

博士論文

Macrophages maintain insulin sensitivity and body
weight by interacting with the intestinal microflora
through an Akt-dependent pathway

(マクロファージにおける Akt を介した代謝恒常性維持機
構の検討)

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1. Introduction

Diabetes mellitus is a condition characterized by chronic hyperglycemia, with impairment of insulin action in various tissues including classical insulin-targeted tissues, such as liver, skeletal muscle and fat¹. Hyperglycemia in diabetes causes complications which arise in an acute or chronic (arising in years' time) course². Diabetes has been shown to be a leading cause of cardiovascular disease, and increases risk of cancer and dementia. Diabetic complications are deleterious to a patient's quality of life, and are recognized as a major health issue worldwide². The pathogenesis of type 2 diabetes is associated with defective insulin secretion, or insulin resistance contributing in varying proportions. These are influenced by a heterogeneous mixture of environmental factors such as lifestyle, of note deprivation of physical exercise, a diet containing excess fat, and genetic factors, resulting in cellular processes such as ER stress, oxidative stress, and inflammation^{2,3}.

At the cellular level, insulin signals through the insulin receptor (IR), which autophosphorylates its 95KDa subunit and in turn phosphorylates the insulin receptor substrate (IRS)-1 and IRS-2 proteins⁴, to subsequently activate phosphoinositide 3-kinase (PI3K), phosphoinositide dependent kinase 1 (PDK1) and Akt⁵⁻⁸.

Akt is a serine/threonine protein kinase which relays signals in numerous signaling

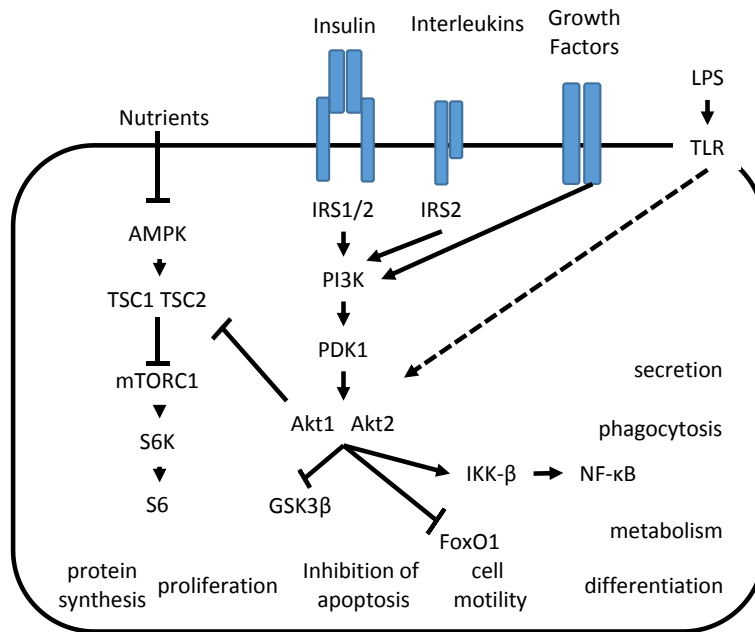


Figure: Schematic illustration of signaling through Akt, and its effects on cell function.

cascades which are activated not only by insulin, but also hormones, colony stimulating factors (CSFs), interleukins, nutrients, and other factors originating in the environment. Upstream signaling events signal through Akt to downstream effectors such as glycogen synthase kinase (GSK)⁹⁻¹¹, Forkhead box O (Foxo) transcription factors¹²⁻¹⁵, and mammalian target of rapamycin (mTOR)^{5,16,17}. Signals mediated by Akt have a regulatory role in cellular processes such as cell division, differentiation, metabolism, cell motility, and exocytosis^{18,19}. Akt is thus in an ideal position to respond to extracellular fluctuations, and mediate cellular responses to maintain homeostasis. In maintaining metabolic homeostasis, the response to feeding is among the most important of physiological responses, and dysregulation of this response is central in the pathogenesis of obesity and type 2 diabetes. Akt has three isoforms, Akt1, Akt2 and Akt3. As Akt1 and Akt2 are ubiquitously expressed, these two isoforms may function in most tissue

