mainly secret IL-4, IL-10, and IL-13. Th2 type cytokine secretion has been associated with protection from progression to autoimmune diabetes in mouse models (80). Th17-like iNKT cells produce IL-17a, IL-21, and IL-22. It is reported that Th17-like iNKT cells are enriched in the peripheral lymph nodes and lungs. This subset of iNKT cells is known to respond to microbial infection and contribute to airway hyperreactivity in the lungs (45). In iNKT cells, production of certain type of cytokines seems to be mainly regulated by many factors including species of CD1d loaded lipid antigens, the types of antigen presenting cells, and ligand stability (73). As there are no particular markers for each type of iNKT cell subsets

so far, the best way to clarify the function of iNKT cells is to check the cytokine production of those iNKT cells exposed to specific local environment.

4. CD1d

1) CD1 family

CD1 genes are MHC class I-like family proteins. In human, CD1 family consists of five genes; CD1a, CD1b, CD1c, CD1d, and CD1e (20). These genes can be divided into group1 including CD1a, CD1b, and CD1c and group 2 consists of CD1d depending on their sequences. As CD1e has a unique structure and cellular localization, it can be divided in separate groups. Group 1 CD1 restricted T cells have similar properties with MHC-restricted T cells. In contrast, there are type I and type II NKT cells in group 2 CD1d-restricted T cells. Because there is only CD1d gene among CD1 family in mouse, many researches about NKT cells are progressed within CD1d-restricted type I NKT cells so far.

2) Binding lipids of CD1d

CD1d molecules have the similar structure to MHC class I molecules consisting of two hydrophobic binding grooves. These grooves are well fitted to various CD1d binding lipids, acting as antigens to CD1d-restricted NKT cells.

Many lipid ligands have been reported to activate iNKT cells by being loaded on CD1d molecules of antigen presenting cells. CD1d binding lipid ligands can be categorized into ceramide-based glycolipids (glycosphinglipids) and glycerol-based lipids (phospholipids) (9). Moreover, CD1d molecules can bind both of foreign and self-lipid ligands. As foreign lipid ligands, α-linked glucosyl or galactosyl diacylglycerols in *Borrelia burgdorferi* and *Streptococcus pneumonias* are reported (46, 47). Many researchers are trying to find the self-lipid ligands of iNKT cells as self-lipid bound CD1d is necessary for development of iNKT cells. So far, some self-lipids are identified as follows: isoglobotrihexosylceramide (iGb3), β-glucosylceramide (β-GC), and plasmalogen lysophosphatidylethanolamine (pLPE). iGb3 was identified as a potential lipid ligand in the study with β-hexosaminidase B deficient mice which were predicted to have low level of iGb3 (95). However, there are controversies since iGb3 synthase deficient mice have shown normal development of iNKT cells and human has no iGb3 synthase (18, 70). β-GC is reported that its accumulation is elevated by activation of bacterial infection-mediated TLR signaling, and acts as antigen for iNKT cells (10). In addition, pLPE is recently identified as self-lipid ligand for CD1d molecules. pLPE is a glycerol-based lipid with a single fatty acid chain attached through a vinyl ether linkage. Mice deficient in the glyceronephosphate O-acyltransferase (GNPAT) which is an essential enzyme for plasmalogen synthesis displayed reduction in thymic maturation of iNKT cells and fewer iNKT cells in thymus and peripheral organs (23). Many research groups are still investigating the self-lipid ligands for iNKT

cells in various pathophysiological states. In this aspect, the investigations of the obesity-related lipid ligands become crucial for understanding adipose tissue inflammation in obesity.

3) Cellular localization of CD1d

CD1d is constitutively expressed by APCs such as dendritic cells, macrophages, and B cells. Double positive thymocytes which are the essential cell type for iNKT cell development are also CD1d expressing cell types. Furthermore, CD1d is expressed on hepatocytes, Kupffer cells, and endothelial cells lining liver sinusoids where the highest frequencies of NKT cells are resided in mouse (4).

CD1d is dynamically recycled between inside of the cells and plasma membrane. After synthesis of CD1d proteins, CD1d associate with β2-microglobulin (β2m). This complex binds phospholipids such as phosphatidylinositol to maintain their structural stability. Microsomal triglyceride transfer protein (MTTP) mediates phospholipid binding to CD1d in ER, which facilitates CD1d-lipid complex trafficking to plasma membrane. Surface CD1d is dynamically endocytosed and entered endosomal and lysosomal compartments, where CD1d bound lipids are exchanged with foreign or self-lipid ligands of CD1d by saposin proteins. This lipid ligands-loaded CD1d is presented on the cell surface to activate iNKT cells (92).

5. Purpose

In obesity, low-grade chronic inflammation has been implicated in insulin resistance. Particularly, expanded adipose tissues induced by excess energy intake have been characterized with accelerated inflammatory response in obesity. To date, many studies have been focused on understanding roles of various immune cells including macrophages and several lymphocytes in adipose tissue inflammation and insulin resistance in obesity. Most of these studies have been compared lean animals with severe obese animals at relatively late stage of obesity. However, it is crucial to understand the initial process of adipose tissue inflammation to verify the causal factors of obesity-induced adipose tissue inflammation. Therefore, I have examined early response of adipose tissue inflammation to find out early responding immune cell types in obesity. With the identification of early responding immune cells and their regulatory mechanisms, it is possible to speculate the causes of adipose tissue inflammation in the beginning of obesity. Upon short term HFD (< 1week), I observed that inflammatory response and adipocyte size increased significantly. Thus, I hypothesized that the HFD-induced metabolic changes in adipocytes could mediate adipose tissue inflammation via regulation of immune cell activities. Here, I have characterized the changes of adipose tissue immune cells including macrophages and T cells which would influence adipose tissue inflammation at early stage of obesity upon HFD. Also, I have investigated the adipocyte-derived mediator that can regulate the activity of early responding immune cells in adipose tissue.

In the first chapter, I have characterized the roles of iNKT cells, innate-like T cell population which recognizes lipid antigens, as an early responding cell type in adipose tissue inflammation in obesity. In the second chapter, I have studied the roles of adipocyte expressing CD1d as the regulator of iNKT cells in adipose tissue inflammation. Taken together, I would like to propose that adipocyte CD1d is able to regulate iNKT cells which would contribute to adipose tissue inflammation in obesity.

CHAPTER ONE:

Invariant natural killer T cells protect against high fat diet induced adipose tissue inflammation.

Abstract

Systemic low-grade chronic inflammation has been intensively investigated in obese subjects. Recently, various immune cell types, such as macrophages, granulocytes, helper T cells, cytotoxic T cells and B cells, have been implicated in the pathogenesis of adipose tissue inflammation. However, it remains unclear what would be initiating factors to promote adipose tissue inflammation. To assess this, early response of adipose tissues upon HFD have been characterized. There were significant changes in adipocytes as well as several immune cells in adipose tissue in the early stage of HFD feeding. Notably, iNKT cells were found as one of the early responding lymphocytes in obese adipose tissue. I demonstrated that adipose iNKT cells decreased in numbers not only in the early stage of obesity, but also genetically obese/diabetic model mice. Furthermore, upon HFD, adipose tissue iNKT cells were activated, and induced into apoptosis as well as proliferation. To verify the roles of iNKT cells in adipose tissue inflammation and insulin resistance, iNKT cell-deficient Jα18 knockout mice have been characterized. Upon HFD feeding, iNKT cell deficient mice became more obese and exhibited increased adipose tissue inflammation. In addition, I found out that expression level of IL-4, but not that of IFN-γ in adipose tissue iNKT cells selectively increased upon HFD. Therefore, these data suggest that adipose tissue iNKT cells would have anti-inflammatory roles, and the reduction of iNKT cells in adipose tissue would mediate acceleration of adipose tissue inflammation in obesity.

Introduction

Obesity is a key risk factor of metabolic syndromes such as hypertension, hyperlipidemia, atherosclerosis and type 2 diabetes. Given that the adipose tissue of obese animals exhibits low-grade chronic inflammation, which is closely associated with metabolic abnormalities (36, 82, 94), recent studies have focused on immune response in adipose tissue. For instance, accumulating evidences indicate that in the adipose tissue of lean animals, anti-inflammatory immune cells such as M2-type macrophages and regulatory T cells play dominant roles in repressing inflammation and help to maintain insulin sensitivity by enhancing Th2-type cytokine (interleukin [IL]-4, IL-10, IL-13) secretion (24, 52, 53, 91). On the other hand, the numbers of pro-inflammatory immune cells such as M1-type macrophages, Th1 cells, and CD8 T cells increase in obese adipose tissue and accelerate adipose tissue inflammation. These pro-inflammatory immune cells aggravate insulin sensitivity through Th1-type cytokine secretion and other, yet unknown, activities (64, 67, 69, 71). Even though various immune cells have been implicated in adipose tissue inflammation and metabolic diseases, the direct regulatory mechanism governing immune response in adipose tissue has not been clearly elucidated yet.

Natural killer T cells (NKT cells) are well known as an immune cell population bridging innate and adaptive immune response (88). There are 3 types of NKT cells, including invariant NKT (type I), non-invariant NKT (type II) and NKT-like cells. Invariant NKT (type I) and non-invariant NKT (type II) cells are

CD1d-dependent, while NKT-like cells are CD1d-independent (32). Invariant NKT (iNKT, type I) cells have a semi-invariant T cell receptor α chain, Vα14Jα18 in mouse and Vα24Jα18 in human (3, 89). iNKT cells are capable of rapid response and secretion of various chemokines and cytokines, including Th1- and Th2-type cytokines (31). Particularly, iNKT cells specifically recognize a variety of lipid antigens loaded on CD1d molecules and do not recognize peptide antigens on major histocompatibility complex (MHC) molecules. α-galactosylceramide (α-GC) is the most potent CD1d-binding lipid antigen for iNKT cell activation (43).

In the present study, I demonstrated that adipose tissue inflammation accompanied with hypertrophied adipocytes starts to increase in the early stage of obesity such as 3 days of HFD feeding. Especially, the number of iNKT cells was significantly reduced in adipose tissue upon 1 week of HFD fed mice. Furthermore, iNKT cell activation, apoptosis, and proliferation increased in adipose tissue upon HFD feeding. In iNKT cell deficient mouse model-Jα18 KO mice, adipose tissue inflammation and insulin resistance were accelerated upon HFD feeding. Therefore, I suggest that iNKT cells would play important roles in the regulation of anti-inflammatory response in adipose tissue in obesity.

Materials and Methods

Animals and treatments

C57BL6/J mice were obtained from Central Lab Animal Inc. (Seoul, Korea) and were housed in colony cages in 12-h light/12-h dark cycles. After a minimum of 1 week for stabilization, 8-week-old mice were fed normal chow diet (NCD) and then were administered a 60% high-fat diet (HFD) for the indicated time periods (Research Diets Inc., New Brunswick, NJ, USA). Then, on the day of sacrifice, all of the HFD-fed mice were compared to age-matched NCD-fed mice. *db/db* mice and *ob/ob* mice were purchased from Central Lab Animal Inc. and sacrificed at 12 weeks of age. Jα18 knockout (KO) mice were generously provided by D.S. Lee. Heterozygous mice were bred to generate KO mice and WT littermates. WT and KO Jα18 mice were maintained on NCD until 8 weeks of age before changing to a 60% HFD for 4 weeks. For the oral glucose tolerance test, mice were fasted for 6 h and basal blood samples were taken, followed by oral glucose administration (3 g/kg). Blood samples were drawn at 15, 30, 45, 60, 90 and 120 min after administration. All experiments with mice were approved by the Institute of Laboratory Animal Resources in Seoul National University.

qRT-PCR.

cDNA was synthesized using reverse transcriptase with dNTP and random

Table 3. Information of qRT-PCR primer sequences

Gene	Forward primer sequence	Reverse primer sequence
mouse *Cyclophilin*	5'-CAGACGCCACTGTCGCTTT-3'	5'-TGTCTTTGGAACTTTGTGTG-3'
mouse *TNF-a*	5'- CATCTTCTCAAAATTCGAGT -3'	5'-TGGGAGTAGACAAGGTACAA -3‘
mouse *IL-6*	5'-TCCACGATTTCCCAGAGAAC -3'	5'-AGTTGCCTTCTTGGGACTGA -3'
mouse *MCP-1*	5'- AGGTCCCTGTCATGCTTCTG -3'	5'-TCTGGACCCATTCCTTCTTG -3'
mouse *caspase-3*	5'-TTGCTGAACGTGAAGCCCATCGAGG-3'	5'-GTCCTTGTAGATCTCCTGGAGCAG-3'
mouse *Adiponectin*	5'-GGCAGGAAAGGAGAACCTGG-3'	5'-AGCCTTGTCCTTCTTGAAGA-3'
mouse *CD11c*	5'-GAGGATTTCAGCATCCCAGA-3'	5'-CACCTGCTCCTGACACTCAA-3'
mouse *SAA*	5'-AGTGATGCCAGAGAGGCTGT-3'	5'-ACCCAGTAGTTGCCCCTCTT-3'
mouse *GLUT4*	5'-GATTCTGCTGCCCTTCTGTC-3'	5'-ATTGGACGCTCTCTCTCCAA-3'
mouse IL-4	5'-GGTCTCAACCCCCAGCTAGT -3'	5'- GCCGATGATCTCTCTCAAGTGAT -3'
mouse IL-10	5'- CCAAGCCTTATCGGAAATGA -3'	5'-TTTTCACAGGGGAGAAATCG -3'
mouse IFN-γ	5'- CGGCACAGTCATTGAAAGCCTA -3'	5'- GTTGCTGATGGCCTGATTGTC -3'

hexamer (Fermentas). These cDNAs were used as templates with specific primers in the presence of dNTPs and *Taq*DNA polymerase. Real time PCR amplification reactions were brought to a final volume of 20 μl and contained 20 ng of cDNA, 0.25 μM of primers and SYBR Green (Bio-Rad). The MyiQ real time PCR detection system (Bio-Rad) was used for PCR amplification in 96-well plates. The relative amounts of each mRNA were calculated by using the comparative threshold cycle (CT) method. *Cyclophilin* mRNA was used as the invariant control. The primer sequence information was added to Table 3.

Fractionation of adipose tissues

Fractionation of adipose tissue was performed as previously described (51, 65). Epididymal adipose tissues were weighed and then chopped and incubated in collagenase buffer for 30 min at 37℃ with shaking. After centrifugation, adipocytes in the supernatant were collected for RNA extraction or primary cell culture. The pelleted stromal vascular cell (SVC) fraction was used for flow cytometry or RNA extraction.

iNKT cell isolation from adipose tissues.

To isolate iNKT cells from adipose tissue, I pooled epididymal adipose tissues from NCD (n=25) and 2 weeks of HFD fed mice (n=15). SVCs were

stained by anti-CD16/32 antibody (eBioscience) followed by APC-conjugated TCRβ and PE-conjugated α-GC -loaded CD1d dimer staining for 30 min at 4℃. After PBS washing, I sorted iNKT cells (TCRβ and α-GC -loaded CD1d dimer double positive cells) and non iNKT T cells (TCRβ positive α-GC -loaded CD1d dimer negative cells) by FACS Aria.

Immunohistochemitry

Whole-mount immunohistochemistry was performed as previously described (41). Epididymal adipose tissues were removed and fixed with 1% paraformaldehyde for 1 h and washed. The samples were then blocked with 1% BSA, incubated for 1 h and then stained with primary antibody (perilipin 1:1000, CD11b 1:1000, CD11c 1:1000) overnight at 4℃. After washing for 1 h, the samples were incubated with fluorescence-labeled secondary antibody for 4 h at room temperature (RT) and washed. Following staining with 4′,6-diamidino-2-phenylindole (DAPI) containing Vectorshield solution, samples were observed using a Zeiss LSM510NLO confocal microscope.

Flow cytometry.

Splenocytes were prepared by mincing spleen tissues and then lysing red blood cells (RBCs). Adipose tissue SVC pellets were incubated with RBC lysis

buffer prior to centrifuging (2000 rpm, 5 min) and resuspended in phosphate-buffered saline (PBS). SVCs were incubated with CD16/32 (eBioscience, San Diego, CA, USA), as blocking antibody, for 10 min at 4℃ prior to staining with fluorescence-labeled primary antibodies for 30 min at 4℃. Phycoerythrin (PE)-conjugated α-GC--loaded CD1d dimer (BD Bioscience, Franklin Lakes, NJ, USA) was prepared as previously described (40). The PE-conjugated immunoglobulin G (IgG) monoclonal antibody (mAb), CD1d dimer, CD3ε mAb, T cell receptor (TCR)-β mAb and CD4 mAb were purchased from BD Biosciences and CD8 mAb from eBioscience. The activation markers (CD44 mAbs) were purchased from eBioscience. For macrophage analysis, I stained SVCs with CD11b (BD Bioscience), F4/80 (eBioscience) and CD11c (eBioscience) mAbs for 20 min at 4℃. Cells were gently washed with and resuspended in PBS. SVCs were analyzed using the fluorescence-activated cell sorting (FACS) CantoII instrument (BD Bioscience). For the detection of iNKT cell apoptosis, I used the Annexin V staining kit purchased from BD Bioscience. For the detection of iNKT cell proliferation, I used FITC-conjugated Ki67 mAb (eBioscience).

Statistics

The results are shown as means ± SEM. All statistical analysis was performed by the Student's *t* test or ANOVA in Excel (Microsoft); P-values < 0.05 were considered significant.

Results

Short-term HFD (ST-HFD) feeding increases body weight, fat mass, and adipocyte size

To investigate the initiating factors in adipose tissue inflammation, I examined several metabolic parameters in adipose tissues at the early stage of HFD. C57BL/6J mice were fed 60% HFD for 0, 3, 7 days and body weight, epididymal adipose tissue mass, SVC number, and adipocyte size were measured at each time point. All mice were sacrificed on the same day. Body weight, epididymal adipose tissue mass, SVC numbers significantly were elevated after only 3 days of HFD feeding (Fig. 2A-C). In addition, adipocyte size was significantly increased (Fig. 2D and E). Thus, these data suggest that adipose tissues rapidly respond to HFD feeding.

Adipocytes increase inflammatory gene expression upon ST-HFD

To verify the inflammatory response of each cell types in diet-induced obesity (DIO), I fractionated adipose tissues into adipocytes and SVCs, and checked inflammatory gene expression involving TNF-α, IL-6, and MCP-1 upon HFD. Although the basal levels of inflammatory gene expression were higher in SVCs than adipocytes, the induction folds of inflammatory genes were more significant in adipocyte fraction (Fig. 3). These data indicate that adipocytes dynamically respond to HFD feeding through elevation of inflammatory response as well as expansion of their size.

Figure 2.

Adipose tissues rapidly respond to short term (ST) HFD. C57BL/6J male mice were fed NCD until they reached the age of 8 weeks. At the age of 8 weeks, they were fed HFD for 0 days (D0), 3 days (D3), 7 days (D7). All the mice were sacrificed and analyzed at the same time. A: Body weight (gram) of the mice. n=5 at each time point. B: Epididymal adipose tissue mass. n=5 at each time point. C: The number of SVCs in adipose tissues. n=5 at each time point. D, E: Whole mount immunohistochemistry of epididymal adipose tissues and qualitative changes of adipocyte size. n=5 at each time point. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with D0.

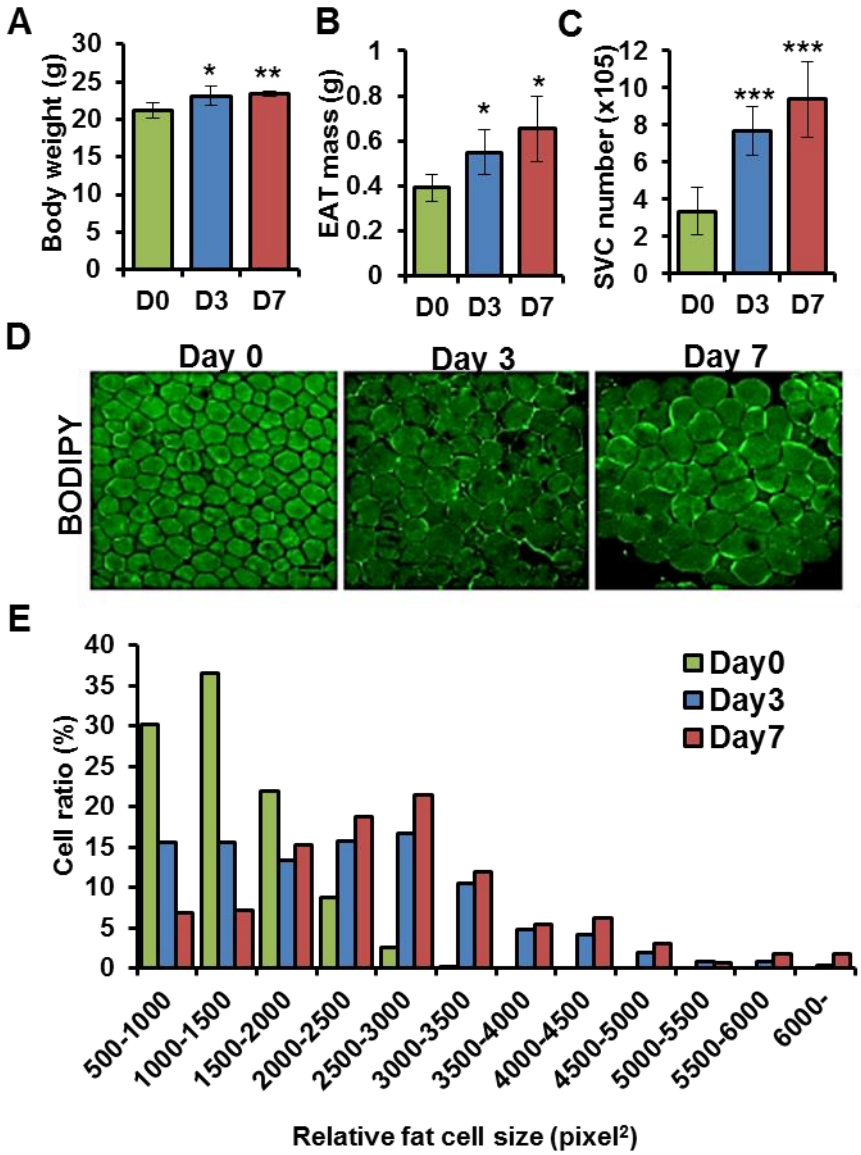
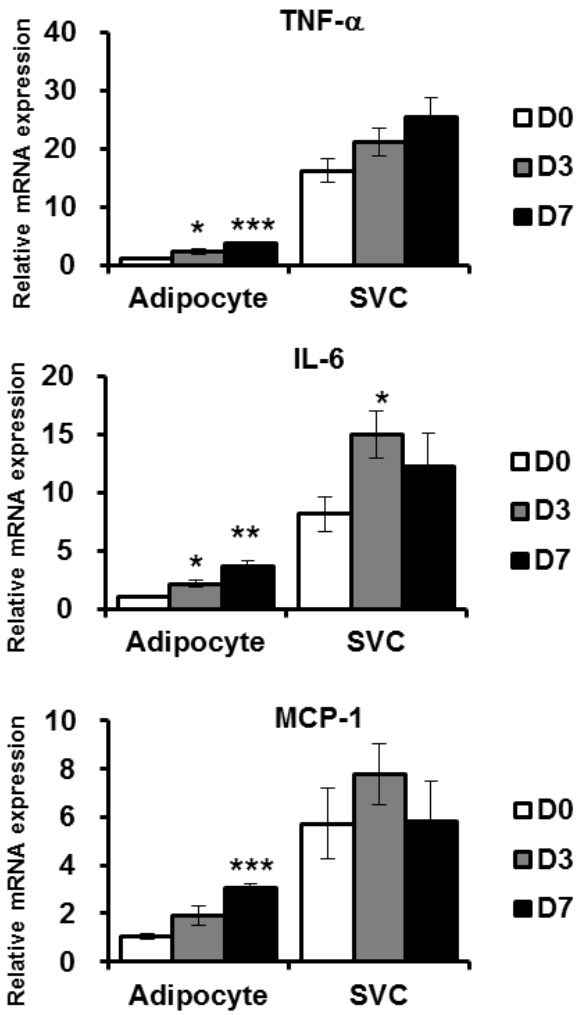

A
Body weight (g)
30
25
20
15
10
5
0
*
**
D0
D3
D7
B
EAT mass (g)
1
0.8
0.6
0.4
0.2
0
*
*
D0
D3
D7
C
SVC number (x105)
12
10
8
6
4
2
0
***
***
D0
D3
D7
D
BODIPY
Day 0
Day 3
Day 7
E
Cell ratio (%)
40
35
30
25
20
15
10
5
0
Day0
Day3
Day7
500-1000
1000-1500
1500-2000
2000-2500
2500-3000
3000-3500
3500-4000
4000-4500
4500-5000
5000-5500
5500-6000
6000-
Relative fat cell size (pixel²)

Figure 3.