types during responses effecting metabolic homeostasis. The deletion of Akt1 in mice results in growth retardation but normal glucose tolerance and protection from obesity while on a high fat diet²⁰. Mice with a genetic deletion of Akt2, on the other hand, exhibit a metabolic phenotype resembling diabetes²¹. A human family possessing an inhibitory mutation in Akt2 presented insulin resistance and diabetes, further highlighting the potential importance of Akt signaling in maintaining glucose homeostasis²². The third isoform Akt3 is required for normal brain growth, but systemic effects of its deletion have not been reported in the literature²³. Activation of Akt has been reported to be impaired in skeletal muscle of insulin resistant subjects^{24,25}. Expression of constitutively active Akt in 3T3L1 adipocytes results in increased glucose uptake through increased GLUT4 translocation, whereas a dominant negative form reduces GLUT4 translocation^{26,27}. From studies utilizing constitutively active Akt in muscle and adipose tissue, Akt was implicated to be the enzyme responsible for relaying signals from the insulin receptor to numerous downstream targets to differentially activate processes such as glucose uptake, glycogen synthesis, and protein synthesis in these tissues types²⁸. In the liver, deletion of Akt dysregulates glucose homeostasis, but interestingly glycemic changes in response to insulin in the fed state have been reported to be preserved when Foxo is deleted simultaneously^{29,30}. The precise function of Akt in classical insulin sensitive tissues that influence homeostasis has thus remained a topic of controversy.

We and others have observed the involvement of inflammation in the dysregulation of metabolic homeostasis, particularly in diet induced obese states where inflammation has been observed in various tissue³¹⁻³⁶. Macrophages have a central role in inflammation³⁷. Pattern recognition receptors such as Toll-like receptors mediate innate immunity³⁸⁻⁴¹, and macrophages employ secreted cytokines to relay signals to cells, such as T and B lymphocytes, which function in adaptive immunity for a more specific response to antigens⁴². Toll-like receptors recognize bacterial substances and induce inflammatory responses in macrophages, which go on to induce specific responses by lymphocytes to eliminate invading bacteria. Lipopolysaccharides (LPS) are part of the cell wall in gram negative bacteria, and are recognized by TLR4, and induce downstream signals including the PI3K/Akt pathway in association with the adaptor molecule MyD88^{41,43}. These signals have been implicated in the pathogenesis of obesity, although the actual players *in vivo* have proved elusive^{44,45}. In recent years, anti-inflammatory Th2 cytokines have been reported to participate via induction of anti-inflammatory macrophages in the maintenance of energy homeostasis through browning of white adipose tissue, illustrating the potential role of immunologic functions other than inflammation in the regulation of metabolism^{46,47}. Signals mediated by the Th2 cytokines IL-4 and IL-13 have been shown to be important in this process. Akt has been implicated in the regulation of inflammatory responses, although inflammatory Th1 and Th2 functions were

observed depending on the isoform discussed⁴⁸. The secretion of inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) have been reported to fluctuate in response to feeding, although the functional role of these fluctuations *in vivo* are not yet clear^{49,50}.

Kinases involved in the insulin cascade in myeloid cells, including macrophages have been explored on a molecular basis in the context of metabolism. Myeloid specific insulin receptor knockout mice did not present alterations in glucose tolerance or body weight while fed on a normal chow diet, but was protected from insulin resistance on a high fat diet. Indeed, euglycemic hyperinsulinemic clamp studies revealed decreased hepatic glucose output and increased peripheral glucose disposal in the knockout mice⁵¹. Myeloid specific deletion of Raptor, a component of mammalian target of rapamycin complex 1 (mTORC1), resulted in decreased infiltration of macrophages in adipose tissue and liver while on a high fat diet, and decreased expression of inflammatory cytokines in these tissues. Based on the findings of decreased phosphorylation of eIF2 α and ATF6 in the myeloid cells, decreased ER stress response was a possible contributing factor to this phenotype⁵². Myeloid specific deletion of Inhibitor of kappa kinase beta (IKK β) resulted in the attenuation of hyperglycemia in a glucose tolerance test and an insulin tolerance test, and also led to decreased hepatic glucose output and peripheral glucose disposal⁵³.