Inflammatory gene expression is induced in adipocytes upon ST-HFD feeding. Both adipocyte and SVC fractions were separated from epididymal adipose tissues, and subjected to analysis of mRNA levels of inflammatory genes such as TNF-α, IL-6, and MCP-1. n=5. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with D0.


TNF-α
Relative mRNA expression
40
30
20
10
0
*
***
Adipocyte
SVC
D0
D3
D7
IL-6
Relative mRNA expression
20
15
10
5
0
*
**
*
Adipocyte
SVC
D0
D3
D7
MCP-1
Relative mRNA expression
10
8
6
4
2
0
***
Adipocyte
SVC
D0
D3
D7

Macrophage infiltration increases in adipose tissues upon ST-HFD

Adipose tissue inflammation involves an increase in the number of adipose tissue macrophage (ATM) polarized toward pro-inflammatory phenotype. These ATMs are often referred to as M1-like polarization in which ATMs express high levels of CD11c and macrophage markers such as F4/80 and CD11b (63). In contrast, CD206+ macrophages represent anti-inflammatory macrophages, called as M2 macrophages. As shown in Fig. 4, flow cytometry analyses revealed that the F4/80 and CD11b double-positive ATMs were elevated in adipose tissue upon 1 week of HFD (Fig. 4B), and a large proportion of these macrophages also expressed CD11c. This increase in CD11c-positive ATM content was also present when the data were normalized to total epididymal adipose tissue mass, showing that pro-inflammatory macrophages accumulate in adipose tissue shortly after HFD (Fig. 4C). Moreover, CD206+ macrophages also increased in number upon HFD feeding when normalized % of SVCs and cell number per fat mass. To observe the localization of macrophages in adipose tissues, I performed whole-mount immunohistochemistry by using anti-CD11b and CD11c (Fig. 5). After 1 week of HFD, the number of CD11c+ macrophages increased and these cells mainly located around crown-like structures. Therefore, these results indicate that the polarization of ATMs toward pro-inflammatory phenotype occurs concomitantly with accumulation of ATM in adipose tissues.

Figure 4.

Macrophages infiltrate in adipose tissues upon ST-HFD. A: Gating regions for analysis of total macrophages (CD11b+F4/80+ double positive cells), M1-like macrophages (CD11b+F4/80+CD11c+ triple positive cells), and M2-like macrophages (CD11b+F4/80+CD206+ triple positive cells). B: Percent of total macrophages, M1-like macrophages, and M2-like macrophages among SVCs. C: Cell number of total, M1-like, and M2-like macrophages per fat mass (gram). n=5, *$P < 0.05$ and **$P < 0.01$ compared with D0.

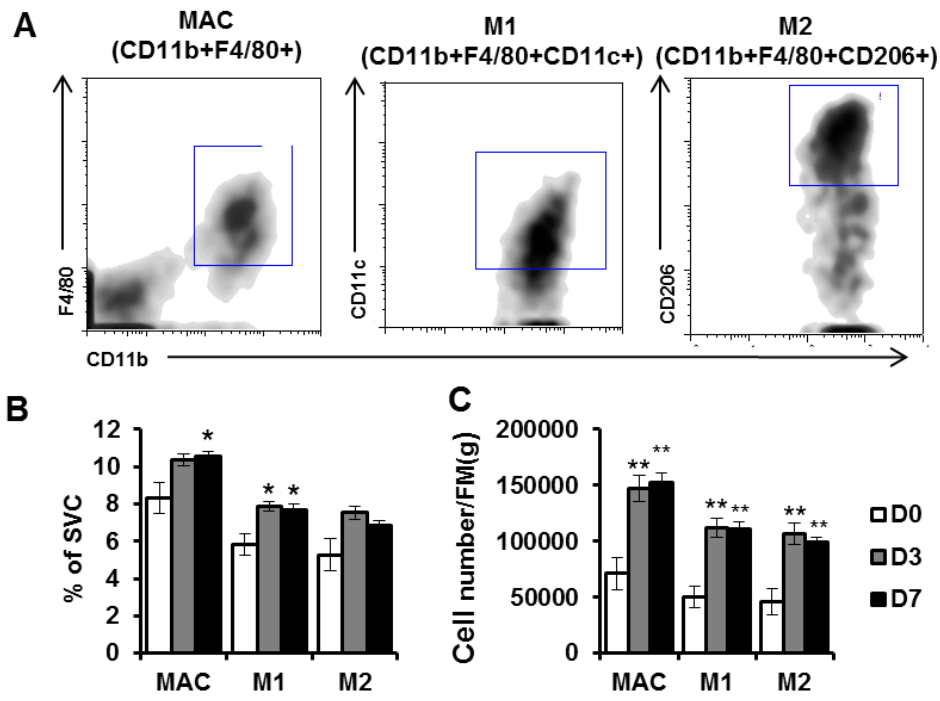
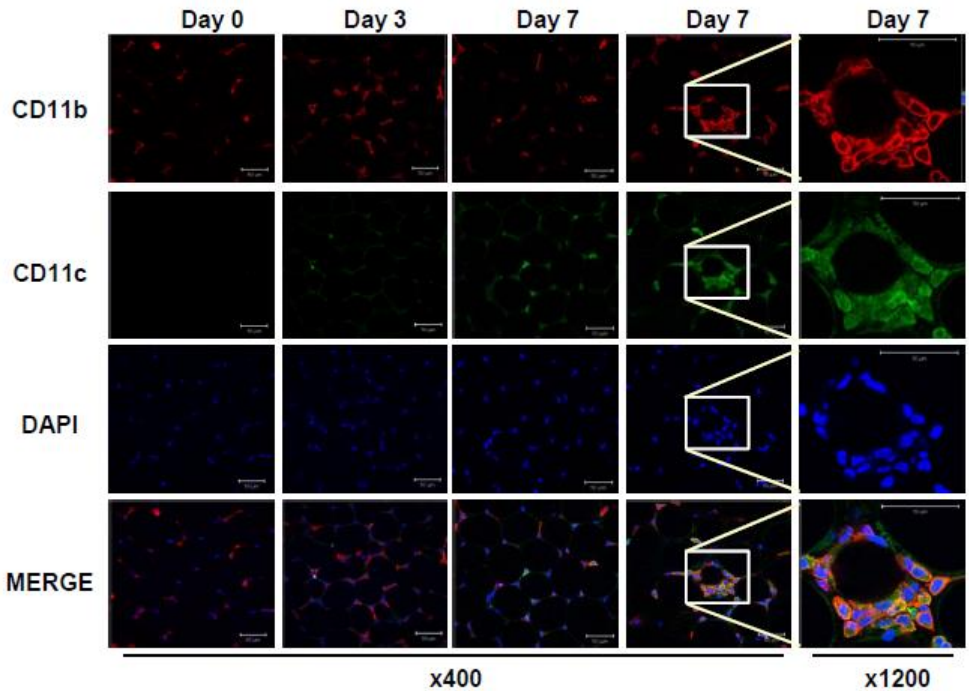

A
MAC
(CD11b+F4/80+)
M1
(CD11b+F4/80+CD11c+)
M2
(CD11b+F4/80+CD206+)
F4/80
CD11b
CD11c
CD206
B
% of SVC
12
10
8
6
4
2
0
MAC
M1
M2
C
Cell number/FM(g)
200000
150000
100000
50000
0
MAC
M1
M2
D0
D3
D7

Figure 5.

Macrophages infiltrate in adipose tissues upon ST-HFD – immunohistochemistry analysis. Whole-mount immunohistochemistry analysis of epididymal adipose tissue from 0, 3 or 7 days of HFD-fed mice. Tissues were stained with DAPI (blue) or antibodies against CD11b (red) and CD11c (green), and visualized by multiphoton confocal microscopy. Scale bar denotes 50 μm.


Day 0
Day 3
Day 7
Day 7
Day 7
CD11b
CD11c
DAPI
MERGE
x400
x1200

The numbers of T cells are not changed in ST-HFD except NKT cells

T cell population can be classified into CD4 T cells, CD8 T cells, and NKT cells. In obese subjects, it has been reported that anti-inflammatory T cells such as regulatory T cells are reduced, whereas pro-inflammatory T cells including Th1 cells and CD8 T cells increases in number (24, 64, 71). To find the early responding cell types among T cells, I characterized the changes of T cell populations in the early stage of HFD feeding. Total T cells were gated by CD3ε single positive cells among lymphocytes gated by FSC and SSC (Fig. 6A). CD4 T cells and CD8 T cells were defined as CD3+CD4+ cells and CD3+CD8+ cells, respectively. For detection of NKT cells, CD3 and NK1.1 mouse antibodies were used as NKT specific markers. Upon ST-HFD, percent of total T cells, CD4 T cells, and CD8 T cells among lymphocytes were not altered (Fig. 6B-D). However, the number of NKT cells significantly decreased upon 3 days of HFD feeding (Fig. 6E). To compare between early response and late response of T cell population in adipose tissues of HFD-induced obese mice, I characterized changes in T cell population during HFD feeding for 1, 2, 7, and 21 weeks. As shown in Fig 7, there was no significant change in the number of CD4 T cells until long term of HFD feeding. In contrast, the number of CD8 T cells significantly increased in 21 weeks of HFD feeding. In addition, NKT cells were reduced regarding of HFD and aging. These data show that only NKT cells are changed among T cell population in adipose tissues upon ST-HFD feeding and imply that NKT cells would be one of the contributors to early response of adipose tissue inflammation.

Figure 6.

T cells except NKT cells are not changed in ST-HFD. A: Gating region of total T cells (CD3+), CD4 T cells (CD3+CD4+ double positive cells), CD8 T cells (CD3+CD8+ double positive cells), and NKT cells (NK1.1+CD3+ double positive cells) in adipose tissues. C-D: Percent of total T cells, CD4 T cells and CD8 T cells in adipose tissues of HFD fed mice for 0, 3, 7 days. E: Percent of NKT cells in adipose tissues of HFD fed mice for 0, 3, 7 days. n=5, *$P < 0.05$ compared with D0.

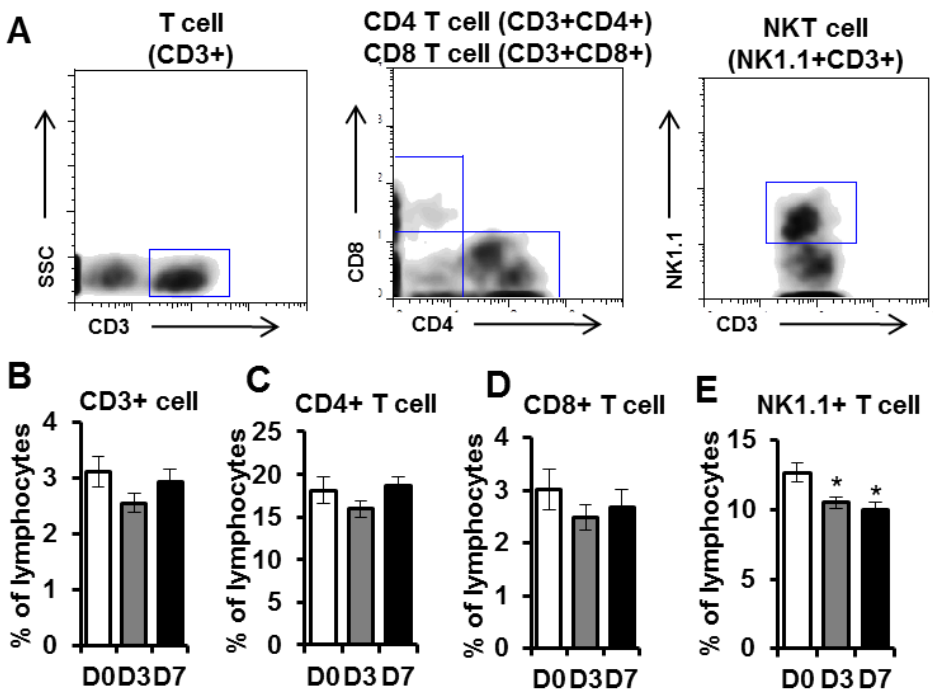
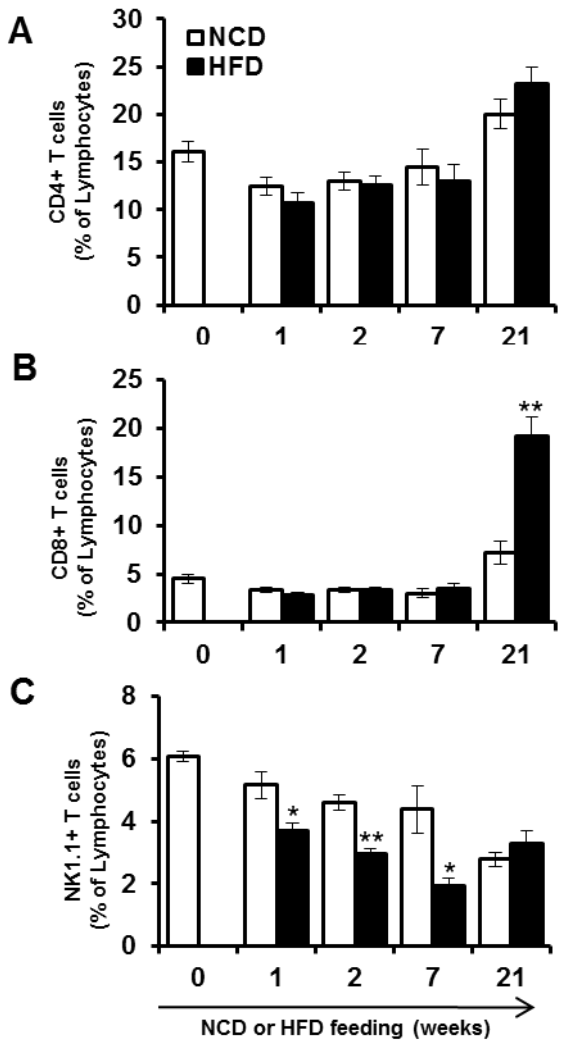

A
T cell
(CD3+)
SSC
CD3
CD4 T cell (CD3+CD4+)
CD8 T cell (CD3+CD8+)
CD8
CD4
NKT cell
(NK1.1+CD3+)
NK1.1
CD3
B
CD3+ cell
% of lymphocytes
0
1
2
3
4
D0 D3 D7
C
CD4+ T cell
% of lymphocytes
0
5
10
15
20
25
D0 D3 D7
D
CD8+ T cell
% of lymphocytes
0
1
2
3
4
D0 D3 D7
E
NK1.1+ T cell
% of lymphocytes
0
5
10
15
*
*
D0 D3 D7

Figure 7.

Quantitative changes in T cell populations in adipose tissues during HFD feeding. C57BL/6J male mice were fed NCD until they reached the age of 8 weeks. At the age of 8 weeks, they were fed HFD for 0 days (D0), 3 days (D3), 7 days (D7). NCD and HFD fed mice were sacrificed at each time point. A-C: Percent of CD4 T cells, CD8 T cells, and NKT cells among lymphocytes in adipose tissues. n=4~5, *$P < 0.05$ compared with NCD fed group.

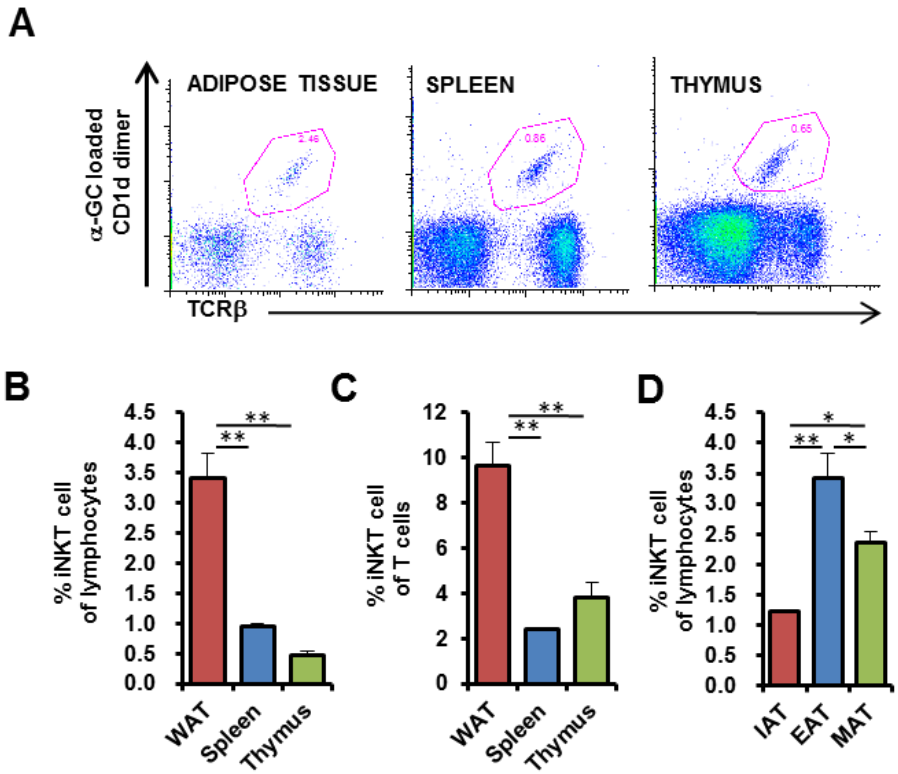

A
NCD
HFD
CD4+ T cells
(% of Lymphocytes)
30
25
20
15
10
5
0
0
1
2
7
21
B
CD8+ T cells
(% of Lymphocytes)
25
20
15
10
5
0
**
0
1
2
7
21
C
NK1.1+ T cells
(% of Lymphocytes)
8
6
4
2
0
*
**
*
0
1
2
7
21
NCD or HFD feeding (weeks)

iNKT cells are abundant in adipose tissue

NKT cells are categorized into 3 types, invariant NKT cell (iNKT, type I), non-invariant NKT cells (type II), and NKT-like cells. Based on the results which NK1.1+ T cell population was reduced upon HFD, I tested iNKT cell (type I) population in adipose tissues. Very recently, the NKT cell population in adipose tissue has been characterized either by the presence of NK1.1+CD3+ cells or by Vα14Jα18 mRNA expression in adipose tissue (15, 66). However, the abundance of the iNKT cell population, especially that of type I NKT cells expressing Vα14Jα18 TCR chains, has not been thoroughly investigated in the adipose tissue of lean or obese mice. To examine the iNKT cell population in adipose tissue, I quantitatively analyzed the proportion of anti-TCRβ and α-GC-loaded CD1d dimer double positive cells by flow cytometry (Fig. 8A). In epididymal adipose tissue, iNKT cells occupied about 3~4% of the total lymphocyte population, which was relatively higher than the ratio of iNKT cells in other immune organs such as the spleen and thymus. When the percentage of iNKT cells was normalized with either total lymphocytes or total T cells in adipose tissue, spleen, and thymus, I noted that the relative percentage of iNKT cells in adipose tissue was significantly higher than the one in the spleen and thymus (Fig. 8B and C). Additionally, when I analyzed the ratio of iNKT cells in several fat depots including inguinal, epididymal and mesenteric adipose tissue, epididymal fat tissue exhibited the highest frequency of iNKT cells (Figure 8D), implying that adipose tissue is relatively enriched with iNKT cells.

Figure 8.

iNKT cells are present in adipose tissue. 8-week-old C56BL6/J male mice were used for preparation of adipose tissue, spleen and thymus; n = 5. ***A***: iNKT cells were detected by staining of α-GC -loaded CD1d dimer and TCR . The iNKT cell population is represented as a dot blot graph. ***B, C***: Percentages of iNKT cells among lymphocytes and T cells in EAT, spleen and thymus. Lymphocyte population was gated by FSC and SSC. ***D***: Percentages of iNKT cells among lymphocytes in fat depots (inguinal [IAT], epididymal [EAT] and mesenteric [MAT] adipose tissue). *P < 0.05 and **P < 0.01.


A
ADIPOSE TISSUE
SPLEEN
THYMUS
α-GC loaded CD1d dimer
TCRβ
2.46
0.86
0.65
B
% iNKT cell of lymphocytes
WAT
Spleen
Thymus
C
% iNKT cell of T cells
WAT
Spleen
Thymus
D
% iNKT cell of lymphocytes
IAT
EAT
MAT

iNKT cell numbers decrease in the adipose tissue of obese mice

To investigate the correlation between iNKT cells in adipose tissue and obesity, I examined the populations of iNKT cells in the adipose tissue of lean and obese *db/db* or *ob/ob* mice. The percentage of iNKT cells among adipose lymphocytes was significantly lower in obese animal models than in lean mice (Fig. 9). In addition, I examined the dynamics of the iNKT cell population in fat tissues during HFD consumption. There was a HFD-dependent decrease in iNKT cell percent of lymphocytes in adipose tissue consistent with decrement of NK1.1+ T cells (Fig. 9B). Furthermore, in HFD fed mice, the percentage of iNKT cells in circulating lymphocytes in blood was not significantly altered in HFD fed mice as well (Fig. 9C). Interestingly, during short-term HFD feeding (1~2 weeks), the percentage of iNKT cells in fat tissues significantly decreased. Therefore, I decided to analyze in detail the population of iNKT cells in short-term (1 week) HFD-fed mice, to unveil changes in adipose tissue in the early stages of obesity. Body weight and fat mass increased upon short-term HFD in adult mice (Fig. 10A and B). On the other hand, the percentages of other T cell populations, such as $CD4^+$ T cells and $CD8^+$ T cells, in fat tissue did not change upon short-term HFD (Fig. 10C and D). In adipose tissue, the percentage of iNKT cells calculated based on the total number of lymphocytes or SVCs was significantly diminished (Fig. 10E and F). Furthermore, the absolute number of iNKT cells per gram of fat mass was significantly lower in fat tissue (Fig.10G). Surprisingly, the reduction of iNKT

Figure 9.

iNKT cell numbers are lower in adipose tissue of obese mice. ***A***: Percentage of iNKT cells among lymphocytes in the adipose tissue of C57BL6/J lean mice, *db/db* mice and *ob/ob* mice; n = 5. ***B***: 8-week-old C57BL6/J male mice were fed normal chow diet (NCD) or a 60% high-fat diet (HFD) for 1, 2, 4 and 7 weeks. The iNKT cell population was measured at each time point. C: Percentage of iNKT cells in blood of HFD fed mice. n=4~5, *P < 0.05, compared to NCD fed mice.