The role of PDK-1 in macrophages was reported to be different from that of preceding

findings regarding components of the insulin cascade, with observations of chronic inflammation and insulin resistance in the adipose tissue of myeloid specific PDK-1 knockout mice on a high fat diet. Myeloid specific constitutively nuclear targeted Foxo1 reproduced these results suggesting that impaired nuclear export signaling from PDK1 to Foxo1 is an important factor in chronic inflammation and subsequent insulin resistance induced by obesity⁵⁴. IRS-2 relays signals from the IL-4/IL-13 receptor heterodimer, which relays anti-inflammatory signals from Th2 cytokines in macrophages, and defective IRS-2 signaling have been shown in models of type 2 diabetes⁵⁵.

As reviewed above, signaling in macrophages has been reported to influence homeostasis in various ways. Since Akt relays signals from, and signals to these molecules, Akt in macrophages may play a crucial role in maintaining metabolic homeostasis by responding to extracellular stimuli that fluctuate during states that influence metabolic homeostasis, such as feeding. We have attempted to elucidate the role of Akt in macrophages using myeloid specific conditional knockout mice, and to discuss its significance in obesity and type 2 diabetes. To our knowledge, our report is the first to assess impact of myeloid specific deletion of Akt1 and Akt2 *in vivo*.

2. Materials and Methods

Mice

Mice were maintained under a 12 hour light-dark cycle. Procedures of *in vivo* experiments using mice, and methods of animal care were approved by the Animal Care Committee of the University of Tokyo. Wild type C57BL/6J mice were purchased from Oriental Yeast. As a normal chow diet, we used Oriental MF diet (Oriental Yeast), consisting of 5.1% fat, 55.3% nitrogen free extract, 23.1% (v/v) protein, 5.8% ash, 2.8% fiber, and 7.9% water, fed *ad libitum*. Diet induced obesity (DIO) was induced in mice by feeding them with a high fat diet (HFD, High Fat Diet 32 (CLEA Japan)), consisting of 32.0% fat, 29.4% nitrogen free extract, 25.5% protein, 4.0% ash, 2.9% fiber, and 6.2% water, fed *ad libitum*. As a myeloid specific Akt deletion model, we bred mice of three lineages, transgenic mice possessing the cre recombinase gene downstream of the Lysozyme M promoter⁵⁶, and Akt1-floxed mice and Akt2-floxed mice, with lox P sequences flanking Exon 4, 5 of Akt1²⁰, and Exon 4, 5 of Akt2⁵⁷, respectively. In the series of studies we used mice with loxP sequences flanking Akt1 and Akt2, with LysMDKO mice possessing the Cre recombinase as the knockout mice and those without the Cre recombinase as f/f control mice. Manipulation of intestinal microflora with vancomycin was conducted using 0.5 g/L of vancomycin (Wako) dissolved in drinking water.

Sampling of tissues

Mice were euthanized by cervical dislocation, and tissue were quickly removed and frozen instantly in liquid nitrogen. Anesthetics consisting of medetomidine hydrochloride (0.75 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg) were used when necessary. For sampling after insulin stimulation, 5 units per body of regular insulin (Humulin R, Eli Lilly) was diluted in normal saline and administered via the inferior vena cava following anesthetization, and tissues were sampled 5 minutes later as stated above.

Analysis of glucose and insulin tolerance

Insulin tolerance tests were conducted using 1.0-2.0 U/kgBW of insulin, administered to mice fed *ad libitum* followed by measurements of plasma glucose concentrations every 20 minutes. Glucose tolerance tests were conducted using 1.0-2.0 g/kgBW of glucose orally or intra-peritoneally, followed by plasma glucose measurements 0, 15, 30, 60, 120 minutes later. Plasma glucose concentrations were measured using a portable measurement device (SKK).

Primary cultures

Bone marrow derived macrophages (BMDMs) were induced using bone marrow from the femur and tibia of mice washed out using sterile phosphate buffered saline (PBS), filtered

using a 100 μm filter to keep contaminating connective tissue out of the culture, and seeded at 1.0×10^4 cells per cm^2 . The cell suspension was cultured in Dulbecco's Modified Eagles Medium (DMEM, Gibco) in the presence of 0.2 ng/ml CSF-1. Stromal vascular fraction of adipose tissue was derived by mincing adipose tissue followed by digestion by collagenase H (Sigma) for 30 minutes at 37 $^{\circ}\text{C}$, and seeded at 1.0×10^4 cells per cm^2 in DMEM. For culture of liver macrophages, mice were anesthetized, infused with medium until contaminating blood was washed out, and subsequently digested using collagenase (Wako) and dispase (Wako). The resulting cell suspension was then filtered through a 100 μm filter, and seeded at 1.0×10^5 cells per cm^2 . Western blots and gene expression assays were performed with samples obtained from cultures starved overnight, stimulated with the indicated stimulant for the indicated time, and frozen immediately afterward with liquid nitrogen. Immunofluorescence assays were performed with primary cells cultured on glass plates, and fixed using 4% paraformaldehyde immediately after stimulation. Recombinant mouse IL-4, and IL-13 (R and D systems), recombinant insulin (Humulin R, Eli Lilly and company) and Lipopolysaccharide (LPS, from E. coli O111, Wako) were used as stated. PI3K inhibitor LY294002 (R and D systems), GSK3 inhibitor SB216763 (R and D systems), and Rapamycin (cell signaling) were used as stated.

Gene expression analysis

RNA was purified from cell cultures using an RNeasy mini kit (Qiagen) or tissue samples using standard phenol chloroform methods. DNA microarrays were performed upon request to Hokkaido Microsystems using 80Kx6 Agilent microarrays. Data were analyzed using GeneSpring software (Agilent technologies). For real time PCR (RT-PCR), RNA samples were then reverse transcribed using a High Capacity Reverse Transcription kit (Applied Biosystems). Gene expression was quantitated using primers listed elsewhere and a SYBR Green master mix (ABI), or a Taqman probe (ABI), and PCR was conducted using a 7900HT real time PCR system (Applied Biosystems) and the relative expression levels were normalized by the expression level of Ppia (encoding Cyclophilin A). The composition of intestinal microflora was analyzed by primers listed elsewhere using fecal DNA extracted using QIAamp DNA Stool Mini Kit (QIAGEN), and a SYBR Green master mix (ABI).

Adenoviral infection

The adenovirus vector expressing IL-10, generated using an Adenovirus Expression Vector Kit, Code No. 6170 (Takara), was a generous gift from Dr. Kazuyuki Tobe, Toyama University. Adenoviruses were infected to HEK 293 cells at 70-80% confluency, and cell supernatants were collected after 2-3 days or after all cells lost adhesion. After centrifugation to dispose of membranous particles, viral particles were concentrated by centrifugation at

85,000 g and 150,000 g in a cesium chloride concentration gradient in this order in a SW28Ti Rotor and SW41Ti Rotor respectively using a Beckman ultra centrifugation device (Beckman Coulter). Virus containing buffer was then dialyzed using a Dialysis Cassette (Pierce) in 10% glycerol in PBS. Viral particles were quantified using the 260 nm absorption of the resulting virus containing buffer.

Biochemical assays

Protein samples were obtained from frozen tissue by homogenizing with a standard liver buffer (10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride at pH 7.4) containing 1% NP-40, a proteinase inhibitor (Roche), or cell cultures using the same reagents, followed by centrifugation at 223,000g in a 70Ti rotor using an ultracentrifugation device stated above to remove contaminating membranous particles. Western blots were performed using samples mixed with standard Laemmli solution (50% glycerol, 25% β -mercaptoethanol) at a 1:4 ratio, and heated to 95 °C for 10 minutes. Samples were electrophoresed in 8% polyacrylamide gels at 15 mA for 30 minutes, and 30 mA for 70 minutes. Transfer to nitrocellulose membranes was performed at 100 V for 2 hours. Blocking was done using western blocking reagent (Roche) in TBS-Tween. Primary antibodies (Cell Signaling) were diluted at 1:1000 unless stated otherwise. Secondary alkaline phosphatase

conjugated antibodies (GE healthcare) were diluted 1:2000 unless stated otherwise. Bands were detected using Pierce Western Blotting Substrate Plus (Thermo Scientific). IL-10 in cell culture supernatants were assessed using mouse IL-10 ELISA kit (R and D systems), insulin in serum samples using high sensitive insulin ELISA kit (Morinaga).