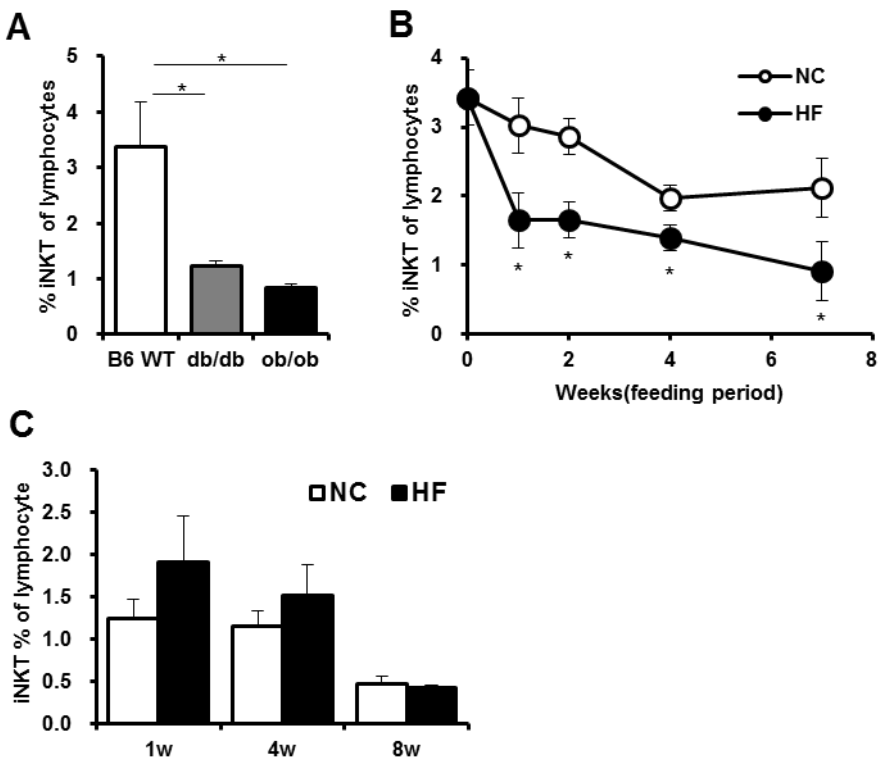
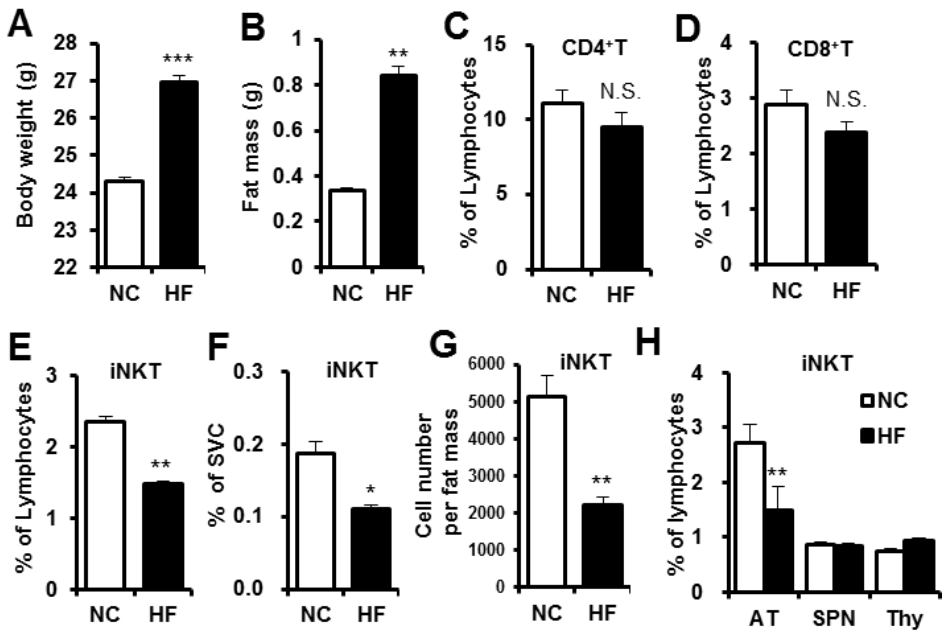

A
% iNKT of lymphocytes
B6 WT
db/db
ob/ob
B
% iNKT of lymphocytes
NC
HF
Weeks(feeding period)
C
iNKT % of lymphocyte
NC
HF
1w
4w
8w

Figure 10.

iNKT cell numbers are reduced in adipose tissues of 1 week of HFD fed mice. C57BL6/J mice were fed NCD and HFD for 1 week. ***A***: Body weight (gram). ***B***: Fat mass (gram). ***C***: Percentage of $CD4^+$ T cells among adipose tissue lymphocytes. ***D***: Percentage of $CD8^+$ T cell among adipose tissue lymphocytes. ***E***: Percentage of iNKT cells among adipose tissue lymphocytes. ***F***: Percentage of iNKT cells among adipose tissue SVCs. ***G***: Absolute iNKT cell number per gram of fat mass. ***H***: Percentage of iNKT cells among lymphocytes in adipose tissue, spleen and thymus. n = 5. *P < 0.05, **P < 0.01, and N.S.; no significant.


A
Body weight (g)
28
27
26
25
24
23
22
***
NC
HF
B
Fat mass (g)
1
0.8
0.6
0.4
0.2
0
**
NC
HF
C
CD4+T
% of Lymphocytes
15
10
5
0
N.S.
NC
HF
D
CD8+T
% of Lymphocytes
4
3
2
1
0
N.S.
NC
HF
E
iNKT
% of Lymphocytes
3
2
1
0
**
NC
HF
F
iNKT
% of SVC
0.3
0.2
0.1
0.0
*
NC
HF
G
iNKT
Cell number per fat mass
6000
5000
4000
3000
2000
1000
0
**
NC
HF
H
iNKT
% of lymphocytes
4
3
2
1
0
**
NC
HF
AT
SPN
Thy

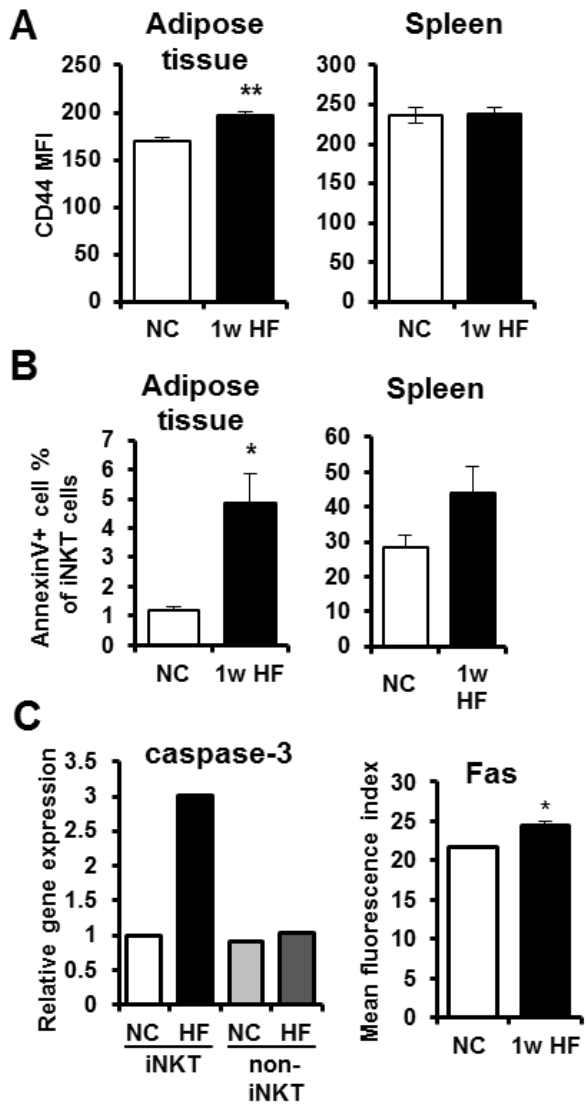
cells upon short-term HFD was specific to adipose tissue but not observed in the spleen and thymus, indicating that the decrease in iNKT cells in adipose tissue appears to be independent of any defects in iNKT cell development (Fig. 10H).

iNKT cells undergo activation-induced cell death upon HFD

I also examined the expression levels of T cell activation/memory markers such as CD44 on iNKT cells and observed that these were up-regulated in the adipose tissue of short-term HFD-fed mice (Fig. 11A). For further characterization of the decrease in adipose tissue iNKT cell population upon short-term HFD, I carefully examined the degree of apoptosis in adipose tissue iNKT cells. As shown in Figure 11B, annexin V-positive iNKT cell greatly increased up to about 4-fold following 1 week of HFD. To investigate in more detail about apoptosis of iNKT cells, I analyzed apoptosis-related mRNA and protein expression in iNKT cells in adipose tissue. The level of Caspase-3 mRNA and Fas protein expression on the surface of adipose tissue iNKT cells were elevated in HFD fed (1 week) mice (Fig. 11C), implying that short-term HFD-induced iNKT cell apoptosis is associated with iNKT cell activation during changes in fat tissue in early obesity.

Figure 11.

Activation induced cell death of iNKT cells upon 1 week of HFD feeding. A: Expression levels of activation/memory markers (CD44) on iNKT cells in adipose tissue and spleen. B: Percentage of Annexin V-positive cells among iNKT cells in adipose tissue and spleen. C: The level of caspase-3 mRNA and cell surface expression of Fas protein of iNKT cells in adipose tissue. *$P < 0.05$ and **$P < 0.01$.


A
Adipose tissue
Spleen
CD44 MFI
250
200
150
100
50
0
**
NC
1w HF
300
250
200
150
100
50
0
NC
1w HF
B
Adipose tissue
Spleen
AnnexinV+ cell % of iNKT cells
7
6
5
4
3
2
1
0
*
NC
1w HF
60
50
40
30
20
10
0
NC
1w HF
C
caspase-3
Relative gene expression
3.5
3
2.5
2
1.5
1
0.5
0
NC HF
iNKT
NC HF
non-iNKT
Fas
Mean fluorescence index
30
25
20
15
10
5
0
*
NC
1w HF

iNKT cell activation, apoptosis, and proliferation is induced in ST-HFD as well as LT-HFD

To investigate the response of iNKT cells during not only ST-HFD but also long term HFD (LT-HFD) feeding, I compared the number of iNKT cells, expression of activation/memory marker (CD44), apoptosis, and proliferation after 2, 4, and 8 weeks of HFD fed mice. The ratio of iNKT cells in lymphocytes (% of lymphocytes) significantly decreased (Fig. 12A). The total number of iNKT cells in adipose tissues per mouse was not significantly different upon 2 and 4 weeks of HFD, but it increased after 8 weeks of HFD (Fig, 12B). When iNKT cell number was normalized by fat mass (gram), it decreased (Fig. 12C). Moreover, consistent with 1 week of HFD, the levels of CD44 expression and apoptosis of iNKT cells increased by 2, 4, and 8 weeks of HFD (Fig. 13A and B). iNKT cell proliferation which was analyzed by Ki67 staining, was gradually elevated during HFD feeding (Fig. 13C). Taken together, iNKT cells appear to undergo activation-induced cell death, and proliferate to compensate the loss of iNKT cell population in adipose tissue upon short term HFD as well as long term HFD feeding.

iNKT cell deficient mice are susceptible to DIO

Although the association of iNKT cells with autoimmune diseases and cancers has been reported (5, 6, 22), the nature of their pathophysiological roles in

Figure 12.

The number of iNKT cells decreases upon 2, 4, and 8 weeks of long term HFD treatment. 8-week-old C56BL6/J male mice were fed NCD or HFD for 2, 4, 8 weeks. A: Percent of iNKT cells among lymphocytes. B: Total iNKT cell number in epididymal adipose tissues per mouse. C: iNKT cell number per fat mass (gram). *P < 0.05 and **P < 0.01.

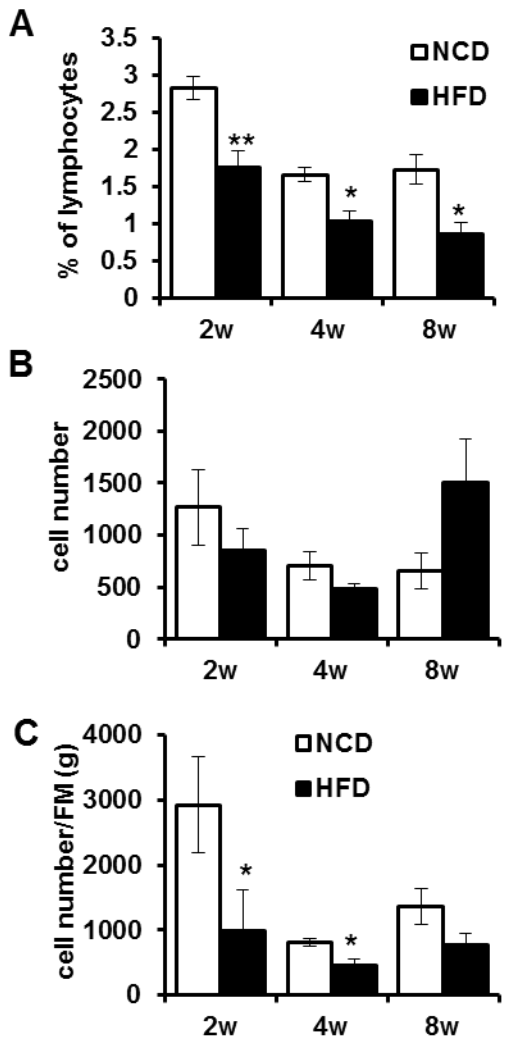
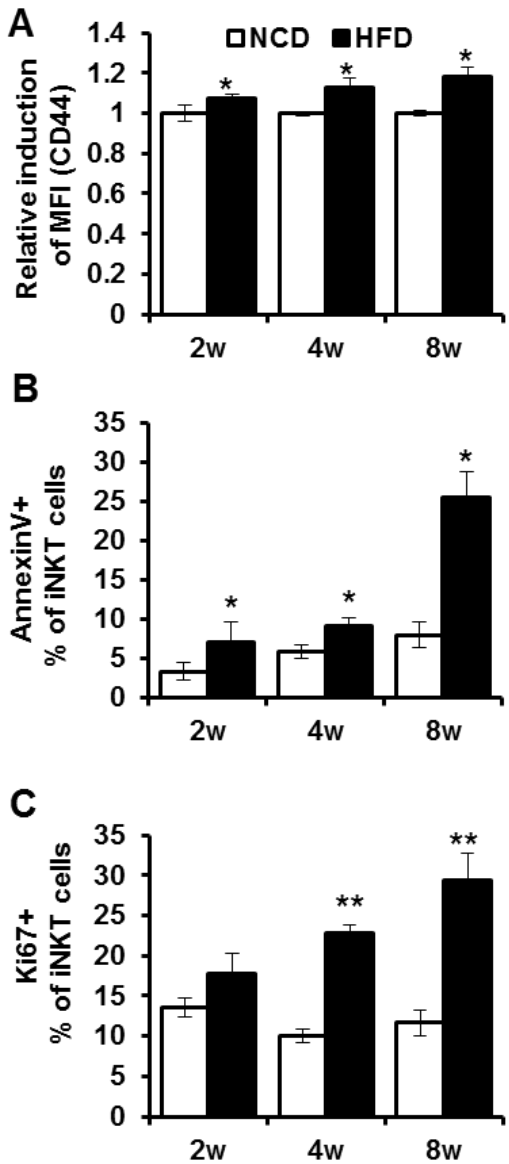

A
3.5
3
2.5
2
1.5
1
0.5
0
% of lymphocytes
NCD
HFD
**
*
*
2w
4w
8w
B
2500
2000
1500
1000
500
0
cell number
2w
4w
8w
C
4000
3000
2000
1000
0
cell number/FM (g)
NCD
HFD
*
*
2w
4w
8w

Figure 13.

CD44 expression, apoptosis, and proliferation of iNKT cells are elevated upon HFD feeding. A: CD44 (memory/activation marker) expression on iNKT cells. B: AnnexinV positive cells among iNKT cells. C: Ki67+ cells among iNKT cells. *P < 0.05 and **P < 0.01.


A
NCD
HFD
Relative induction of MFI (CD44)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2w
4w
8w
B
AnnexinV+ % of iNKT cells
35
30
25
20
15
10
5
0
C
Ki67+ % of iNKT cells

various diseases is still controversial. To get insight into the role of iNKT cells in adipose tissue inflammation in obesity, I have compared obesity-associated phenotypes in WT mice with Jα18 KO mice lacking iNKT cells. There were no significant changes in body weight gain between NCD-fed WT and KO mice. In contrast, upon HFD consumption, even for a short duration, the body weight of Jα18 KO mice was predisposed to obesity (Fig. 14A and B). The mass of the epididymal adipose tissue in HFD-fed Jα18 KO mice was significantly higher than in WT mice, whereas liver mass was not significantly different between two genotypes (Fig. 14C and D). Since it is well established that obesity is a major risk factor for insulin resistance, I assessed glucose tolerance. As shown in Figure 15, Jα18 KO mice revealed a greater degree of glucose intolerance as compared to WT mice upon HFD feeding. Moreover, plasma insulin level was higher in Jα18 KO mice than in WT mice upon HFD consumption. These data indicate that mice deficient in iNKT cells might be susceptible to obesity and glucose intolerance upon HFD.

iNKT cell deficient mice increase adipose tissue inflammation

To determine the effect of iNKT cell deficiency on adipose tissue inflammation, I examined macrophage infiltration and inflammatory gene expression in adipose tissue. Upon HFD, the percentages of $CD11b^{+}F4/80^{+}$ cells and $CD11b^{+}F4/80^{+}CD11c^{+}$ cells among SVCs were significantly elevated in WT

Figure 14.

Jα18 KO mice are more susceptible to body weight gain and fat mass gain upon HFD. 8-week-old WT and Jα18 KO mice were fed NCD or HFD for 4 weeks. ***A:*** Body weight gain was measured every week. ***B:*** Absolute body weight after 4 weeks of HFD. ***C***: Fat mass (gram). D: Liver mass (gram). *P < 0.05 and **P < 0.01, HFD WT vs. HFD KO.

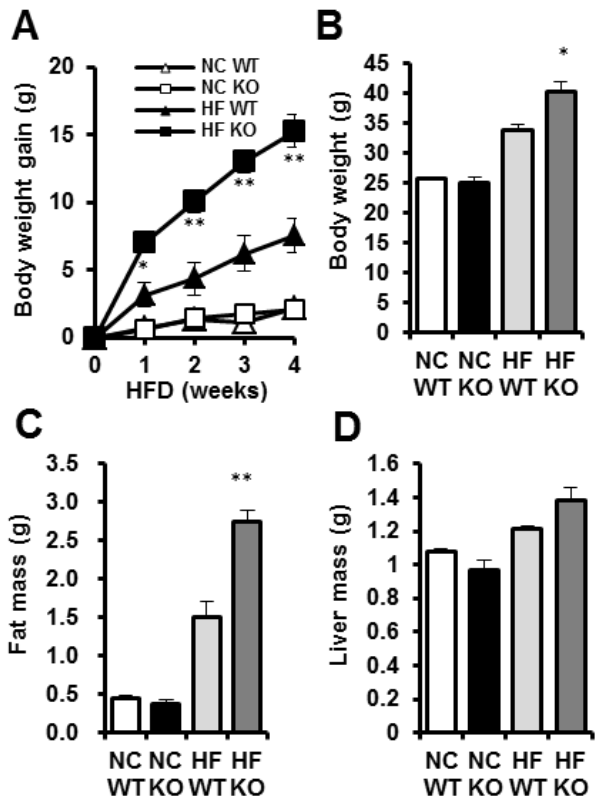
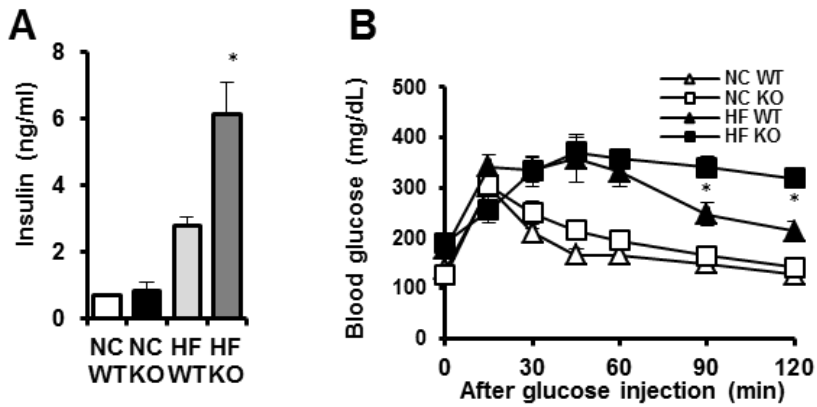

A
B
C
D
NC WT
NC KO
HF WT
HF KO
Body weight gain (g)
HFD (weeks)
Body weight (g)
Fat mass (g)
Liver mass (g)
NC WT
NC KO
HF WT
HF KO

Figure 15.

Jα18 KO mice are more susceptible to glucose intolerance upon HFD. A: Plasma insulin levels (ng/ml). B: Mice were given HFD for 4 weeks and then subjected to the oral glucose tolerance test. Jα18 KO mice increased the level of plasma insulin and glucose intolerance as well. n = 4 at each time point. *P < 0.05, HFD WT vs. HFD KO.


A
Insulin (ng/ml)
8
6
4
2
0
*
NC WT
NC KO
HF WT
HF KO
B
Blood glucose (mg/dL)
500
400
300
200
100
0
0
30
60
90
120
After glucose injection (min)
NC WT
NC KO
HF WT
HF KO
*
*

mice and macrophage accumulation in adipose tissue was further pronounced in Jα18 KO mice (Fig. 16A). The number of adipose tissue macrophages per gram of fat mass significantly increased in Jα18 KO mice (Fig. 16B). To gain insight into the effect of iNKT cell deficiency on macrophage polarization in adipose tissue, I analyzed the proportion of $CD11c^+$ cells (M1-type macrophages) or $CD206^+$ cells (M2-type macrophages) among $CD11b^+F4/80^+$ cells (total macrophages). The percentage of $CD11c^+$ cells was higher in Jα18 KO mice than in WT mice upon HFD. However, there was no significant change in the proportion of M2-type $CD206^+$ cells among CD11b+F4/80+ macrophages (Fig. 16C).