Histological analysis

For immunohistochemical assays, cells were fixed in 4% paraformaldehyde, permeabilized with 0.25% triton for 10 minutes following wash, and blocked using 1.5% bovine serum albumin (BSA). Samples were treated with 0.1% Triton in all subsequent steps. Anti phosphor-Akt antibodies (Cell signaling) were diluted 1:200, F4/80 (Life, goat) 1:500, F4/80 (abcam, rabbit) 1:200, IL-10 (Santa Cruz Biotechnology, rat) 1:50. Secondary anti-rabbit IgG antibodies (Alexa 594 conjugated) were diluted 1:500, and anti rat IgG antibodies (Alexa 488 conjugated) were diluted 1:1000. Cells were counted using Keyence Hybrid Cell Count Software (Keyence). F4/80 positive cells were counted as macrophages. Tissue samples were embedded in paraffin following fixation and deparaffinization, treated with ethanol and water, and treated with Mayer's Hematoxylin Solution (Wako) for 10 minutes, followed by 1% Eosin Y solution for 5 minutes. The samples were then treated with ethanol and xylene.

In immunohistochemical assays, samples were blocked with BSA, and stained using

anti mouse F4/80 antibody (AbD Serotec, rat) at 1:400, and Anti-Rat biotin (BA4001) at 1:300.

ABC reagents, DAB were used according to the manufacturer's protocols.

Cell sorting analysis

FACS analyses of stromal venous fraction cells were performed using cells suspended in Stain buffer (BD Pharmingen) following red cell lyse using lyse buffer (BD Pharmingen), blocked using Fc block (BD Pharmingen), stained using conjugated antibodies for CD11b (BD biosciences, Pacific Blue) and F4/80 (BD biosciences, PE) at dilutions of 1:100. Dead cells were detected using PI. Flow cytometry and cell sorting were performed using a BD FACS Aria II device (BD). We counted F4/80 CD11b double positive cells as macrophages.

Cell migration assays

Cell migration was assessed using a QCM Chemotaxis Cell Migration Assay (Merckmillipore). BMDM cultures were trypsinized and suspended at 1.0×10^5 cells/ml in DMEM, and were applied to inserts with a 5 μ m pore in a 24 well dish, containing the indicated stimulant. Cells that migrated to the lower plate were counted.

Statistical analyses

Data are shown as the mean value \pm SEM. Statistical significance was assessed using an unpaired 2-tailed t-test when the initial null hypothesis was between 2 conditions, or analysis of variance (ANOVA) with post-hoc Tukey's Honest Significant Difference (HSD) using Easy R software where it was between more than 2 conditions⁵⁸. P values < 0.05 were considered statistically significant.

3. Results

Defective Akt stimulation in macrophages of mice with DIO

As Akt is a kinase which may relay signals in macrophages from many extracellular factors perhaps influenced by processes such as feeding and other signaling molecules which have been shown to influence metabolism, we explored the possibility that Akt acts as a signaling hub in macrophages and moreover, is important in the maintenance of metabolic homeostasis. First, we aimed to elucidate the differences of Akt activation in tissue macrophages in the normal state and the obese, HFD fed state. For this end, we cultured stromal vascular cells from white adipose tissue of wild type mice fed on a normal chow diet, and HFD, respectively. In an unstimulated state, more F4/80 (a macrophage marker) positive stromal vascular cells from mice on a high fat diet were phosphor-Akt positive (Figure 1A-B). Moreover,