Among T cell populations, there were no significant changes in total T cell population and CD8 T cell between WT and Jα18 KO mice upon HFD (Fig. 17A and B). On the other hand, the number of CD4 T cell population decreased in HFD fed KO mice relative to HFD fed WT mice (Fig. 17C). Of note, Foxp3+ CD4 T cells which are regulatory T cells (Tregs) were significantly reduced in KO mice upon HFD feeding (Fig. 17D and E). Since Treg cells have been well-known anti-inflammatory immune cell types, reduced Treg cells and increased M1 macrophages are potentially associated with accumulation of adipose tissue inflammatory response in Jα18 KO mice.

I further examined the expression of genes involved in adipose tissue inflammation and insulin resistance. Consistent with the data from FACS analysis, pro-inflammatory genes such as CD11c, serum amyloid A (SAA), TNFα and IL-6

Figure 16.

Macrophage infiltration increased in Jα18 KO mice. ***A*** Percentage of CD11b+F4/80+ and CD11b+F4/80+CD11c+ cells among adipose tissue SVCs. **P < 0.01, HFD WT vs. HFD KO. ***B***: Numbers of CD11b+F4/80+ and CD11b+F4/80+CD11c+ cells per gram of fat mass. *P < 0.05, HFD WT vs. HFD KO. ***C***: Percentages of CD11c+ and CD206+ cells among CD11b+F4/80+ cells in adipose tissue. *P < 0.05, HFD WT vs. HFD KO.

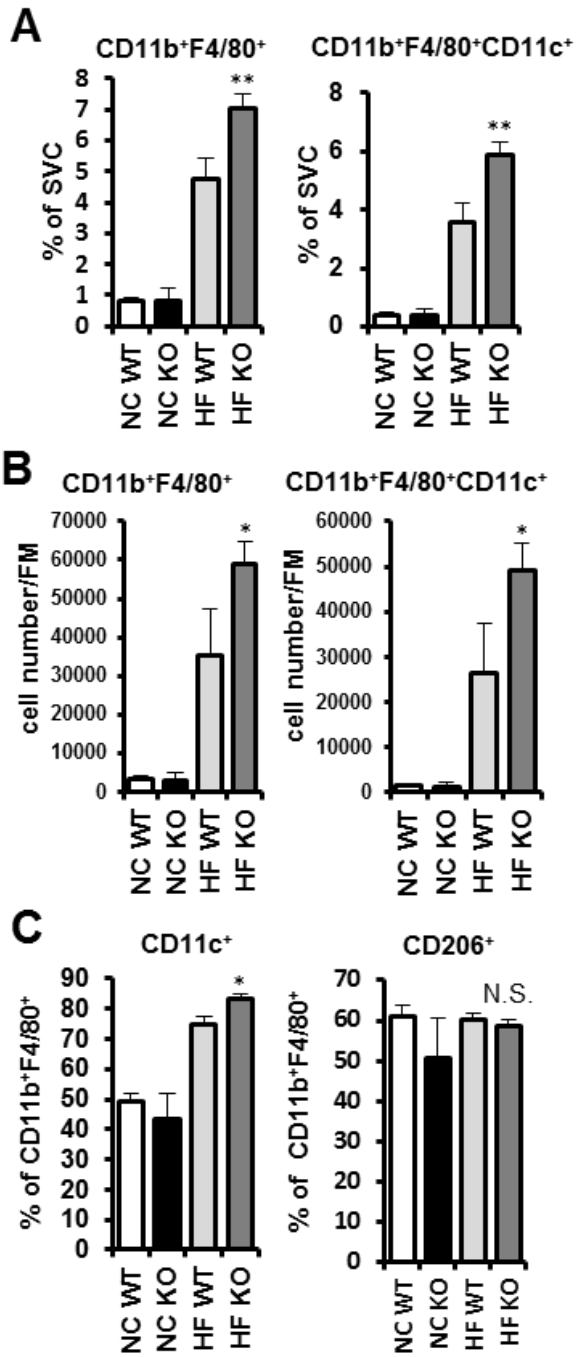
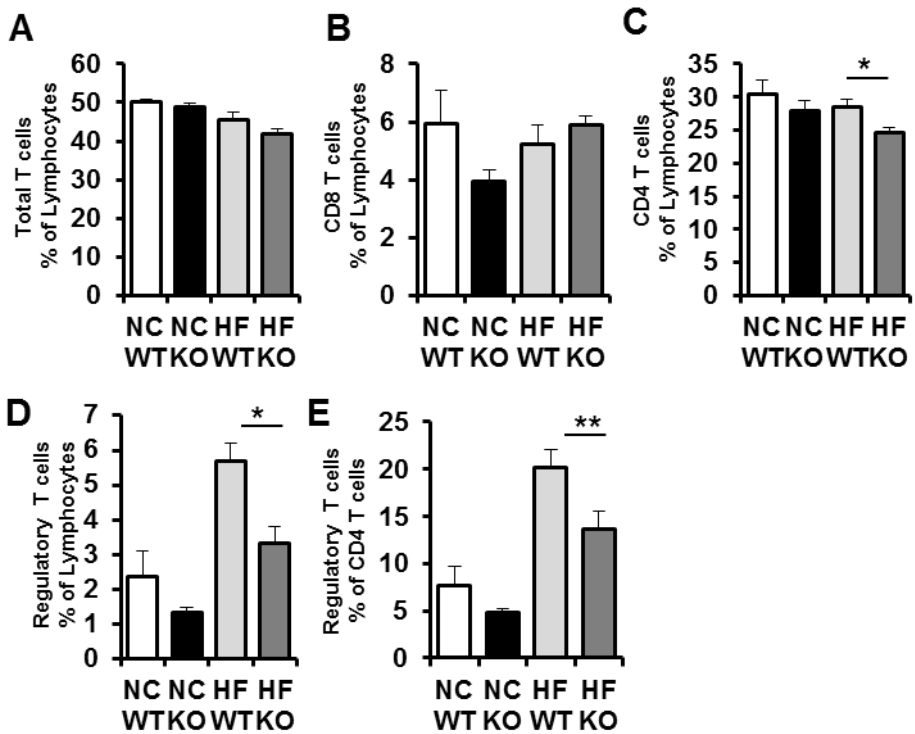

A
CD11b+F4/80+
CD11b+F4/80+CD11c+
% of SVC
**
NC WT
NC KO
HF WT
HF KO
B
CD11b+F4/80+
CD11b+F4/80+CD11c+
cell number/FM
*
C
CD11c+
CD206+
% of CD11b+F4/80+
N.S.

Figure 17.

Regulatory T cells are reduced in adipose tissues of Jα18 KO mice. A-C: Percent of total T cells, CD8 T cells, and CD4 T cells among lymphocytes in adipose tissues. D: Percent of regulatory T (Treg) cells among lymphocytes. E: Percent of Treg cells among CD4 T cells in adipose tissues. *P < 0.05 and **P < 0.01, HFD WT vs. HFD KO.


A
Total T cells
% of Lymphocytes
60
50
40
30
20
10
0
NC NC HF HF
WT KO WT KO
B
CD8 T cells
% of Lymphocytes
8
6
4
2
0
NC NC HF HF
WT KO WT KO
C
CD4 T cells
% of Lymphocytes
35
30
25
20
15
10
5
0
*
NC NC HF HF
WT KO WT KO
D
Regulatory T cells
% of Lymphocytes
7
6
5
4
3
2
1
0
*
NC NC HF HF
WT KO WT KO
E
Regulatory T cells
% of CD4 T cells
25
20
15
10
5
0
**
NC NC HF HF
WT KO WT KO

were upregulated in Jα18 KO mice upon HFD (Fig. 18A). On the contrary, the mRNA expression levels of adiponectin and GLUT4, whose levels are negatively correlated with insulin resistance in adipocytes, decreased in Jα18 KO mice upon HFD (Fig. 18B). Furthermore, serum TNF-α was elevated in Jα18 KO mice as compared to WT mice in HFD fed group (Fig. 18C).

Lipid catabolism-related genes decrease in Jα18 KO mice

To study the mechanisms mediating increased fat mass in HFD fed Jα18 KO mice than WT mice, I checked lipid metabolism-related gene expression. Fatty acid oxidation related genes such as PPARα, Tfam, and NRF-1 were significantly reduced in KO mice (Fig. 19). In addition, mRNA expression levels of lipolysis-related genes involving ATGL, HSL, and CGI-58 significantly decreased in KO mice. Thus, I suggest that reduction in catabolism pathways of lipids would affect increment of fat mass in KO mice.

iNKT cells elevated the expression of Th2-type cytokines upon HFD

In various diseases, the effector functions of iNKT cells are mediated by cytokine secretion such as Th1 type (IFN-γ) and Th2 type (IL-4, IL-10, IL-13), leading to the regulation of the other immune cell activity. To understand the anti-inflammatory function of iNKT cells in diet-induced obese (DIO) mice, I isolated

Figure 18.

Inflammatory response increased in Jα18 KO mice. ***A***: Relative levels of CD11c, SAA, TNF-α and IL-6 transcripts in epididymal adipose tissue by qRT-PCR. ***B:*** Relative levels of adiponectin (AdipoQ) and GLUT4 mRNA in epididymal adipose tissue by qRT-PCR. ***C:*** Serum TNF-α concentration. *P < 0.05, **P < 0.01 and ***P < 0.001, HFD WT vs. HFD KO.

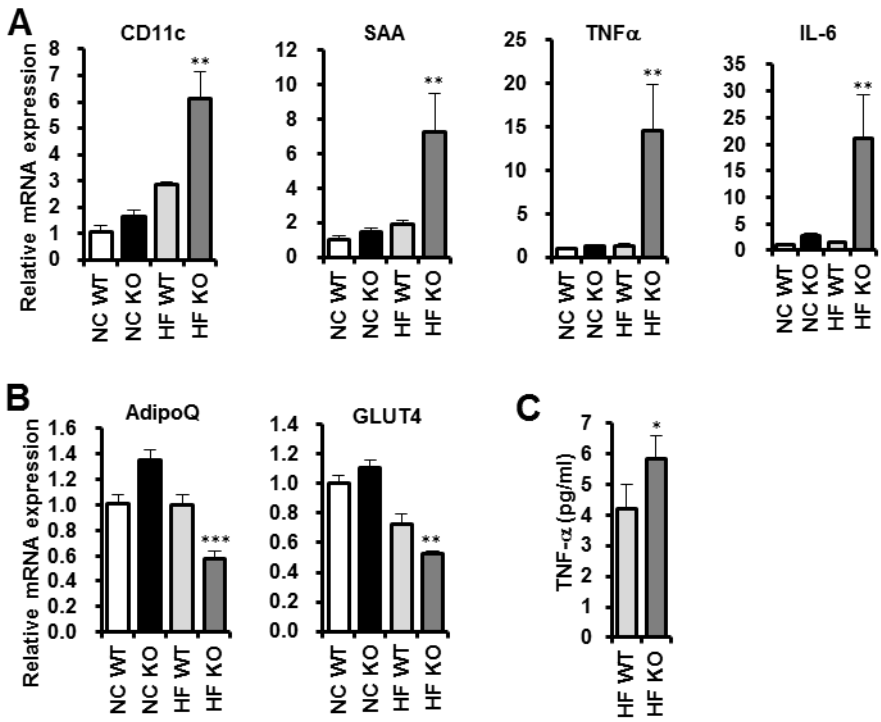
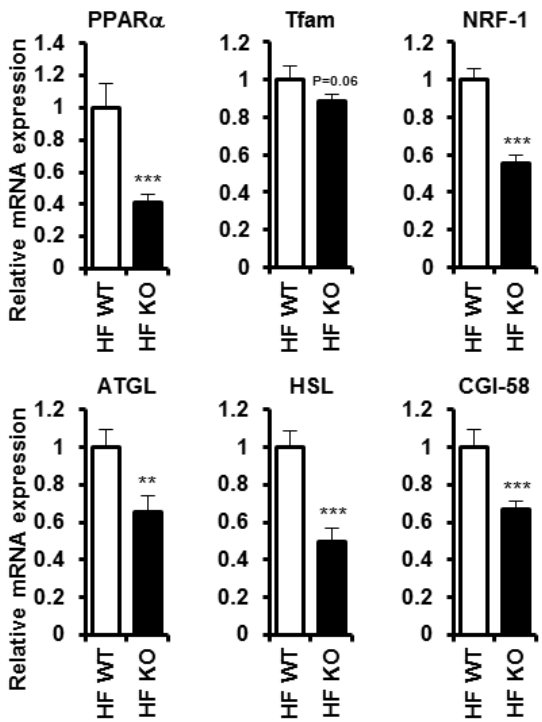

A
CD11c
SAA
TNFα
IL-6
Relative mRNA expression
NC WT
NC KO
HF WT
HF KO
B
AdipoQ
GLUT4
C
TNF-α (pg/ml)

Figure 19.

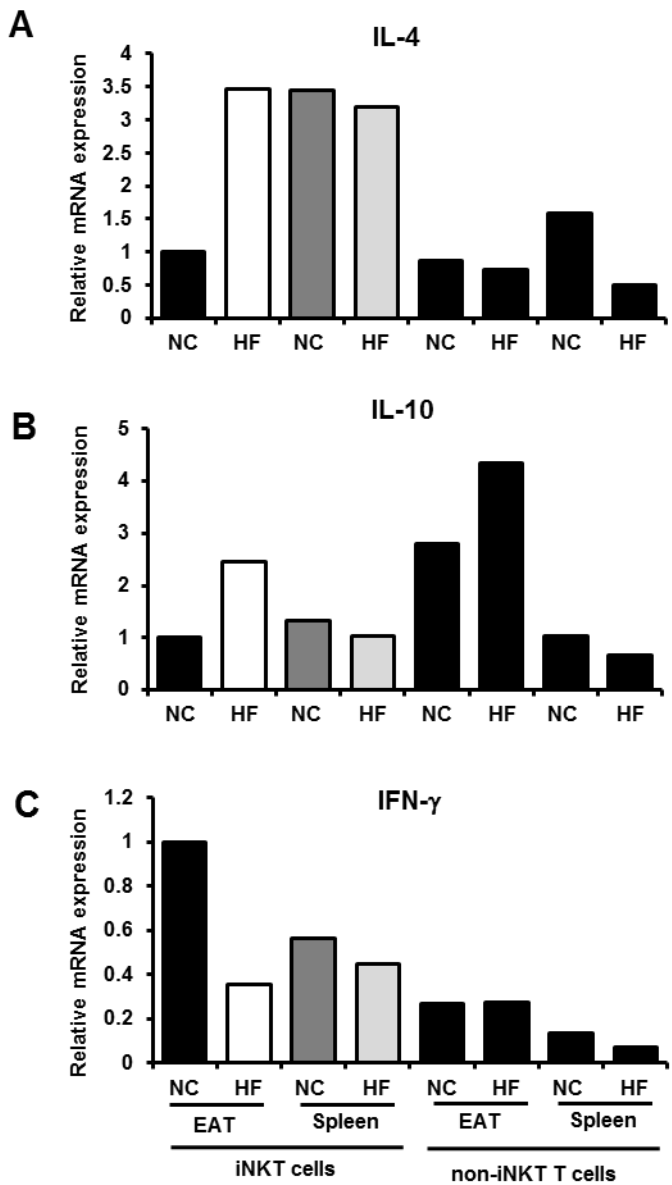
The mRNA expression level of lipid catabolism-related genes decrease in Jα18 KO mice. The mRNA levels of fatty acid oxidation genes such as PPARα, Tfam, and NRF-1, and lipolysis related genes such as ATGL, HSL, and CGI-58 in adipose tissues. n=4~5, **P < 0.01 and ***P < 0.001, HFD WT vs. HFD KO.


PPARα
Tfam
NRF-1
ATGL
HSL
CGI-58
Relative mRNA expression
HF WT
HF KO
P=0.06
***
**

iNKT cells from adipose tissues of NCD fed mice and 2 weeks of HFD fed mice, and examined mRNA level of Th1 type (IFN-γ) and Th2 type (IL-4 and IL-10) cytokines. In adipose tissue, expression of Th2 type cytokines rather than Th1 type cytokines were elevated in iNKT cells upon HFD (Fig. 20). On the other hand, there were no changes present in splenic iNKT cells with respect to expression level of cytokines in DIO. Furthermore, IL-4 and IFN-γ expression in other T cells excluding iNKT cells was retained regardless of types of diet. Thus, induction of IL-4 could contribute to anti-inflammatory function of iNKT cells in obesity-induced adipose tissue inflammation.

Figure 20.

Changes in Th1-type and Th2-type cytokine expression of adipose iNKT cells upon HFD feeding. C57BL/6J male mice were fed NCD or HFD for 2 weeks from 8-week-old. iNKT cells were isolated from pooled epididymal adipose tissues of NCD and HFD fed mice by using FACS Aria. As control cells, splenic iNKT cells were isolated as well. In addition, T cells except iNKT cells (non-iNKT T cells) were used as control cell types. A-C: The mRNA levels of IL-4, IL-10, and IFN-γ in iNKT cells and non-iNKT T cells in adipose tissues and spleen. n=1. (each n is pooled adipose tissues from 30 mice for NCD and 15 mice for HFD)


A
IL-4
Relative mRNA expression
4
3.5
3
2.5
2
1.5
1
0.5
0
NC HF NC HF NC HF NC HF
B
IL-10
Relative mRNA expression
5
4
3
2
1
0
NC HF NC HF NC HF NC HF
C
IFN-γ
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
NC HF NC HF NC HF NC HF
EAT
Spleen
EAT
Spleen
iNKT cells
non-iNKT T cells

Discussion

In obesity, adipose tissue is inflamed with increased macrophage infiltration and exhibits insulin resistance with dysregulation of glucose and lipid metabolism (67). Recently, it has been reported that the HFD induces inflammation rapidly and selectively in adipose tissue (48, 50, 78). Notably, on HFD, adipose tissue inflammation initiates within a week, concurrently with insulin resistance (50). These results suggest that, in adipose tissue, there would be a rapid and sensitive process to recognize the change of energy state and induce inflammation. Although various immune cells have been implicated in adipose tissue inflammation (24, 52, 53, 64, 67, 69, 71, 84, 91), it has not been completely understood the initial process and regulatory mechanisms of adipose tissue inflammation. Based on the data which iNKT cells, T cells respond to various lipid antigens presented on CD1d molecules in antigen-presenting cells, were changed in early stage of obesity, I hypothesized that adipose tissue iNKT cells might dynamically respond to changes in the lipid metabolism of adipose tissue to coordinate whole body energy homeostasis.

Here, I found that, in adipose tissue, ratio of iNKT cells among total lymphocytes was higher than that in spleen and thymus. Moreover, adipose tissue iNKT cells were selectively and sensitively decreased in obese animals such as *db/db*, *ob/ob* and DIO mice. The decrease in iNKT cells in adipose tissue became apparent after 1 weeks of HFD without changes in spleen and thymus. Moreover, activation, apoptosis and proliferation of iNKT cells were induced by HFD feeding.

Since there was no change in the level of activation marker expression of iNKT cells in spleen upon HFD feeding, elevated iNKT cell activation in adipose tissue would be caused by local environmental factors rather than infiltration of systemically activated iNKT cells. In order to understand the roles of iNKT cells in adipose tissue inflammation, I have carefully investigated the metabolic and inflammatory phenotypes of iNKT cell-deficient Jα18 KO mice. Upon short term HFD, Jα18 KO mice gained more body weight and fat mass partly due to the decreased expression of energy metabolism related genes such as PPARα, Tfam, NRF-1, ATGL, HSL and CGI-58 in WAT. Also, iNKT cell deficiency exacerbated glucose intolerance and adipose tissue inflammation by HFD. In last, the mRNA expression level of IL-4 increases in isolated iNKT cells from adipose tissue upon HFD. These results suggest that iNKT cells would suppress adipose tissue inflammation and insulin resistance in obesity.

Among our observations, several lines of evidence suggest that iNKT cells in adipose tissue might exert anti-inflammatory effects. First, in the adipose tissue of iNKT cell-deficient Jα18 KO mice, the pro-inflammatory $CD11c^{+}$ M1 macrophage population was greatly elevated, whereas anti-inflammatory regulatory T cell population was reduced upon HFD feeding. Second, the expression of several pro-inflammatory genes was stimulated in the fat tissue of HFD-fed Jα18 KO mice. Third, the body weight and fat mass of Jα18 KO mice were higher than those of WT mice upon HFD feeding, resulted from reduced levels of energy metabolism genes in WAT of Jα18 KO mice. Nevertheless, the mass of other

peripheral tissues including the liver was not significantly changed. Moreover, HFD-fed Jα18 KO mice exhibited glucose intolerance and higher plasma insulin levels. In further study, adoptive transfer of iNKT cells into obese mice would demonstrate the direct function of iNKT cells in energy metabolism and inflammatory response involving cytokine expression and macrophage infiltration and polarization.

In adipose tissues of Jα18 KO mice, CD4 T cells decreased in adipose tissue of Jα18 KO mice upon HFD although there was no difference in the numbers of total T cells and CD8 T cells between WT and Jα18 KO mice. There are four subpopulations in CD4 T cells including Th1, Th2, Th17, and regulatory T cells. It has been reported that Th2 cells and regulatory T cells play anti-inflammatory roles whereas Th1 cells and Th17 cells are involved in the pro-inflammatory response. Since among CD4 T cells responsible for pro-inflammatory response, Th17 cells have been proven to be implicated in metabolic disorders such as arthrosclerosis (27), it is possible that the number of Th17 cells increases in adipose tissues of Jα18 KO mice upon HFD. Moreover, enhanced mRNA expression level of IL-6 which is crucial cytokine for the differentiation of Th17 cells could stimulate the increment of the number of Th17 cells in adipose tissues of Jα18 KO mice upon HFD (7). However, it is needed to be determined whether the numbers of Th1, Th2, and Th17 cells are affected in Jα18 KO mice in further study.

Several lines of evidences indicate that iNKT cells regulate adipose tissue inflammation. However, there are conflicts among the conclusions from different reports, probably due to analyses of different mouse models in different conditions. Therefore, the role of iNKT cells in adipose tissue inflammation has been remained controversial. For example, recently Ohmura et al. reported that iNKT cells promote adipose tissue inflammation by using beta2-microglobulin knockout mice as iNKT cell deficient mouse model(66). However, beta2-microglobulin KO mice lack CD8 T cells as well as NKT cells and CD8 T cells are well-known for their pro-inflammatory functions. Therefore, their results obtained from this mouse model could be, at least partly, conferred by CD8 T cell deficiency (64). Wu et al. also proposed a pro-inflammatory role of iNKT cells after long term HFD. They analyzed the phenotypes of Jα18 KO and CD1d KO mice after more than 10 weeks of HFD feeding and demonstrated decreased adiposity and adipose tissue inflammation in the KO mice (93), which may conflict with our results. However, this study was focused on the function of iNKT cells in the late-stage of obesity, whereas I mainly analyzed the function of iNKT cells in the early stage of obesity. Depending on the time point (long and short term HFD), it is possible that different lipid antigens could be loaded on CD1d, which will prime iNKT cells either toward anti- or pro-inflammatory cells producing Th2 and Th1 type cytokines, respectively. The roles of iNKT cells and the types of lipid antigens in relation to the time course of obesity have to be elucidated in further study. On the contrary to these two reports, Satoh et al. observed that there is no difference in body weight, fat mass

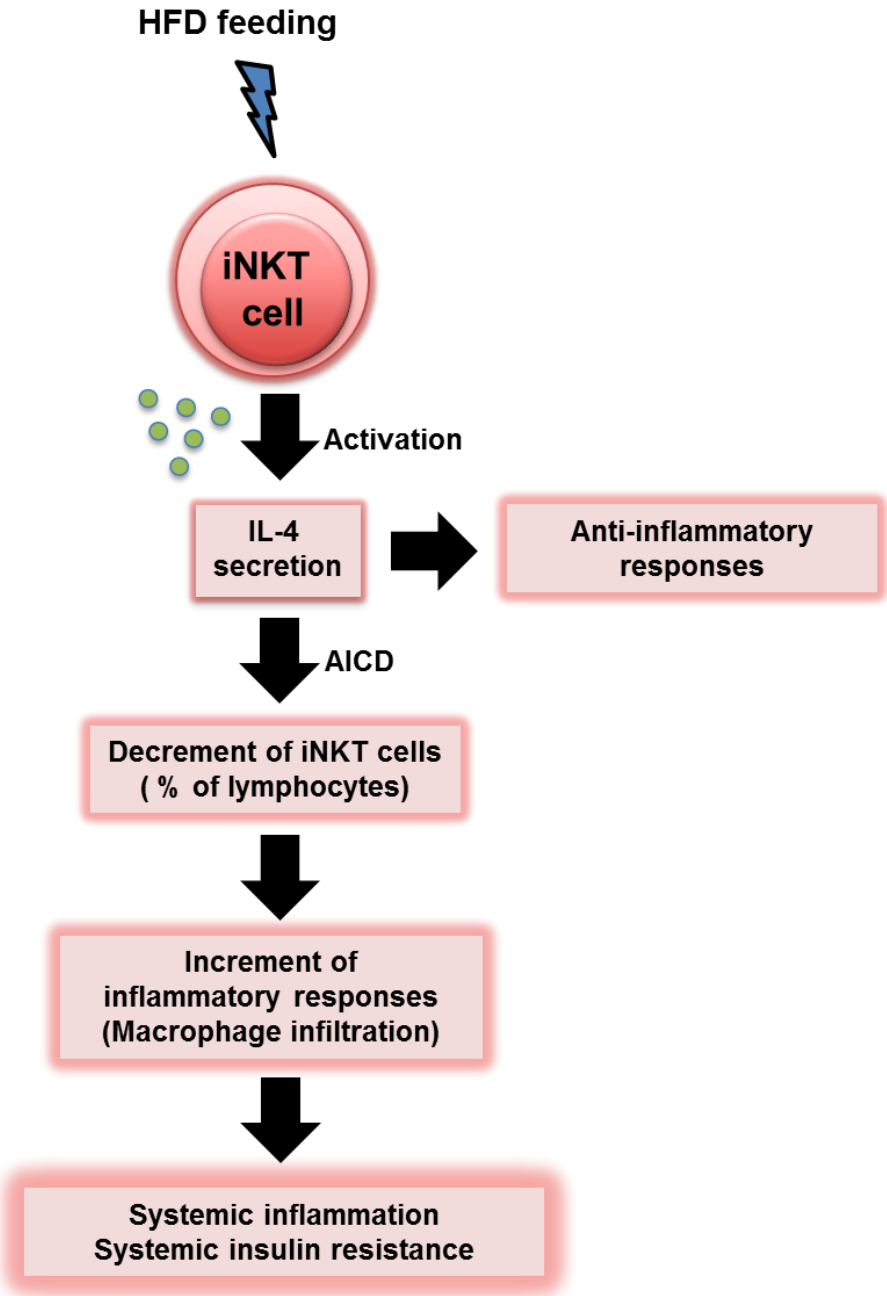
and glucose tolerance between Jα18 KO mice and WT mice after 18 weeks of HFD feeding (75). They compared between Jα18 KO mice with commercial B6 mice, rather than littermate control WT mice. Recently, it is reported that microbiota in guts can affect energy metabolism by HFD and it is well recognized that mice touch and sometimes eat feces frequently in the cage (62). Therefore using commercially purchased WT control mice, but not littermate control mice, maybe have affected differential commensal microbiota in WT and KO mice. In this study, I used littermate WT mice as a control group and observed that Jα18 KO mice showed repeatedly and consistently increase in body weight gain, adiposity and adipose tissue inflammation compared to WT mice after short term HFD feeding. In concordance with our study, recently Ji et al. reported an anti-inflammatory role of iNKT cells (40). They reported impaired insulin sensitivity in CD1d KO mice after 4 days of HFD. Furthermore, Lynch et al. reported that Jα18 KO mice increased body weight gain, fat mass, and adipose tissue inflammation. They performed adoptive transfer of iNKT cells to obese mice, which resulted in decrement of body weight and fat mass, and improved insulin sensitivity (55).

To date, most of the studies that are relevant to immune cells in adipose tissues have focused on their roles in prolonged or severe obesity (24, 53, 64, 91), which may mask the effects of iNKT cells on adipose tissue inflammation at the early stage of obesity or provoke compensatory response to resolve long range of imbalanced energy homeostasis. In contrast, the function of immune cells in the early stages of obesity has not properly addressed, although there are appreciable

changes in adipose tissue inflammation and insulin sensitivity in those phase (48, 50, 78). In the present study, I suggest that upon HFD-induced activation, iNKT cells can secret anti-inflammatory cytokines such as IL-4 which suppress adipose tissue inflammation in the early stage of obesity. However, HFD-induced activation of iNKT cells partially make them undergo cell death, which follows decrement of iNKT cells in adipose tissues. As relatively reduced population of iNKT cells contribute to acceleration of pro-inflammatory response and insulin resistance (Fig. 21).

Figure 21.

Proposed model: a role of iNKT cells in adipose tissue inflammation. HFD induces iNKT cell activation, which allows for the secretion of Th2 type cytokines such as IL-4 and IL-10. These cytokines play important roles in the suppression of pro-inflammatory response. Concomitantly, activated iNKT cells undergo activation-induced cell death in adipose tissues. It results in reduction of anti-inflammatory response, and subsequent accumulation of pro-inflammatory response in adipose tissues of obese subjects. Consequently, systemic inflammation and insulin resistance develop in obesity.


HFD feeding
iNKT cell
Activation
IL-4 secretion
Anti-inflammatory responses
AICD
Decrement of iNKT cells (% of lymphocytes)
Increment of inflammatory responses (Macrophage infiltration)
Systemic inflammation
Systemic insulin resistance

CHAPTER TWO:

CD1d-expressing adipocytes regulate invariant natural killer T cell Response

Abstract

In obesity, chronic and low grade of adipose tissue inflammation plays a role in insulin resistance. Many immune cells including macrophages, eosinophils, mast cells, B cells, and T cells, are involved in inflammation and insulin resistance in adipose tissues. Recently, it has been reported that adipose iNKT cells would contribute to anti-inflammatory response in obesity-induced adipose tissue inflammation. However, the regulatory mechanisms of iNKT cell activity have not been clearly elucidated yet. As iNKT cells recognize lipid antigens loaded on CD1d molecules, I hypothesized that adipocytes, which are active for lipid metabolism, could regulate iNKT cell activity in adipose tissues of obese subjects. Here, I discovered that CD1d molecules were highly expressed in adipose tissues, mainly in adipocyte fraction. Adipocyte CD1d is capable to activate iNKT cells *in vitro*. In addition, CD1d expression in adipocytes was regulated by PPARγ, a master regulator transcription factor of adipogenesis. In differentiated adipocytes, the level of CD1d mRNA expression decreased concomitantly with reduction of PPARγ expression in severely obese adipose tissues. In adipocyte-specific CD1d KO mice, iNKT cell number and proliferation were reduced compared to WT mice upon HFD feeding. Taken together, I suggest that adipocyte expressing CD1d could regulate iNKT cell response in obese adipose tissues.

Introduction

Recent findings, notably on adipokines and adipose tissue inflammation, have revised the concept of adipose tissues being a mere storage depot for body energy. Instead, adipose tissues are emerging as endocrine and immunologically active organs with multiple effects on the regulation of systemic energy homeostasis (68). Notably, compared with other metabolic organs such as liver and muscle, various inflammatory response are dynamically regulated in adipose tissues and most of the immune cells in adipose tissues are involved in obesity-mediated metabolic complications, including insulin resistance (50). Recently, it has been reported the key roles of innate (neutrophils, macrophages, mast cells, eosinophils) and adaptive (regulatory T cells, type 1 helper T cells, CD8 T cells, B cells) immune cells in adipose tissue inflammation and metabolic dysregulation in obesity (17, 79). In particular, the roles of natural killer T cells, one type of innate lymphocytes, in adipose tissue inflammation were studied.

Although there are discrepancies on the roles of type I NKT (iNKT) cells in adipose tissue inflammation in obesity, I and other groups demonstrated the anti-inflammatory roles of iNKT cells (37, 41, 55, 77, 92). According to these studies, the number of iNKT cells is reduced in obese adipose tissue, and iNKT cell deficient mice exhibit acceleration of tissue inflammation and insulin resistance. When iNKT cells from lean mice were transferred to obese mice, they protected the mice from weight gain, adipocyte hypertrophy, and insulin resistance (55).

When α-galactosylceramide, potent lipid ligands for CD1d, were injected to obese mice, there were decreases in body weight and blood glucose level as well as increases in anti-inflammatory cytokines such as IL-4 and IL-10 followed by improved insulin sensitivity. . However, it remains unclear how iNKT cell activity is regulated in adipose tissue in obesity.

iNKT cells specifically recognize a variety of lipid antigens loaded on CD1d molecules and do not recognize peptide antigens on major histocompatibility complex (MHC) molecules. For example, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, isoglobotrihexosylceramide (iGb3), β-glucosylceramide (β-GC), and plasmalogen lysophosphatidylethanolamine (pLPE) have previously been reported to be lipid antigens of CD1d (12, 60). In particular, α-galactosylceramide (α-GC) is the most potent CD1d-binding lipid antigen for iNKT cell activation (43). It is a MHC class I-like glycoprotein and has a lipid-binding hydrophobic groove (2). CD1d is mainly expressed on professional antigen-presenting cells such as dendritic cells, macrophages, B cells and hepatocytes as well (92).

Adipocyte constitutes one of the major cell types responsible for the regulation of dynamic lipid metabolisms in response to various energy states. Notably, their lipid metabolism and consequent lipid metabolites are significantly altered in obesity. There are compelling evidences to suggest that altered lipid metabolism and lipid metabolites play critical roles in the regulation of insulin sensitivity in obese and diabetic animals (14, 16, 26, 34, 35, 74, 81, 85). These

recent findings led us to hypothesize that lipid metabolites produced by adipocytes might be presented by CD1d molecules on the plasma membrane of adipocytes; the recognition of lipid-CD1d complexes in adipose tissue would subsequently modulate iNKT cell activity. Therefore, I investigated whether adipocytes bearing CD1d molecules act as antigen-presenting cells to regulate iNKT cell activities in adipose tissue. In this study, I have revealed the role of adipocyte CD1d molecules in iNKT cell activation as well as in adipose tissue inflammation.

Materials and Methods

Animals and treatments

C57BL6/J mice were obtained from Central Lab Animal Inc. (Seoul, Korea) and were housed in colony cages in 12-h light/12-h dark cycles. After a minimum of 1 week for stabilization, 8-week-old mice were fed normal chow diet (NCD) and then were administered a 60% high-fat diet (HFD) for the indicated time periods (Research Diets Inc., New Brunswick, NJ, USA). Then, on the day of sacrifice, all of the HFD-fed mice were compared to age-matched NCD-fed mice. CD1d knockout (KO) mice were generously provided by S.H. Park. All experiments with mice were approved by the Institute of Laboratory Animal Resources in Seoul National University.

Adipocyte specific CD1d KO mice

C57BL/6J mice expressing the cre recombinase under the control of adipocyte specific promoter of adiponectin were generously provided by G.Y. Gou. CD1d floxed mice were purchased from the Jackson Laboratory. First, heterozygous CD1d floxed mice with adiponectin cre recombinase were generated by mating CD1d floxed mice with cre mice. Then cre inserted or non-inserted homozygous CD1d floxed mice were generated by mating heterozygous CD1d floxed with non cre inserted mice with heterozygous CD1d floxed with cre inserted

mice. 8-week-old mice were fed normal chow diet (NCD) and then were administered a 60% high-fat diet (HFD) for the 4 weeks (Research Diets Inc., New Brunswick, NJ, USA). Then, on the day of sacrifice, all of the HFD-fed mice were compared to age-matched NCD-fed mice.

Isolation of adipocytes, macrophages, and endothelial cells from adipose tissues

To isolate various cell types from adipose tissue, I pooled epididymal adipose tissues from 20-weeks of NCD (n=10) fed mice. After collagenase treatment and centrifugation, adipocytes in the supernatant were collected. The pelleted stromal vascular cell (SVC) fraction was used for isolation of macrophages and endothelial cells. SVCs were stained by anti-CD16/32 antibody (eBioscience) followed by APC-Cy7-conjugated CD11b, PE-conjugated CD11c, PE-Cy7 conjugated CD31, and PerCP-conjugated CD45 staining for 30 min at 4℃. After PBS washing, I sorted CD11b+CD11c+ cells, CD11b+CD11c- cells, CD11b-CD11c+ cells, and CD45-CD31+ cells by FACS Aria.

qRT-PCR.

The methods of qRT-PCR are provided in chapter one. The primer sequence information was added to Table 4.

Table 4. Information of qRT-PCR primer sequences

Gene	Forward primer sequence	Reverse primer sequence
mouse *Cyclophilin*	5'-CAGACGCCACTGTCGCTTT-3'	5'-TGTCTTTGGAACTTTGTGTG-3'
mouse *CD1d*	5'-TCCTAGAGGCAGGGAAGTCA-3'	5'-AGCATTTGGCAGGAAATCAC-3'
mouse *PPARγ*	5'-TTGCTGAACGTGAAGCCCATCGAGG-3'	5'-GTCCTTGTAGATCTCCTGGAGCAG-3'
mouse *Adiponectin*	5'-GGCAGGAAAGGAGAACCTGG-3'	5'-AGCCTTGTCCTTCTTGAAGA-3'
mouse *CD11c*	5'-GAGGATTTCAGCATCCCAGA-3'	5'-CACCTGCTCCTGACACTCAA-3'
mouse *CD31*	5'-ATCAGCTGCCAGTCCGAAAA -3'	5'-CAACTTCATCCACTGGGGCT -3'
mouse *CD11b*	5'-GACTCAGTGAGCCCCATCAT -3'	5'-AGATCGTCTTGGCAGATGCT -3'
Mouse *F4/80*	5'-GCTGCACCTCTGTGCCTTT-3'	5'-CAGGTATGCCATGATGCTTG-3'
human *GAPDH*	5'-CCACTCCTCCACCTTT-3'	5'-ACCACCCTGTTGCTGT-3'
human *CD3ε*	5'-TGAGGGCAAGAGTGTGTGAG-3'	5'-TAGTCTGGGTTGGGAACAGG-3'
human *CD1d*	5'-GTGTAGCTCCCACCCCAGTA-3'	5'-AGCCTGTATGGGTGAAGTGG-3'
human *Vα24*	5'-CTGGAGGGAAAGAACTGC-3'	5'-TGTCAGGGAAACAGGACC-3'

Immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described (41). Epididymal adipose tissues were removed and fixed with 1% paraformaldehyde for 1 h and washed. The samples were then blocked with 1% BSA, incubated for 1 h and then stained with primary antibody (perilipin 1:1000, CD1d 1:500) overnight at 4℃. After washing for 1 h, the samples were incubated with fluorescence-labeled secondary antibody for 4 h at room temperature (RT) and washed. Following staining with 4′,6-diamidino-2-phenylindole (DAPI) containing Vectorshield solution, samples were observed using a Zeiss LSM510NLO confocal microscope.

3T3-L1 cell line differentiation

3T3-L1 fibroblasts were passaged in high-glucose DMEM plus 10% bovine calf serum (BCS). After reaching confluence, 3T3-L1 cells were incubated with high-glucose DMEM plus 10% fetal bovine serum (FBS), 0.5mM 3-isobutyl-1-methylxanthine (IBMX), 1μM dexamethasone, and 1μg/ml insulin. After 2 days, 3T3-L1 cells were transferred to high glucose DMEM plus 10% FBS and 1μg/ml insulin for 2 days. Then, differentiated 3T3-L1 adipocytes maintained with high glucose DMEM plus 10% FBS.

Co-culture experiments

Primary adipocytes were prepared by the fractionation of mouse epididymal adipose tissue or by 3T3-L1 cell line differentiation. DN32.D3 hybridoma cells (provided by S.H. Park and D.H. Chung) were used as CD1d-restricted NKT cells. In case of α-GC treatment experiment, I pre-treated α-GC (100 ng/ml) to adipocytes and washed with PBS and then co-cultured with DN32.D3 cells. Adipocytes and DN32.D3 cells were co-cultured with or without a transwell membrane (0.4 μm pore size). The IL-2 cytokine concentration was measured by an IL-2 ELISA kit (eBioscience). I transfected negative control siRNA and CD1d siRNA into differentiated 3T3-L1 adipocytes by electroporation. siRNA sequences are provided in Table 5. To assess the attachment of 3T3-L1 and DN32.D3 cells, I pre-stained DN32.D3 with CellTracker Red CMPTX (Invitrogen, Carlsbad, CA, USA) and then spread it onto the adipocytes. After 24 h of incubation, unattached cells were washed out by vigorous PBS washing and mounted with DAPI containing Vectorshield solution.

Human samples

Human adipose tissue samples from 24 obese and overweight nondiabetic patients were provided by the Obesity Research Center at King Saud University and were analyzed for the expression of CD1d and Vα24 mRNA by quantitative reverse transcription polymerase chain reaction (qRT-PCR). This work is under a

Table 5. Information of siRNA sequences

Gene	Sense	Antisense
Negative control	CCUACGCCACCAAUUUCGU	ACGAAAUUGGUGGCGUAGG
CD1d	CUGCGGGCUGUGAAAUGUA	UACAUUUCACAGCCCGCAG

program to study adipocyte biology and adipokines. The whole project was approved by the College of Medicine Ethics Committee, King Saud University.

Statistics

The results are shown as means ± SEM. All statistical analysis was performed by the Student's *t* test or ANOVA in Excel (Microsoft); P-values < 0.05 were considered significant.

Results

Adipocytes express high levels of CD1d

While most T cells recognize peptides loaded on MHC molecules, iNKT cells recognize lipids or glycolipids bound to the MHC-like glycoprotein CD1d. Since iNKT cells are abundant and activated in adipose tissue, I decided to test the expression pattern of CD1d in adipose tissue. Northern blotting and qRT-PCR analyses clearly indicated that CD1d mRNA was highly expressed in adipose tissue (Fig. 22A and B). Among several fat depots, the level of CD1d mRNA was higher in epididymal and mesenteric adipose tissue than in inguinal adipose tissue (Fig. 22C)

Next, I fractionated adipose tissue into adipocytes and SVCs composed of endothelial cells (CD45-CD31+), M1-like macrophages (CD45+CD11b+CD11c+), M2-like macrophages (CD45+CD11b+CD11c-), and CD11c single positive cells (CD45+C1b-CD11c+) (Fig. 23A). Then, I compared the levels of CD1d mRNA in these fractions. The mRNA level of markers used in cell sorting was also analyzed in each sample (Fig. 23B). The expression level of CD1d mRNA was more than 10-fold higher in the adipocyte fraction than in the other cell types included in SVC fraction (Fig. 23C). By contrast, other antigen presenting molecules such as MHC class I and MHC class II molecules were not dominantly expressed in adipocyte fraction. In order to determine the subcellular localization of CD1d in adipocytes, I carried out whole mount immunohistochemistry. As shown in Fig. 24, CD1d

Figure 22.

CD1d is highly expressed in adipose tissue. Tissues were obtained from 8-week-old C57BL6/J male mice. ***A***: CD1d mRNA expression levels in thymus (THY), spleen (SPN), liver (LIV), epididymal adipose tissue (EAT), kidney (KID), lung (LNG), intestine (INT) and heart (HRT) were detected by Northern blot analysis. ***B***: qRT-PCR data of CD1d mRNA levels in each tissue. ***C***: CD1d mRNA expression level in fat depots. **P < 0.01 and ***P < 0.001.

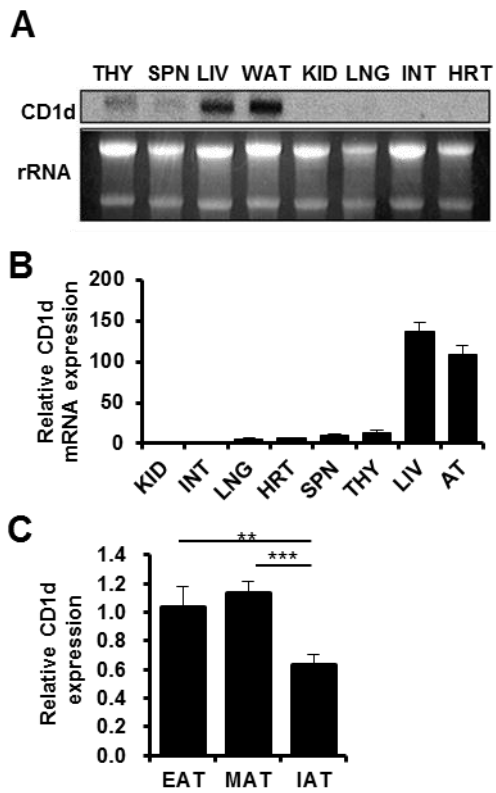

A
THY SPN LIV WAT KID LNG INT HRT
CD1d
rRNA
B
Relative CD1d mRNA expression
200
150
100
50
0
KID INT LNG HRT SPN THY LIV AT
C
**
***
Relative CD1d expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
EAT MAT IAT

Figure 23.

CD1d mRNAs are mainly expressed in adipocyte. Epididymal adipose tissues were obtained from 33-week-old C57BL6/J male mice. After separation of adipocytes and SVCs by centrifugation, SVCs were further sorted into endothelial cells, M1-like macrophages, M2-like macrophages, and CD11c single positive cells. A: Gating region for isolation of each cell population. For endothelial cells, CD45-CD31+ cells were sorted from SVCs. CD45+CD11b+CD11c+ cells for M1-like macrophages, CD45+CD11b+CD11c- for M2-like macrophages, and CD45+CD11b-CD11c+ cells for CD11c single positive cells were used. B:The mRNA levels of marker genes such as CD31, CD11b, CD11c, F4/80, and arginase-1. C: The mRNA levels of antigen presenting molecules involving CD1d, MHC class I, and MHC class II. n=3 (each n is pooled adipose tissue from 10 mice).