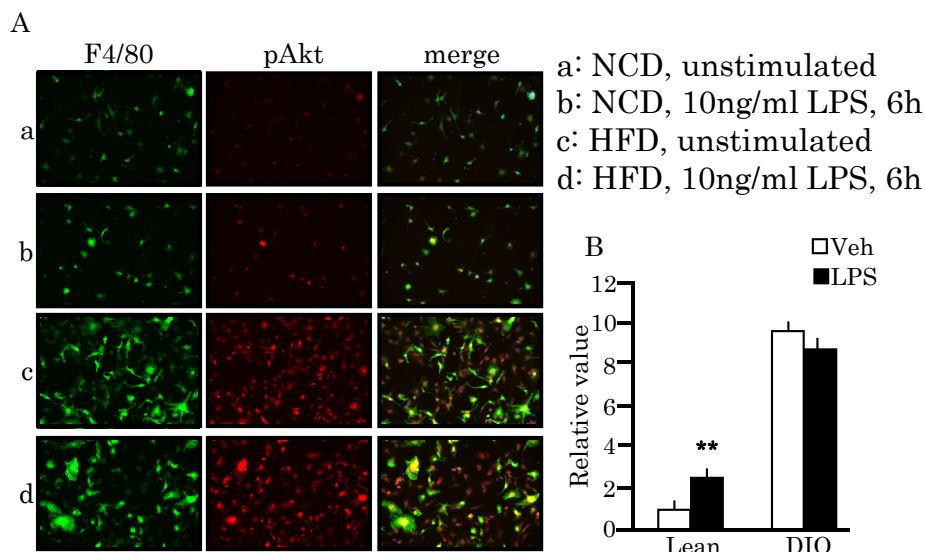


Figure 1: Akt phosphorylation in tissue macrophages obtained from models of obesity. A: Analysis of Akt signaling in tissue macrophages using stromal vascular fraction (SVF) cells obtained from wild type mice fed a normal chow diet (NCD) or a high fat diet (HFD) as a model of diet induced obesity. Cultured SVF cells were stimulated with 10ng/ml LPS or vehicle for 6 hours (a-d is designated in upper right. The blue bar is equivalent to 50 μ m), and assayed by immunofluorescence of phosphor-Akt (red) and F4/80 (green). B: Quantification of phosphor-Akt F4/80 double positive cells using hybrid cell count software (The number of double positive areas was normalized to the number of F4/80 positive areas. Results are shown as relative values to unstimulated SVF cells in normal NCD fed wild type mice. n= 7-10 fields per condition). **: P<0.01

when an innate immune response was induced by LPS stimulation, F4/80 and phosphor-Akt double positive stromal vascular cells increased in mice on a normal chow diet, but remained virtually unchanged in mice with DIO (Figure 1A-B), suggesting Akt could no longer respond to extracellular stimuli in DIO.

Impaired downstream signaling through Akt in macrophages of LysMDKO mice

These data raised the possibility that under normal conditions, Akt activation in tissue macrophages in response to innate stimuli may play a role in maintaining homeostasis, and that dysregulation of this response in DIO causes pathological changes. To assess this hypothesis,