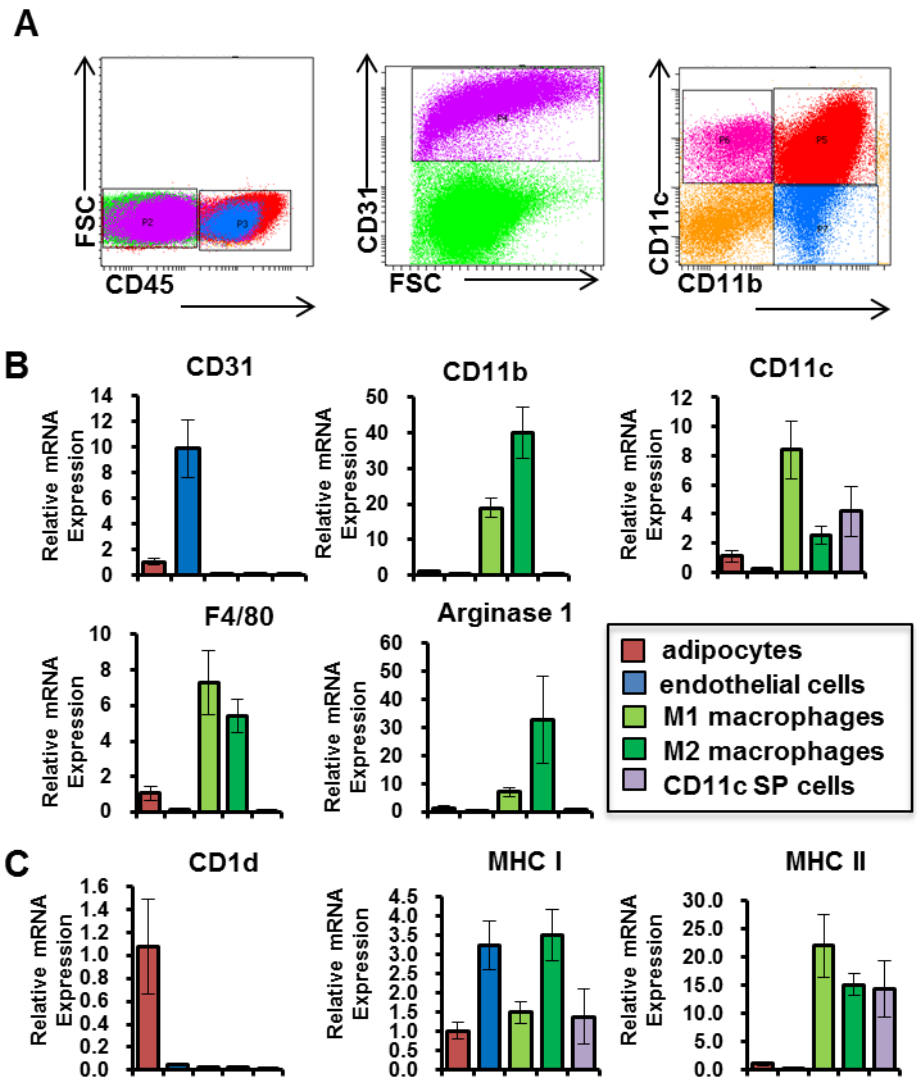
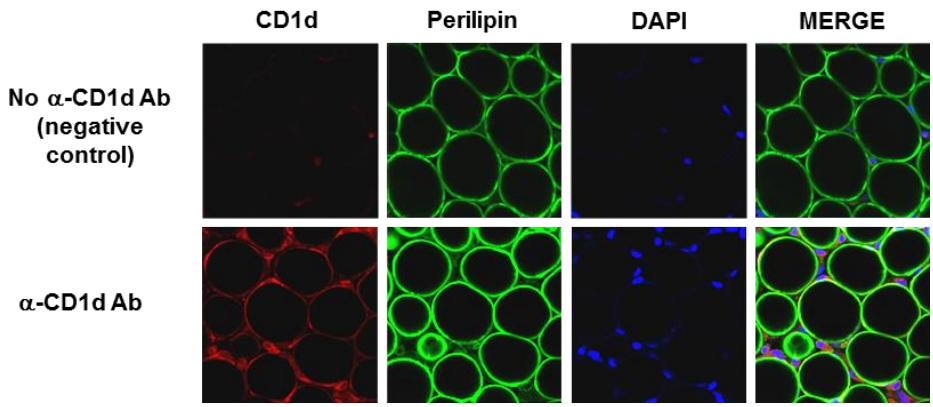

A
FSC
CD45
P2
P3
CD31
FSC
P4
CD11c
CD11b
P6
P5
P7
B
CD31
CD11b
CD11c
F4/80
Arginase 1
Relative mRNA Expression
adipocytes
endothelial cells
M1 macrophages
M2 macrophages
CD11c SP cells
C
CD1d
MHC I
MHC II

Figure 24.

CD1d is highly expressed in adipocytes – Immunohistochemistry. Localization of CD1d in adipocytes assessed by histological analysis of epididymal adipose tissues. CD1d (red), perilipin (green) and DAPI (blue) are seen.


CD1d
Perilipin
DAPI
MERGE
No α-CD1d Ab (negative control)
α-CD1d Ab

was abundantly expressed on the plasma membranes of adipocytes. In addition, the increased level of CD1d mRNA was observed in differentiated 3T3-L1 adipocytes (Fig. 25). However, neither CD1d knockdown nor its overexpression altered adipogenesis (Fig. 26). Taken together, these data suggest that mature adipocytes express high levels of CD1d on their cell surfaces, probably to facilitate interaction with iNKT cells and thereby modulate immune response in adipose tissue.

Adipocytes activate iNKT cells through CD1d

To test whether adipocyte CD1d is able to stimulate iNKT cells in adipose tissue, I carried out co-culture experiments with differentiated 3T3-L1 adipocytes and the iNKT cell hybridoma cell line DN32.D3 under various experimental conditions. I designed two different methods of co-culture, including the direct physical contact system (Contact) and the cell-impermeable transwell system (transwell) that blocks physical interaction. To monitor iNKT cell activation, α-GC, a potent iNKT cell-activating lipid antigen, was treated to load onto CD1d in adipocytes. Since IL-2 is secreted by activated T cells, I determined the concentration of secreted IL-2 in the culture media of either the contact or transwell systems. The IL-2 secretion was higher in iNKT cells in contact system than in iNKT cells in transwell system, implying that adipocytes are able to stimulate iNKT cell activation via direct cell–cell contact rather than via indirect cytokine secretion (Fig. 27A).

Figure 25.

The mRNA level of CD1d increases during adipogenesis. The mRNA levels of CD1d, PPARγ and adiponectin increased during 3T3-L1 adipocyte differentiation.

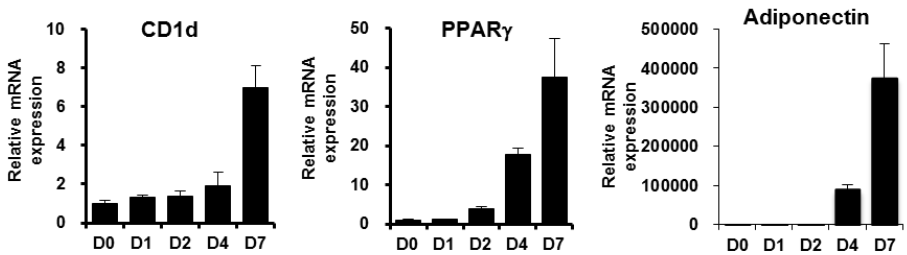
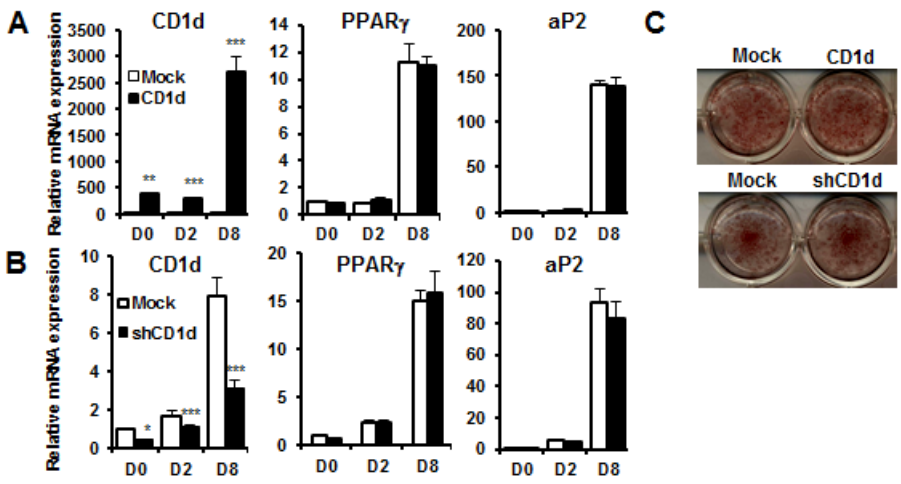

CD1d
Relative mRNA expression
10
8
6
4
2
0
D0 D1 D2 D4 D7
PPARγ
Relative mRNA expression
50
40
30
20
10
0
D0 D1 D2 D4 D7
Adiponectin
Relative mRNA expression
500000
400000
300000
200000
100000
0
D0 D1 D2 D4 D7

Figure 26.

Adipogenesis is not affected by CD1d expression in adipocytes. A. qRT-PCR data of CD1d, PPARγ, and aP2 in CD1d overexpressing stable cells. **B.** qRT-PCR data of CD1d, PPARγ, and aP2 in CD1d shRNA overexpressing stable cells. **C.** Oil-red O staining at day 8 of adipogenesis. *P < 0.05 and ***P < 0.001.


A
CD1d
PPARγ
aP2
Relative mRNA expression
Mock
CD1d
D0
D2
D8
B
shCD1d
C
Mock
CD1d
shCD1d

To clarify the role of adipocyte CD1d in iNKT cell activation, I suppressed adipocyte CD1d by using siRNA, followed by direct co-culture with iNKT cells. IL-2 secretion level significantly reduced in CD1d-suppressed adipocytes (Fig 27B). Next, I explored the role of adipocyte CD1d in cell–cell contact with iNKT cells. As shown in Figure 27C, iNKT cells physically interacted with CD1d-expressing adipocytes, whereas iNKT cell attachment was notably diminished in CD1d-suppressed adipocytes. In addition, the IL-2-producing activity of primary adipocytes isolated from the adipose tissues of $CD1d^{+/-}$ and $CD1d^{-/-}$ mice was determined by co-culture with iNKT cells. Primary adipocytes from $CD1d^{+/-}$ mice potentiated IL-2 secretion whereas CD1d-deficient adipocytes from $CD1d^{-/-}$ mice failed to induce IL-2 secretion in iNKT cells (Fig. 27D).

Since I observed that CD44 (activation/memory marker) expression on iNKT cells were augmented in adipose tissue after short-term HFD consumption, I studied the involvement of adipocytes in iNKT cell activation upon short-term HFD. I isolated primary adipocytes from mice that were given NCD or HFD for 1 week and co-cultured them with DN32.D3 cells with or without α-GC pre-treatment. Although the level of adipocyte CD1d mRNA was not significantly different between NCD and HFD group, primary adipocytes isolated from HFD-fed mice led to higher degree of iNKT cell activation as compared to those from NCD-fed mice (Fig. 28). These data imply that primary adipocytes from short-term HFD-fed mice appear to be more potent than those from NCD-fed mice in stimulating iNKT cells in adipose tissue. Together, these results propose that adipocytes indeed

Figure 27.

Adipocyte CD1d regulates iNKT cell activity. ***A***: DN32.D3 hybridoma cells were used as iNKT cells. Adipocytes and DN32.D3 cells were directly mixed and co-cultured in contact, or cultured separated by a transwell membrane, which is cell-impermeable with a 0.4-μm pore size. IL-2 secretion by DN32.D3 cells was induced by co-culturing with differentiated 3T3-L1 adipocytes which α-GC (100 ng/ml) pre-treated for 4 hours. ***B***: CD1d expression in negative control (NC) and CD1d siRNA transfected adipocytes (left). IL-2 secretion by DN32.D3 cells upon co-culturing with siRNA-transfected 3T3-L1 adipocytes (right). Adipocytes were pre-treated with α-GC (100 ng/ml) for 4 h and, after PBS washing, co-cultured with DN32.D3 cells for 6 h. ***C***: Immunocytochemistry analysis of DN32.D3 cells attached to 3T3-L1 adipocytes. Adipocytes were transfected with control siRNA or CD1d siRNA and co-cultured with DN32.D3 cells for 24 h. After washing with PBS, attached red fluorescence-labeled DN32.D3 cells were monitored. ***D***: The amount of secreted IL-2 was determined by ELISA analysis. Primary adipocytes were isolated from the adipose tissues of $CD1d^{+/-}$ or $CD1d^{-/-}$ mice and co-cultured with DN32.D3 cells for 24 h with or without α-GC (100 ng/ml). n = 5; *$P < 0.05$ and **$P < 0.01$.

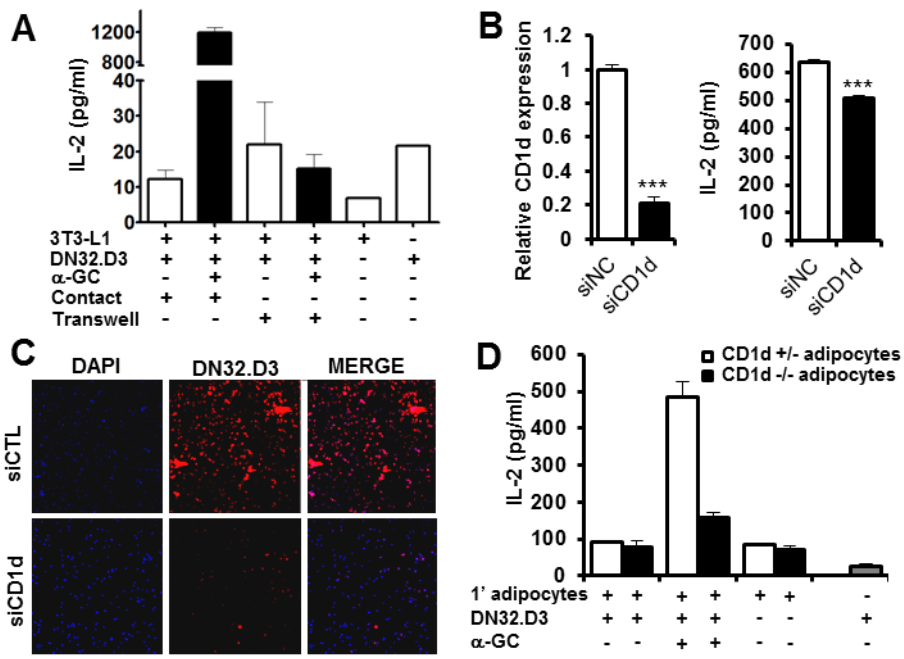
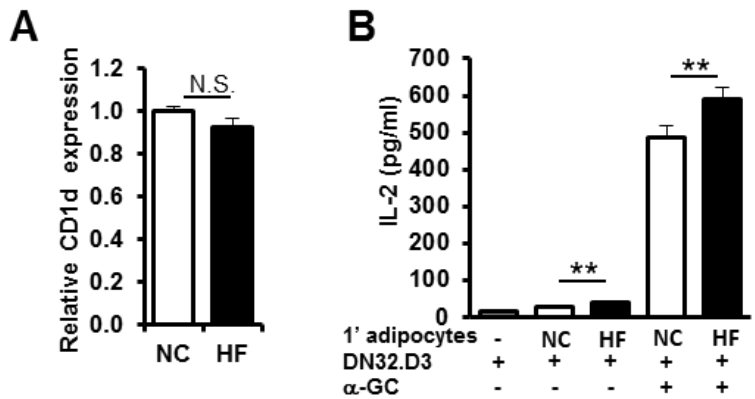

A
IL-2 (pg/ml)
1200
800
40
30
20
10
0
3T3-L1 + + + + + -
DN32.D3 + + + + - +
α-GC - + - + - -
Contact + + - - - -
Transwell - - + + - -
B
Relative CD1d expression
1.2
1
0.8
0.6
0.4
0.2
0
***
siNC
siCD1d
IL-2 (pg/ml)
700
600
500
400
300
200
100
0
***
siNC
siCD1d
C
DAPI
DN32.D3
MERGE
siCTL
siCD1d
D
CD1d +/- adipocytes
CD1d -/- adipocytes
IL-2 (pg/ml)
600
500
400
300
200
100
0
1' adipocytes + + + + + + -
DN32.D3 + + + + - - +
α-GC - - + + - - -

Figure 28.

Adipocytes from 1 week HFD fed mice induce iNKT cell activation. Primary adipocytes were isolated from mice given NCD or HFD for 1 week. ***A:*** CD1d mRNA level of adipocytes from NCD-fed mice (white), or from HFD-fed mice (black). ***B:*** iNKT cells were activated by treatment with α-GC (100 ng/ml) pre-treated adipocytes. Adipocytes and DN32.D3 cells were co-cultured for 36 h. n = 5; *P < 0.05 and **P < 0.01.


A
Relative CD1d expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
N.S.
NC
HF
B
IL-2 (pg/ml)
700
600
500
400
300
200
100
0
**
**
1' adipocytes - NC HF NC HF
DN32.D3 + + + + +
α-GC - - - + +

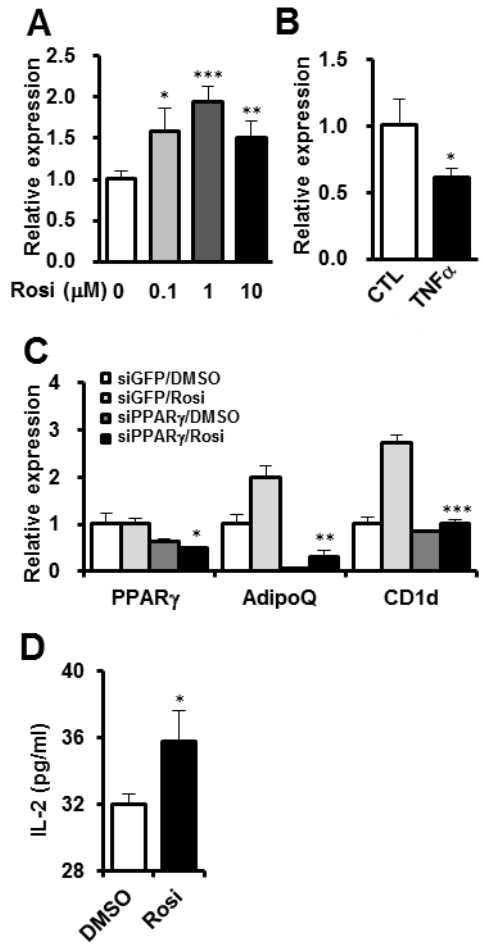
have the ability to activate iNKT cells via physical interaction with adipocyte CD1d, especially upon short term HFD.

Adipocyte CD1d expression is regulated by peroxisome proliferator-activated receptor

I next investigated the regulatory mechanism of CD1d expression in adipocytes. Several transcription factors such as peroxisome proliferator-activated receptor (PPAR)-γ, CCAAT/enhancer-binding protein (CEBP)-β, retinoic acid receptor (RAR) and the Ets family have been reported to be involved in the regulation of CD1d expression (29, 83, 87). PPARγ, the master regulator of adipogenesis, is abundantly expressed in adipocytes. To examine the role of PPARγ in CD1d expression in adipocytes, I determined the expression level of adipocyte CD1d mRNA upon rosiglitazone, a synthetic agonist of PPARγ and tumor necrosis factor (TNF)-α, a functional inhibitor of PPARγ. In adipocytes, the level of CD1d mRNA increased upon rosiglitazone treatment, but TNF-α treatment inhibited CD1d expression (Fig. 29A and B). To confirm whether PPARγ is indeed involved in adipocyte CD1d expression, I investigated the effect of PPARγ knockdown on the expression of CD1d mRNA in adipocytes. Unlike the cells treated with control siRNA, CD1d mRNA expression was not induced by rosiglitazone in cells with PPARγ knockdown (Fig. 29C). Then I tested whether increased CD1d expression in adipocytes by activated PPARγ affected iNKT cell activity. When iNKT cells

Figure 29.

Expression of adipocyte CD1d is stimulated by PPARγ activation. Differentiated 3T3-L1 adipocytes were used as the adipocyte source. ***A, B***: Level of CD1d mRNA in adipocytes treated with rosiglitazone (0.1, 1 μM) or TNF-α(10 ng/ml) for 24 h. ***C***: Effect of PPARγ knockdown on CD1d expression. Differentiated 3T3-L1 adipocytes were transfected with siRNAs (negative control or PPARγ) by electroporation. After 12 h, the cells were treated with rosiglitazone (10 μM). mRNA levels of PPARγ, adiponectin and CD1d were determined by qRT-PCR. ***D***: IL-2 secretion by DN32.D3 cells stimulated by adipocytes treated with rosiglitazone (10 M). *P < 0.05, **P < 0.01 and ***P < 0.001.


A
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
*
***
**
Rosi (μM) 0
0.1
1
10
B
Relative expression
1.5
1.0
0.5
0.0
*
CTL
TNFα
C
Relative expression
4
3
2
1
0
siGFP/DMSO
siGFP/Rosi
siPPARγ/DMSO
siPPARγ/Rosi
*
**
***
PPARγ
AdipoQ
CD1d
D
IL-2 (pg/ml)
40
36
32
28
*
DMSO
Rosi

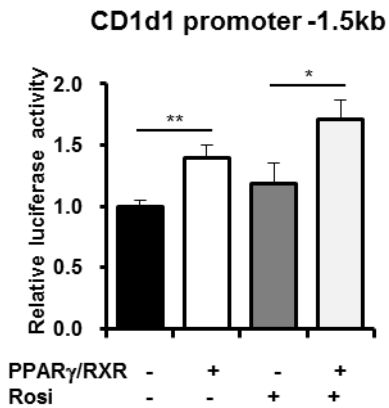
were co-cultured with adipocytes in the presence of rosiglitazone, the secretion level of IL-2 slightly but substantially increased (Fig. 29D). Moreover, when I performed the luciferase reporter assay with the mouse CD1d promoter, the CD1d promoter was transactivated by PPARγ (Fig. 30). Together, these data imply that PPARγ would regulate iNKT cell activity in adipose tissue.

Adipocyte CD1d and PPARγ expression levels decrease in adipose tissue of obese subjects

The finding that the iNKT cell population decreased in the adipose tissue of obese mice prompted us to examine the level of CD1d mRNA in several obese animal models. In concordance with the decrease in iNKT cell population in obesity, CD1d expression in the adipocyte fraction was also significantly reduced in the adipose tissue of *db/db*, *ob/ob* and long-term (16 weeks) HFD-fed obese (diet-induced obesity, DIO) mice (Fig. 31A). Given that the PPARγ mRNA was reduced in the adipocyte fraction of obese mice, it appears that CD1d expression in adipocytes might be positively correlated with PPARγ (Fig. 31B). Consistent with the mouse models, the mRNA expression levels of Vα24 and CD1d in human visceral adipose tissue was negatively correlated with the body mass index (BMI) (Fig. 32). These results support the idea that the decrease in the iNKT cell population in adipose tissue might be accompanied by reduced CD1d expression in adipocytes in severe obesity.

Figure 30.

Expression of adipocyte CD1d is stimulated by PPARγ activation – luciferase assay. Luciferase assay of mouse CD1d promoter with or without PPARγ/RXR and rosiglitazone in H293T cell line. Two kinds of CD1d promoter were tested which is including -1.5 kb and -1.0 kb. PPARγ binding sequence (DR-1 sequence) is located about -1.2kb of CD1d1 promoter. *P < 0.05 and **P < 0.01.


CD1d1 promoter -1.5kb
Relative luciferase activity
2.0
1.5
1.0
0.5
0.0
**
*
PPARγ/RXR - + - +
Rosi - - + +

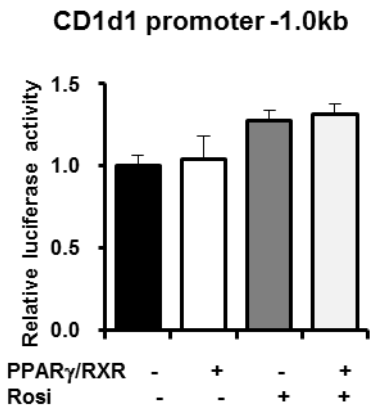

CD1d1 promoter -1.0kb
Relative luciferase activity
1.5
1.0
0.5
0.0
PPARγ/RXR - + - +
Rosi - - + +

Figure 31.

Expression levels of adipocyte CD1d and PPARγ decrease in adipose tissue in obesity. Primary adipocytes were isolated from the adipose tissue of obese mice. ***A***: Relative levels of CD1d mRNA and ***B:*** relative levels of PPARγ mRNA in adipocytes between *db/+* and *db/db* mice, WT and *ob/ob* mice and lean and DIO mice. ***P < 0.001.

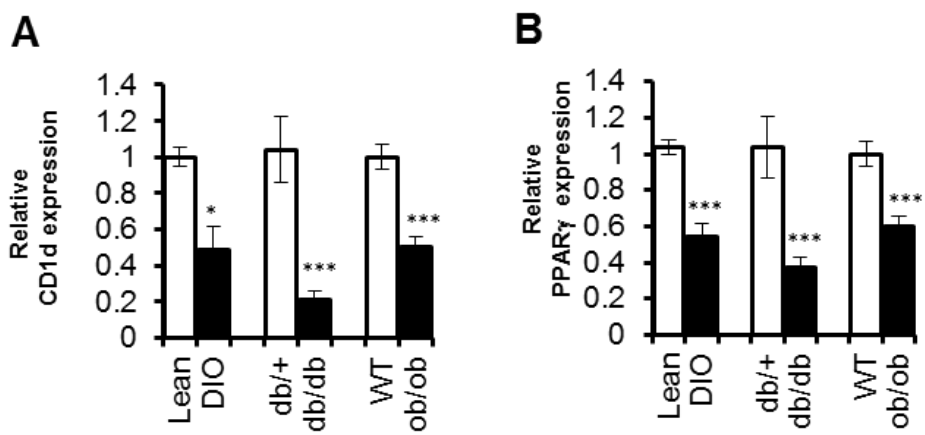
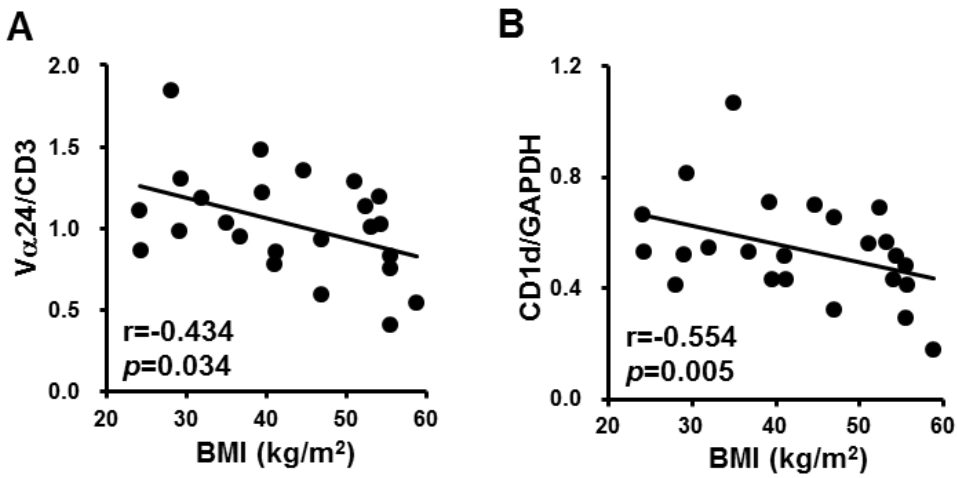

A
Relative CD1d expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
***
***
Lean
DIO
db/+
db/db
WT
ob/ob
B
Relative PPARγ expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
Lean
DIO
db/+
db/db
WT
ob/ob

Figure 32.

The expression levels of Vα24 and CD1d are reduced in the adipose tissue of obese humans. ***A:*** Vα24 mRNA expression normalized by CD3ε mRNA and ***B***: CD1d mRNA expression normalized by GAPDH, depending on BMI.


A
Vα24/CD3
2.0
1.5
1.0
0.5
0.0
r=-0.434
p=0.034
20
30
40
50
60
BMI (kg/m²)
B
CD1d/GAPDH
1.2
0.8
0.4
0.0
r=-0.554
p=0.005
20
30
40
50
60
BMI (kg/m²)

In severely obese adipose tissues, the level of adipocyte-expressing CD1d and NKT cell proliferation are reduced.

Based on the data demonstrating that CD1d mRNA expression of adipocytes in severely obese adipose tissue was reduced as shown in fig. 31 and 32, I speculated the possible role of adipocyte CD1d in the regulation of the number of iNKT cells in adipose tissues. Thus, I checked CD1d protein expression on the surface of adipocytes and iNKT cell proliferation in severely obese adipose tissues in HFD fed mice for 16weeks. In parallel with the data which showed decreased level of CD1d mRNA expression in 16 weeks of HFD fed mice, the surface expression level of CD1d on adipocytes was significantly diminished (Fig. 33A and B). In addition, the proliferation of iNKT cells was also reduced in these mice (Fig. 33C). Therefore, I suggest that the level of CD1d expression and iNKT cell proliferation could be positively correlated in adipose tissues in obese mice.

Adipose iNKT cells are reduced in adipocyte specific CD1d KO mice

To examine the role of CD1d on adipocytes in the regulation of iNKT cell response to DIO *in vivo*, I generated adipocyte-specific CD1d knockout mice by using cre/loxP system (Fig. 34). To knockout CD1d gene in adipocyte specifically, I used the adipocyte specific cre mice that cre recombinase is expressed under control of the adiponectin promoter. When CD1d1 floxed mice (Jackson Lab.) bred to cre mice, exon 2-6 are deleted. Since the homologous CD1d2 gene has a

Figure 33.

Positive association between CD1d expression on adipocytes and proliferation of iNKT cells in long term (16 weeks) HFD. ***A***: Primary adipocytes from adipose tissues of NCD and 16 weeks of HFD fed mice were isolated and surface expression of CD1d on adipocytes were measured by flow cytometry. ***B:*** Mean fluorescence intensity of CD1d expression on adipocytes of NCD and HFD fed mice. ***C***: Ki67 positive cells among iNKT cells. **P < 0.01 and ***P < 0.001.

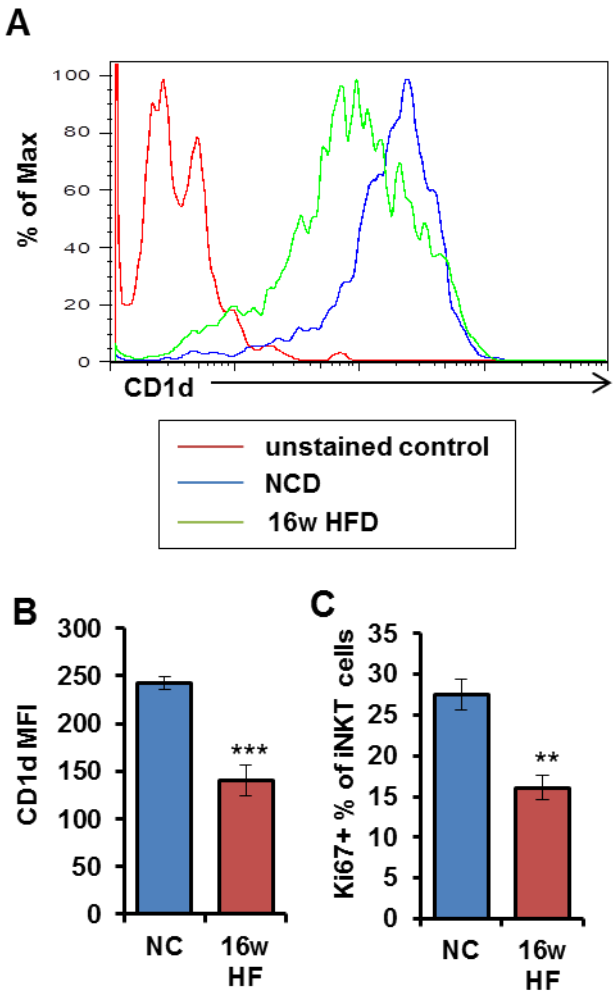
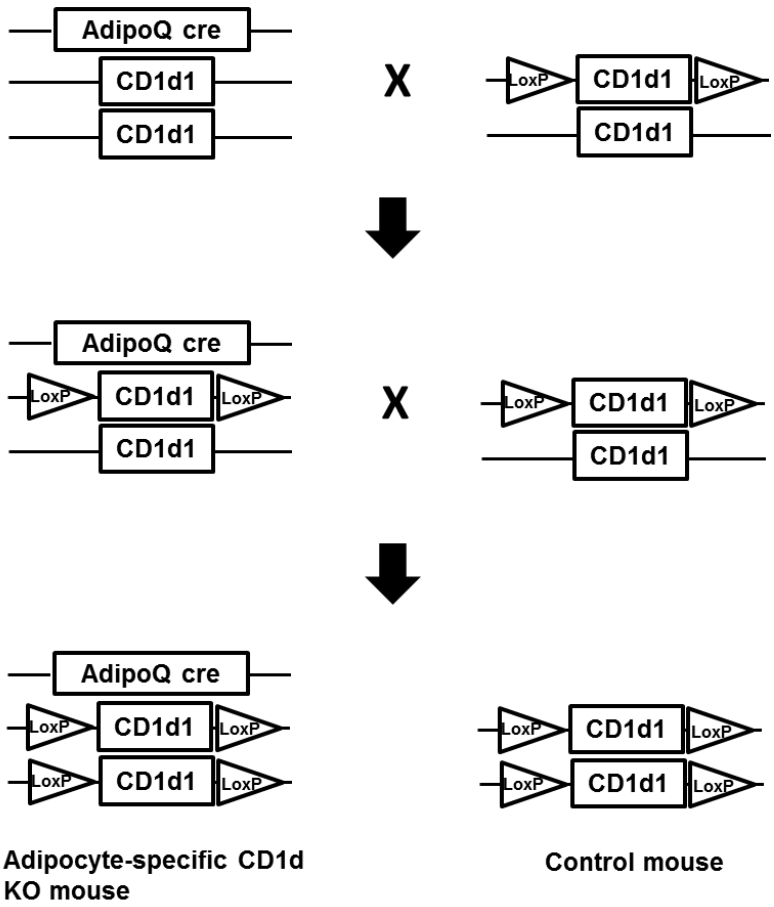

A
100
80
60
40
20
0
% of Max
CD1d
unstained control
NCD
16w HFD
B
300
250
200
150
100
50
0
CD1d MFI
***
NC
16w HF
C
35
30
25
20
15
10
5
0
Ki67+ % of iNKT cells
**
NC
16w HF

Figure 34.

Mating scheme for producing adipocyte-specific CD1d KO mice. To generate adipocyte-specific CD1d KO mice, C57BL/6J mice expressing the cre recombinase under the control of adipocyte specific promoter of adiponectin and CD1d floxed mice were used. First, heterozygous CD1d floxed mice with adiponectin cre recombinase were generated by mating CD1d floxed mice with cre mice. Then, cre inserted or non-inserted homozygous CD1d floxed mice were generated by mating heterozygous CD1d floxed non cre inserted mice with heterozygous CD1d floxed with cre inserted mice.


AdipoQ cre
CD1d1
CD1d1
X
LoxP
CD1d1
LoxP
CD1d1
AdipoQ cre
LoxP
CD1d1
LoxP
CD1d1
X
LoxP
CD1d1
LoxP
CD1d1
AdipoQ cre
LoxP
CD1d1
LoxP
LoxP
CD1d1
LoxP
LoxP
CD1d1
LoxP
Adipocyte-specific CD1d KO mouse
Control mouse

frameshift mutation preventing expression of a functional protein, blocking expression of CD1d1 results in the complete absence of CD1d protein at the cell surface (1).

To test that CD1d gene is specifically deleted in adipose tissues of these mice, I compared CD1d expression in various tissues. The expression of CD1d mRNA was selectively reduced in adipose tissues including brown adipose tissues (BAT), inguinal adipose tissue (IAT), and epididymal adipose tissue (EAT) (Fig. 35A). Immunohistochemistry data also showed greatly reduced CD1d expression in adipocytes (Fig. 35B). Thus, I suppose that these adipocyte specific CD1d knockout mice can be used for the investigation of interactions between adipocyte CD1d and iNKT cells in adipose tissues.

Adipocyte-CD1d KO mice and their littermate mice (WT) were subjected to the comparison of the number of iNKT cells in adipose tissues upon NCD and HFD feeding. Although it was not statistically significant, the percentage of iNKT cells among lymphocytes was reduced in WT mice upon HFD (Fig. 36A). In NCD fed mice, the percent of iNKT cells among lymphocytes decreased in KO mice. Also, iNKT cell number per fat mass was significantly reduced in adipose tissues of KO mice compared with WT mice upon NCD and HFD feeing (Fig. 36B). In addition, when I checked the proliferation of iNKT cells, HFD fed KO mice displayed reduction in iNKT cell proliferation relative to HFD fed WT mice (Fig. 36C). However, proliferation of other T cells such as regulatory T cells was retained regardless of genotypes (Fig. 36D). Therefore, I suggest that adipocyte

Figure 35.

CD1d expression is specifically reduced in adipose tissues KO mice. A: CD1d mRNA expression in tissues of WT and adipocyte-specific CD1d KO mice. CD1d mRNA expression level in KO tissues were normalized to the mRNA level of each tissues from WT mice. B: DAPI (blue), CD1d (red), and perilipin (green) staining of epididymal adipose tissues of WT and KO mice.

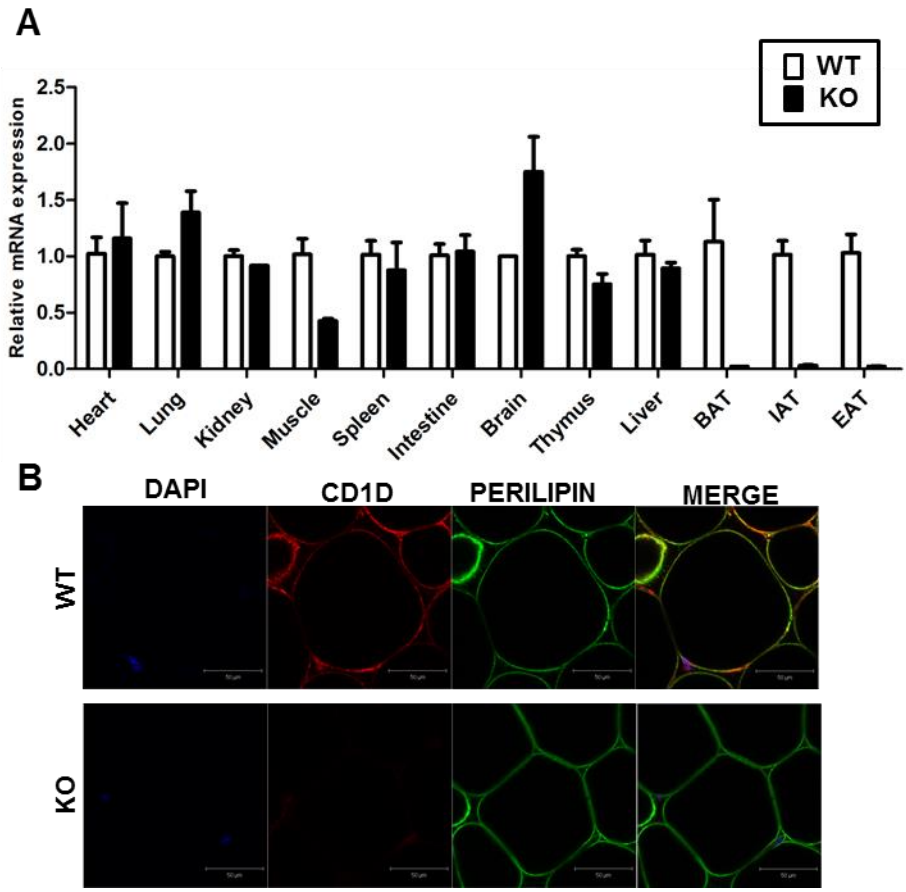
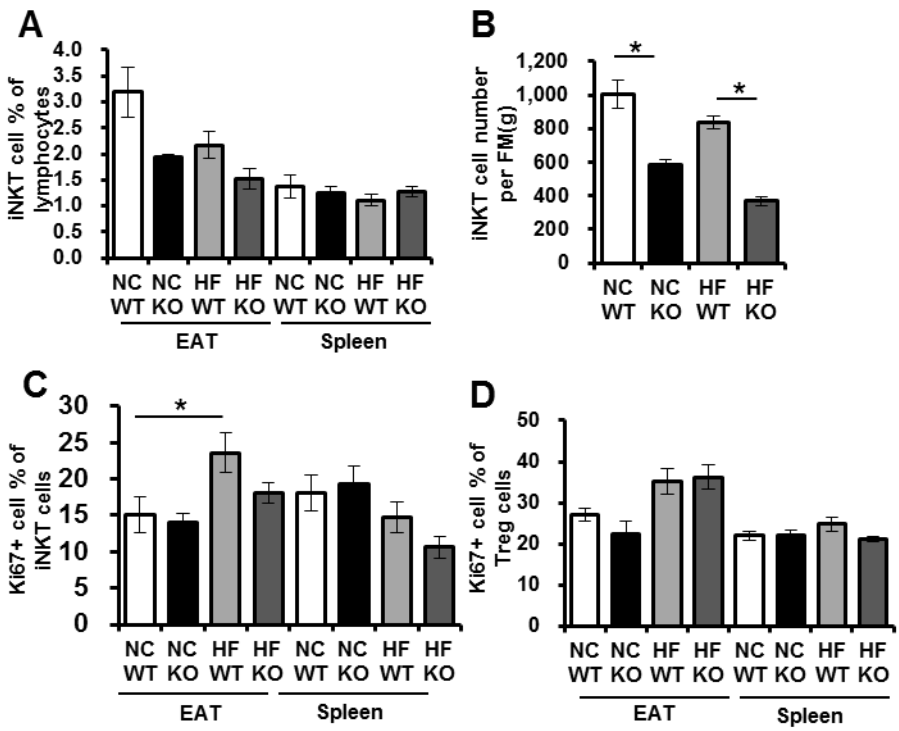

A
WT
KO
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
Heart
Lung
Kidney
Muscle
Spleen
Intestine
Brain
Thymus
Liver
BAT
IAT
EAT
B
DAPI
CD1D
PERILIPIN
MERGE
WT
KO

Figure 36.

The number of iNKT cells is reduced in adipose tissues of adipocyte-specific CD1d KO mice. WT and adipocyte-specific CD1d KO mice were fed HFD for 4 weeks from the age of 8 weeks. A: Percent of iNKT cells among lymphocytes in epididymal adipose tissue (EAT) and spleen. B: iNKT cell number per fat mass (gram). C: Percent of Ki67 positive cells among iNKT cells in adipose tissue and spleen. D: Percent of Ki67 positive cells among Treg cells in adipose tissue and spleen. *$P < 0.05$.


A
iNKT cell % of lymphocytes
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NC WT
NC KO
HF WT
HF KO
NC WT
NC KO
HF WT
HF KO
EAT
Spleen
B
iNKT cell number per FM(g)
1,200
1,000
800
600
400
200
0
*
*
NC WT
NC KO
HF WT
HF KO
C
Ki67+ cell % of iNKT cells
30
25
20
15
10
5
0
*
NC WT
NC KO
HF WT
HF KO
NC WT
NC KO
HF WT
HF KO
EAT
Spleen
D
Ki67+ cell % of Treg cells
50
40
30
20
10
0
NC WT
NC KO
HF WT
HF KO
NC WT
NC KO
HF WT
HF KO
EAT
Spleen

CD1d would regulate iNKT cell number by modulating proliferation of iNKT cells in adipose tissues.

Discussion

Among various immune cells, invariant NKT cells are suggested as anti-inflammatory cells in adipose tissue inflammation although there are controversies (37, 41, 55, 93). The number of iNKT cells was reduced in the early stage of obesity such as 1 week of HFD. Moreover, iNKT cell deficient mice exhibited aggravated adipose tissue inflammation and systemic insulin resistance. However, the regulatory mechanisms of iNKT cells in adipose tissues have not been studied.

In the present study, to investigate the mechanism by which iNKT cells are modulated in adipose tissue of obese animals or individuals, I examined the expression of CD1d in adipose tissues. Surprisingly, CD1d was highly expressed in differentiated adipocytes. Moreover, primary adipocytes from short-term HFD-fed mice potently stimulated iNKT cell activation. However, the mRNA levels of CD1d and Vα24 decreased in adipose tissues of obese human subjects, suggesting the association between the level of adipocyte CD1d and the number of iNKT cells in adipose tissues. Furthermore, adipocyte specific CD1d KO mice exhibited reduced number and proliferation of iNKT cells in adipose tissues. These results suggest that the activity and proliferation of iNKT cells could be regulated by adipocytes expressing CD1d.

CD1d is expressed on the surface of dendritic cells, macrophages, endothelial cells and hepatocytes (11). It has been reported that the liver is the organ showing the highest CD1d expression (28). However, the expression of

CD1d in adipose tissue, especially in adipocytes, has not been studied. Here, I discovered, unexpectedly, that CD1d mRNA expression in adipose tissue was very highly expressed. Furthermore, the major source of CD1d in adipose tissue was the adipocyte fraction, not the SVC fraction. In addition, I found that PPARγ is involved in the regulation of CD1d mRNA expression in adipocytes. Since PPARγ activation with rosiglitazone exerts beneficial effects on insulin sensitivity and anti-inflammation (19, 44), it is conceivable that CD1d, as one of potential target genes of PPARγ, might be associated with beneficial effects on adipose tissue inflammation or metabolism.

Adipocytes are active in lipid metabolisms. In this respect, it appears that the species of lipids presented on adipocyte CD1d molecules may determine iNKT cell activity in adipose tissue. Several lipids and lipid derivatives such as various phospholipids, iGb3, β-glucosylceramide (β-GC), and plasmalogen lysophosphatidylethanolamine (pLPE) have been reported as CD1d-binding antigens (12). CD1d is also considered as a reporting molecule as it reflects the state of cellular lipid metabolism. One of the distinct characteristics of iNKT cells is their ability to secrete Th1-type cytokines or Th2-type cytokines depending on the species of CD1d-loaded lipid antigens that they encounter, which eventually influence iNKT cell activity (25). In obesity, adipocytes play crucial roles in managing increased lipid metabolism by processing and storing various lipid species in adipose tissue. Thus, it is possible that certain lipid metabolites could be presented on adipocyte CD1d molecules and the lipid-CD1d complex would be

exposed on the adipocyte surface, leading to the modulation of iNKT cell activity in fat tissue. In the case of hepatic inflammation, iNKT cells encounter hepatocytes and become activated upon recognizing specific lipid antigens loaded on hepatocyte CD1d molecules (86). Similarly, it appears that adipose tissue iNKT cells may patrol around adipocytes and respond to various lipid antigens loaded on adipocyte CD1d in fat tissue to coordinate and adapt to nutritional changes.