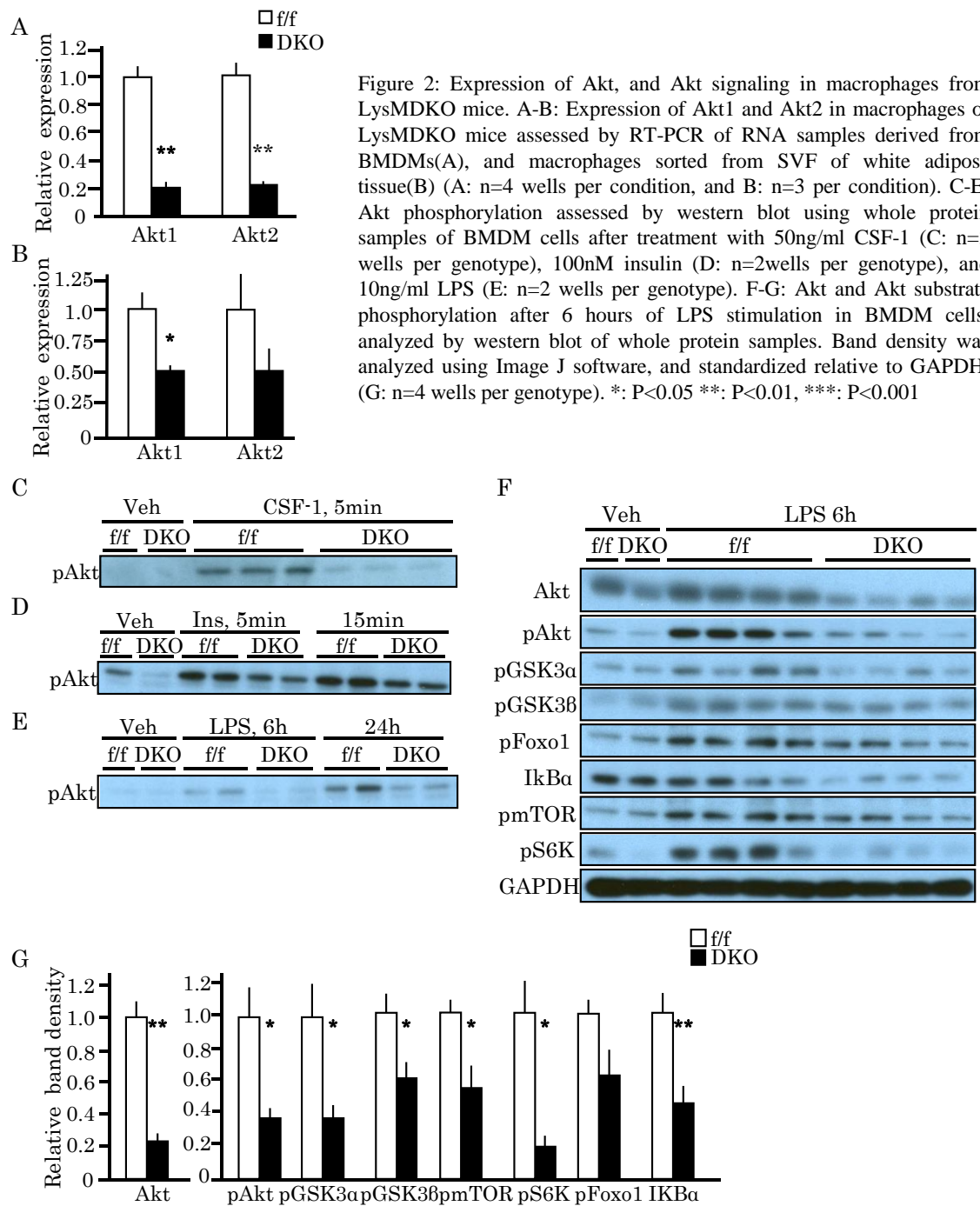
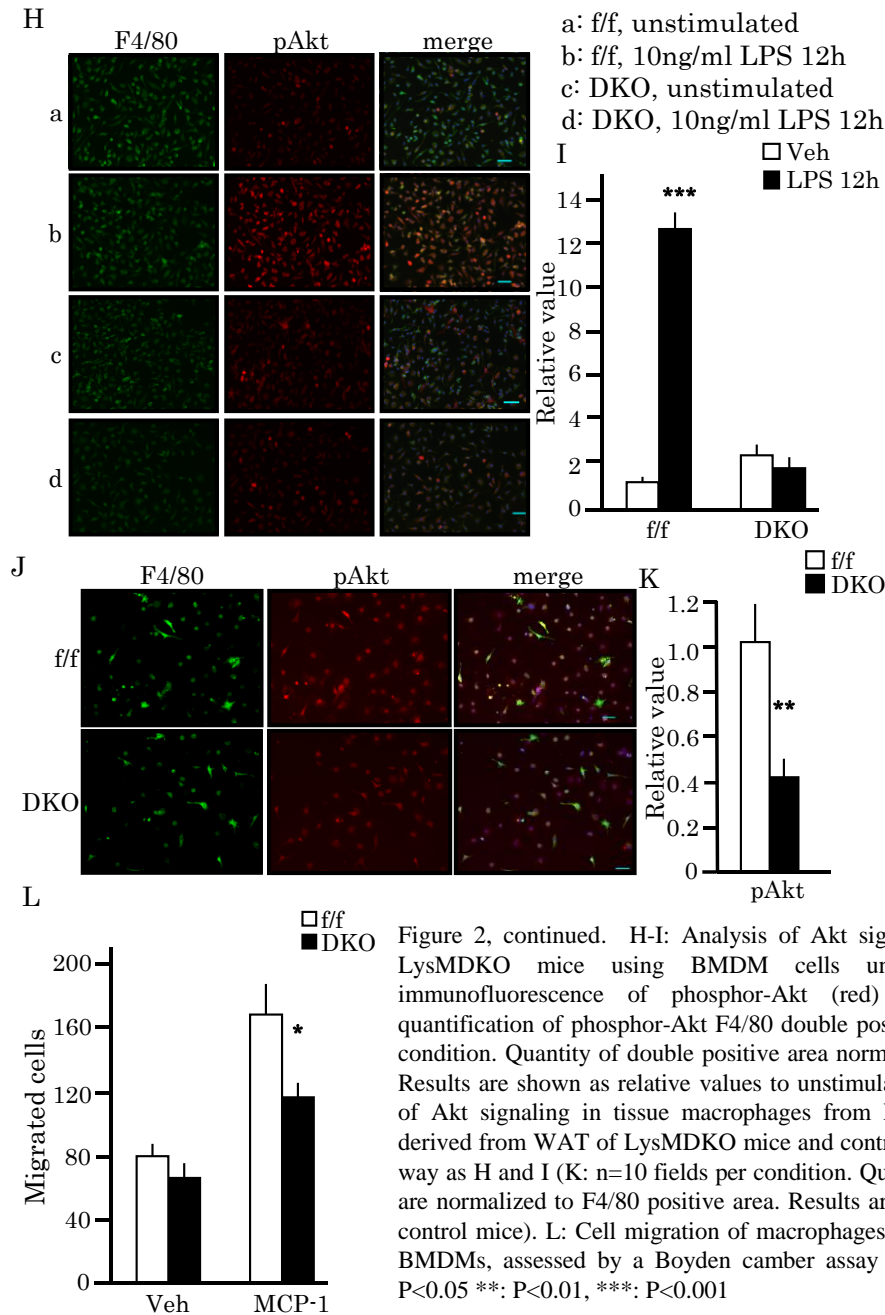