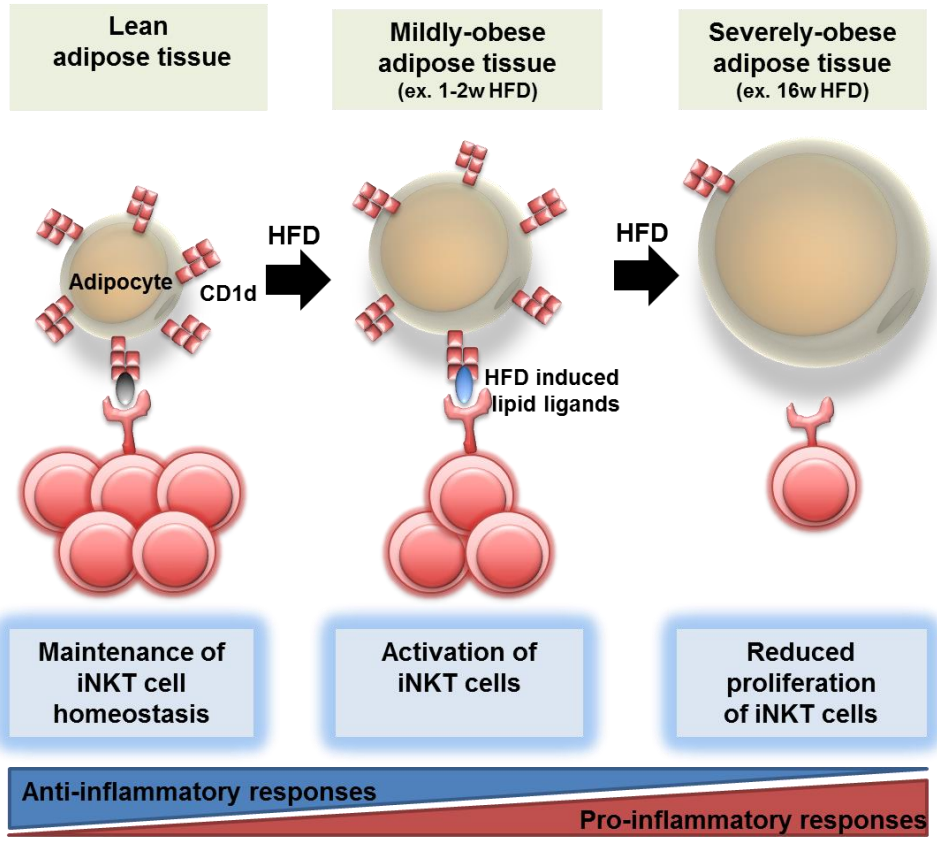
Several data have shown the possibility of adipocytes as antigen presenting cells for adipose iNKT cells. For example, CD1d mRNA level is the highest in adipocyte among adipose tissue-composing cells. Moreover, adipocytes can activate iNKT cells in a CD1d dependent manner. Notably, there is correlative change between iNKT cell response such as proliferation and the level of CD1d mRNA expression of adipocyte after long term HFD feeding. Therefore, I generated adipocyte specific CD1d KO mice to investigate the roles of adipocyte CD1d in the regulation of adipose iNKT cell response. Although further studies are required to find the roles of adipocyte expressing CD1d *in vivo*, decrement of the number and HFD-induced proliferation of iNKT cells implies the contribution of adipocyte CD1d in the regulation of iNKT cell response. It is needed to verify the mechanisms of decrement of iNKT cell proliferation and number in adipose tissues of adipocyte CD1d KO mice. It is possible that deficiency of adipocyte CD1d affect iNKT cell activation or survival. However, it is necessary to further investigate in-depth characterization of iNKT cell apoptosis, activation, and cytokine expression in adipose tissues in adipocyte-specific CD1d KO mice.

Moreover, the effects of adipocyte-specific CD1d KO on adipose tissue inflammation, adipose tissue mass, and systemic insulin tolerance have to be examined in the future study. Moreover, adipose tissue macrophage cannot be excluded as antigen presenting cell for iNKT cells since macrophage is well-known antigen presenting cell for iNKT cells in other organs. Although the mRNA expression level of CD1d is the highest in adipocyte, the co-stimulatory molecules and cytokines expressed on macrophage could enhance the activation of iNKT cells upon HFD feeding in adipose tissue. Therefore, it is necessary to compare the iNKT cell response in adipose tissue between adipocyte specific CD1d KO mice and macrophage specific CD1d KO mice.

In this work, I have newly identified the regulatory mechanism involved in the crosstalk between adipocytes and iNKT cells in adipose tissue. As shown in Fig. 37, I propose the role of adipocyte expressing CD1d in the regulation of iNKT cells in adipose tissues. CD1d is highly expressed in adipocytes and maintains homeostasis of iNKT cell population in lean adipose tissues. Upon ST-HFD

Figure 37.

Proposed model – interaction between adipocyte CD1d and iNKT cells. In lean adipose tissues, CD1d is highly expressed in adipocytes. However, HFD induces presentation of HFD-induced lipid antigens to iNKT cells and activate iNKT cells. In severely obese adipose tissues, CD1d expression of adipocytes is reduced, which allows for a decrease in iNKT cell proliferation. This results in diminished anti-inflammatory response in obese adipose tissues. Therefore, adipocyte expressing CD1d could function as one of the crucial mediators for governing homeostasis of iNKT cells and adipose tissue inflammation in obesity.


Lean
adipose tissue
Mildly-obese
adipose tissue
(ex. 1-2w HFD)
Severely-obese
adipose tissue
(ex. 16w HFD)
Adipocyte
CD1d
HFD
HFD
HFD induced
lipid ligands
Maintenance of
iNKT cell
homeostasis
Activation of
iNKT cells
Reduced
proliferation
of iNKT cells
Anti-inflammatory responses
Pro-inflammatory responses

feeding, adipocyte CD1d mediates iNKT cell activation by presentation of HFD-induced lipid ligands. However, in severely obese adipose tissues, accumulation of inflammatory response reduces the surface expression of CD1d. This reduction of CD1d expression in adipocytes results in repression of proliferation and function of iNKT cells which mediate suppression of adipose tissue inflammation. Consequently, it would contribute to the accumulation of pro-inflammatory response in severely obese adipose tissues. Therefore, I believe that adipocyte CD1d molecules play a pivotal role in sensing and mediating metabolic changes and then present to iNKT cells, which would eventually affect fine-tuning between the innate and adaptive immune response to confer systemic energy homeostasis. The exact regulatory mechanisms of iNKT cell response in adipose tissue by adipocyte CD1d and the identification of obesity-related lipid antigens remain to be investigated.

Conclusion and Perspectives

1. Adipose tissues are primarily responding tissues to mediate HFD-induced inflammation

In obesity, chronic adipose tissue inflammation is known as one of the key contributors to insulin resistance. Recently, a number of studies have demonstrated the roles of various immune cells in adipose tissue inflammation. Innate immune cells including macrophages, mast cells, eosinophils, and neutrophils and adaptive immune cells including B cells, Th1 cells, Treg cells, CD8 T cells play either anti-inflammatory or pro-inflammatory roles in obesity-induced adipose tissue inflammation (17, 58). Regarding adipose tissue inflammation, most studies have been revealed with obese animal models such as long term HFD fed DIO mice or *db/db* and *ob/ob* mice. However, it needs to be characterized the initial process of adipose tissue inflammation to verify the initiating/inducing factors of adipose tissue inflammation. Therefore, I have investigated the early responding immune cells and regulatory mechanisms of them in obesity-induced adipose tissue inflammation. In this study, I have examined the changes in adipose tissues upon short term as well as long term HFD. Surprisingly, adipose tissues were rapidly changed in adipocyte size, inflammatory gene expression, and macrophage infiltration even within 3 days of HFD feeding. Furthermore, Lee et al. have reported that the induction of inflammatory response is specifically observed in adipose tissue, but not in other metabolic tissues such as liver and muscle (50).

Thus, I have speculated that adipose tissues may mainly respond to HFD because excess lipids are often transported and stored in adipocytes. Taken together, I suggest that adipose tissue is a rapidly responding and initiating organ to mediate HFD-induced inflammatory response, compared to other metabolic tissues.

Although inflammatory response induced by short term (< 1 week) HFD feeding may not be a key causal factor of systemic insulin resistance, accumulation of pro-inflammatory response in adipose tissues would be an important contributor to insulin resistance in later stage of obesity. During short term (< 1 week) HFD, not only the number of pro-inflammatory macrophages but also anti-inflammatory macrophages increased in adipose tissues, implying that adipose tissues appear to suppress pro-inflammatory response through induction of ant-inflammatory response in the early stage of HFD. Nevertheless, pro-inflammatory response is dominated over anti-inflammatory response by chronic excess energy input, leading to systemic insulin resistance in late stage of obesity. Therefore, it is important to investigate the underlying mechanisms involved in the regulation of anti-inflammatory response in adipose tissue of obese subjects.

2. Anti-inflammatory roles of iNKT cells in adipose tissue inflammation

iNKT cells are innate-like lymphocytes which rapidly respond and produce large quantities of cytokines. iNKT cells are known to influence on many other immune cells through cytokine production as well as interaction with surface

molecules. For example, activation of iNKT cells can promote neutrophil recruitment, macrophage polarization, T cell activation, and NK cell activation (92).

In obesity, the functions of iNKT cells have received a lot of attention because iNKT cells could recognize lipid species whose amounts increase dramatically in obesity. Although several groups have demonstrated the roles of iNKT cells in adipose tissue inflammation, the precise functions of NKT cells remain controversial. For example, Mantell et al. have suggested that the functions of iNKT cells are dispensable in adipose tissue inflammation because body weight gain, glucose sensitivity, fat mass, and adipose tissue inflammation are not significantly changed in HFD-fed CD1d KO mice (57). In contrast, Wu et al. have demonstrated that iNKT cells augment obesity-related inflammation and insulin resistance in both Jα18 and CD1d KO mice (93) although other studies using these mice have suggested an anti-inflammatory function of iNKT cells in obesity (37, 41, 55, 77). For example, upon HFD, both Jα18 KO mice and CD1d KO mice exacerbated insulin resistance accompanied by increases in body weight, fat mass, and adipose tissue inflammation, compared with WT mice (37, 55). Moreover, adoptive transfer of iNKT cells into obese mice induced loss of body weight, improved glucose tolerance, and decreased adipose tissue inflammation (55). In addition, it has been shown that single or double injections of α-GC are sufficient to induce expression of arginase-1, which is one of the M2 marker genes, and improve insulin sensitivity (40).

In chapter one, I demonstrated anti-inflammatory roles of iNKT cells in agreement with other groups (41, 55). In adipose tissues, HFD challenge induced decrement of iNKT cells concomitant with activation, apoptosis, and proliferation of iNKT cells. To investigate the role of iNKT cells in adipose tissue inflammation, I analyzed iNKT cell deficient Jα18 KO mice. Compared to WT mice, Jα18 KO mice gained more body weight and fat mass upon HFD. In addition, Jα18 KO mice displayed impaired glucose tolerance and increased adipose tissue inflammation. The number of Treg cells, potent anti-inflammatory immune cells, decreased in adipose tissues of Jα18 KO mice upon HFD. To find out the mediators governing anti-inflammatory response of iNKT cells, the mRNA levels of cytokines in adipose iNKT cells were examined. In adipose tissue of HFD fed mice, the mRNA levels of IL-4 and IL-10 genes in iNKT cells increased whereas IFN-γ mRNA was diminished. Together, I suggest that iNKT cells would play a key role in the regulation of anti-inflammatory response in obese adipose tissue.

3. CD1d expressing adipocytes present lipid antigens to iNKT cells

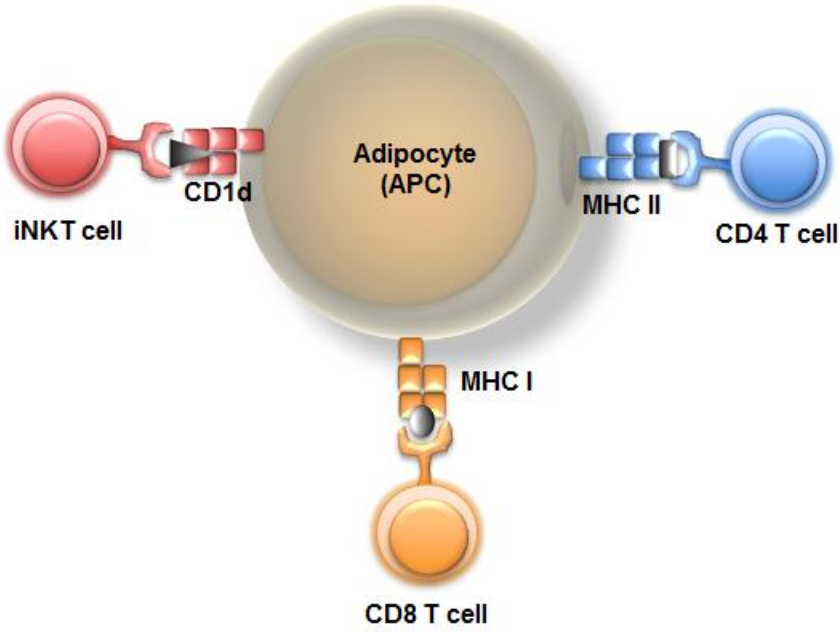
Traditionally, the major functions of adipocytes are to store excess energy, to protect vital organs, and to insulate the body against heat loss. However, accumulating evidence suggests that adipocytes are also endocrine cells that secrete a variety of adipokines such as leptin, adiponectin, and resistin (68). In obesity, adipocytes secrete pro-inflammatory cytokines including TNF-α and IL-6 and

stimulate adipose tissue inflammation. Recently, it has been suggested that adipocytes would act as antigen presenting cells to T cells in adipose tissue inflammation. Although insulin stimulates translocation of MHC class I molecules from the endoplasmic reticulum (ER) to the plasma membrane in rat brown adipocytes, there is no direct evidence for the interaction between adipocytes and CD8 T cells (56). Very recently, it has been reported that adipocytes also express MHC class II molecules and co-stimulatory signal molecules such as CD80 and CD86 (21). MHC class II molecules on adipocytes can functionally activate CD4 T cells in an antigen-specific and contact-dependent manner. Despite of these findings, the contribution of adipocyte-induced activation of T cells in adipose tissue inflammation has not been clearly understood. Morris et al. have suggested that MHC class II molecules on macrophages, but not on adipocytes, could play critical roles in CD4 T cell activation in adipose tissue (61). Therefore, it would be important to investigate the significance of adipocytes as antigen presenting cells in adipose tissue inflammation (Fig. 37).

In chapter two, I demonstrated that CD1d, an antigen-presenting molecule that presents a lipid antigen, was highly expressed in adipose tissues, mainly in adipocytes compared to macrophages. α-GC presentation to iNKT cells though adipocytes was mediated in a CD1d dependent manner. Adipocytes isolated from 1 week of HFD fed mice activated iNKT cells with or without α-GC pre-treatment. Given that the number and proliferation of iNKT cells were reduced in long term HFD, I decided to generate adipocyte specific CD1d KO mice. In adipose tissues

Figure 38.

Model of adipocytes as antigen presenting cells. Adipocytes could act as antigen presenting cells via expression of key molecules for antigen presentation in obese adipose tissue. Adipocytes expressing MHC I could mediate CD8 T cell response whereas those expressing MHC II molecules could regulate CD4 T cell response. In addition, adipocytes could modulate the function and activation of iNKT cells via high expression of CD1d molecules in adipose tissue.


Adipocyte
(APC)
CD1d
iNKT cell
MHC II
CD4 T cell
MHC I
CD8 T cell

of adipocyte specific CD1d KO mice, the number and HFD-induced proliferation of iNKT cells were diminished in adipose tissues. Therefore, I propose that adipocyte CD1d would mediate HFD-induced activation signaling to iNKT cells in adipose tissues.

It has been reported that distinct antigen presenting cell types would affect the cytokine bias of iNKT cells (1). They suggest that presentation of synthetic variants of α-GC with a marked Th2 bias is not affected by dendritic cell specific CD1d KO mice, whereas α-GC, both bias of Th1 and Th2, mediated iNKT cell activation requires CD1d expression by dendritic cells. Thus, it would be interesting to compare the phenotypes of adipocyte specific CD1d KO mice and macrophage or dendritic cell specific CD1d KO mice in diet induced obesity.

To date, a few endogenous antigens of CD1d have been reported (10, 23, 92). For example, plasmalogen LPE, iGb3, and β-glucosylceramide are potential endogenous lipid antigen species that bind to CD1d and induce subsequent activation of iNKT cells. However, the specific pathways mediating lipid antigen-induced activation of iNKT cells in obesity are largely unknown. Thus, it is of particular interest to investigate whether adipocytes would actively modulate iNKT activation through presentation of lipid antigens in addition to the secretion of various cytokines. I have demonstrated that CD1d expression is reduced in obese adipose tissue, which could account for the decrease in iNKT cell number upon HFD feeding (37). Although the role of adipocyte CD1d in antigen presentation is not clear yet, several characteristics sharing between adipocytes and macrophages

suggest the potential activation of iNKT cells through adipocyte CD1d-mediated antigen presentation. For example, both macrophages and adipocytes are able to take up and store lipids in response to nutrient cues. Furthermore, preadipocytes appear to engage in phagocytic and antimicrobial activity (82). Therefore, it is likely that HFD-induced changes in lipid metabolites loaded onto adipocyte CD1d could mediate functional alterations of iNKT cells in obesity.

In conclusion, various immune cells respond to excess energy intake in adipose tissues. It is plausible to speculate that HFD feeding might induce alteration of lipid metabolism in adipocytes, which subsequently present different lipid antigens to immune cells. Then, HFD-induced lipid metabolites-loaded CD1d complex on adipocytes would activate iNKT cells and make them to play anti-inflammatory roles in adipose tissues in the early stage of obesity. However, in late stage of severe obesity, it appears that accumulation of pro-inflammatory response inhibit CD1d expression on adipocytes, which would reduce proliferation of iNKT cells. Taken together, the interaction between adipocytes and iNKT cells would be crucial for maintaining the balance of adipose tissue inflammation and insulin resistance.

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국문초록

최근 연구 결과에 따르면 지방조직은 단순한 에너지 저장 기관으로서뿐 아니라'아디포카인'이라고 불리는 사이토카인의 분비 및 활발한 면역반응 조절을 통하여 전신적 에너지 항상성 조절에도 중요한 역할을 담당한다. 간이나 근육과 같은 다른 대사기관에 비하여 지방조직은 다양한 면역세포가 존재하며 비만동물의 지방조직에서 관찰되는 염증반응 변화에 기여한다. 이러한 지방조직 내 염증반응의 증가는 인슐린 저항성과 그로 인한 대사성 질환을 유발하는데 관여한다. 최근 보고에 따르면 지방조직 내 대식세포, 과립구, helper T 림프구, cytotoxic T 림프구, 그리고 B 림프구와 같은 면역세포가 지방조직 염증반응을 조절한다고 보고된 바 있다. 하지만 아직 비만 동물의 지방조직 내 염증반응이 항진되는 원인에 대해서는 거의 알려진 바가 없다. 따라서 비만에 의한 지방조직 염증반응 유발 인자를 찾기 위하여 생쥐 모델에 고지방식이를 준 후 지방조직 염증반응의 초기 변화를 조사하였다. 흥미롭게도 지질을 항원으로 인식한다고 알려진 invariant Natural Killer T (iNKT) 림프구의 수가 지방조직에서 특이적으로 변하는 것을 발견하였다. 따라서 본 연구를 통해 비만에 의한 지방조직 염증반응 과정에서 iNKT 림프구의 역할 및 iNKT 림프구의 활성화 조절 기전을 규명하고자 하였다.

Chapter one 에서는 비만에 의한 지방조직 염증반응 과정에서 iNKT 림프구의 특성 및 역할을 조사하였다. 먼저 iNKT 림프구가 비만 동물의 지방조직에서 그 수가 감소되어 있는 것을 발견하였다. 비만에 의한 iNKT 림프구의 수적 감소는 고지방식이에 의한 지방조직 내 iNKT 림프구의 활성화로 인한 세포사멸이 주된 원인으로 추정된다. 또한, iNKT 림프구가 결핍된 Jα18 knockout 생쥐의 경우 고지방식이에 의한 비만 유도 및 지방조직 염증반응이 더욱 증가되는 것이 관찰되었다. 따라서 iNKT 림프구는 비만에 의한 지방조직 염증반응 과정에서 항염증기능을 담당할 것으로 추정된다.

Chapter two 에서는 지방조직 내 iNKT 림프구 반응의 조절 기전에 대해 연구하였다. CD1d 는 iNKT 림프구에 지질 항원을 제시해주는 분자이다. 본 연구를 통하여, 지방세포에서 CD1d 의 발현양이 매우 높다는 것과 지방세포의 CD1d 발현을 매개로 한 iNKT 림프구와의 물리적 접촉을 통해 iNKT 림프구를 활성화 시킬 수 있다는 것을 새롭게 발견하였다. 또한 비만한 생쥐와 사람에서 지방조직 내 iNKT 림프구의 수와 CD1d 유전자 발현양이 감소한 것이 관찰되었다. 이러한 지방세포의 CD1d 발현양과 iNKT 림프구의 수적 변화의 상관관계에 대한 결과를 바탕으로 지방세포 특이적 CD1d 결핍 생쥐를 제작하였다. 지방세포 특이적 CD1d 결핍 생쥐의 경우 정상식이와 고지방 식이 섭취군 모두에서 지방조직 내 iNKT 림프구의 수적 감소가

관찰되었으며, 대조군 생쥐에서 보이는 고지방식이에 의한 iNKT 림프구의 증식 증가는 관찰되지 않았다. 따라서, 지방세포는 CD1d 유전자의 발현을 통해 지방조직 내 iNKT 림프구의 반응을 조절할 수 있으며 지방세포와 iNKT 림프구의 상호작용은 비만에 의한 지방조직 염증반응 조절에 관여할 것으로 추정된다.

본 연구의 결과를 종합적으로 살펴볼 때, 과잉의 에너지 섭취는 지방조직 내 지방세포의 CD1d 유전자를 통해 iNKT 림프구의 활성화를 유도하고, 이는 결국 항염증반응을 증가시킴으로써 지방조직 염증반응을 억제하는데 기여할 것으로 생각된다. 하지만 심각한 비만 상황에서는 지방세포의 CD1d 발현양이 줄어듦으로써 iNKT 림프구의 반응이 감소하게 되고, 이는 결국 지방조직의 염증반응 증가에 영향을 주게 될 것이다. 따라서 지방세포와 iNKT 림프구의 상호작용은 비만에 의한 지방조직 염증반응을 조절하는데 중요한 기여를 할 것으로 제안된다.

주요어 : 비만, 지방조직 염증반응, invariant Natural Killer T 림프구, 지방세포, CD1d, 지질 항원 제시

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