Figure 2: Expression of Akt, and Akt signaling in macrophages from LysMDKO mice. A-B: Expression of Akt1 and Akt2 in macrophages of LysMDKO mice assessed by RT-PCR of RNA samples derived from BMDMs(A), and macrophages sorted from SVF of white adipose tissue(B) (A: n=4 wells per condition, and B: n=3 per condition). C-E: Akt phosphorylation assessed by western blot using whole protein samples of BMDM cells after treatment with 50ng/ml CSF-1 (C: n=3 wells per genotype), 100nM insulin (D: n=2wells per genotype), and 10ng/ml LPS (E: n=2 wells per genotype). F-G: Akt and Akt substrate phosphorylation after 6 hours of LPS stimulation in BMDM cells, analyzed by western blot of whole protein samples. Band density was analyzed using Image J software, and standardized relative to GAPDH. (G: n=4 wells per genotype). *: P<0.05 **: P<0.01, ***: P<0.001

we generated myeloid cell-specific Akt1/Akt2 double knockout mice (LysMDKO mice). We first examined the efficacy of tissue specific deletion of Akt in macrophages using primary macrophage cultures. Akt expression in BMDM cells from LysMDKO mice was lowered by about 70-80% (Figure 2A). Macrophages sorted from the stromal vascular fraction of white



adipose tissue using a cell sorter also showed suppressed Akt expression, albeit to a lesser degree than BMDM cells perhaps due to the contamination of cells other than macrophages in the process of cell sorting, indicating that Akt expression was repressed in macrophages *in vivo* and *ex vivo* (Figure 2B). To assess Akt phosphorylation in response to extracellular signals, we

stimulated BMDMs with CSF-1, insulin, or LPS. BMDMs showed decreased Akt phosphorylation when stimulated by all three stimulants (Figure 2C-E). Notably, BMDMs from LysMDKO mice stimulated with LPS showed markedly suppressed signaling to Akt substrates, especially p70 S6K, an effector activated via mTOR (Figure 2F-G). Decreased Akt phosphorylation in macrophages from LysMDKO mice was also observed in immunofluorescence assays using BMDMs upon LPS stimulation (Figure 2H-I), and stromal vascular cells obtained from white adipose tissue (Figure 2J-K), together implying that signaling from TLR4 through Akt is impaired in LysMDKO mice both *in vivo* and *ex vivo*.

As cell migration of macrophages in response to chemokines is a crucial cellular function in many immune processes, we examined the migration of macrophages from LysMDKO mice using a Boyden chamber assay. Less BMDMs from LysMDKO mice migrated toward MCP-1, which implicated impairment of cell function in the context of deficient Akt signaling in macrophages (Figure 2L).

Obese traits in LysMDKO mice fed on a normal chow diet

We went on to explore the effect of deficient Akt signaling in macrophages and resultant insufficiencies in cell function on whole body homeostasis. When fed on a normal chow diet, LysMDKO mice showed increased body weight compared to control mice after 14

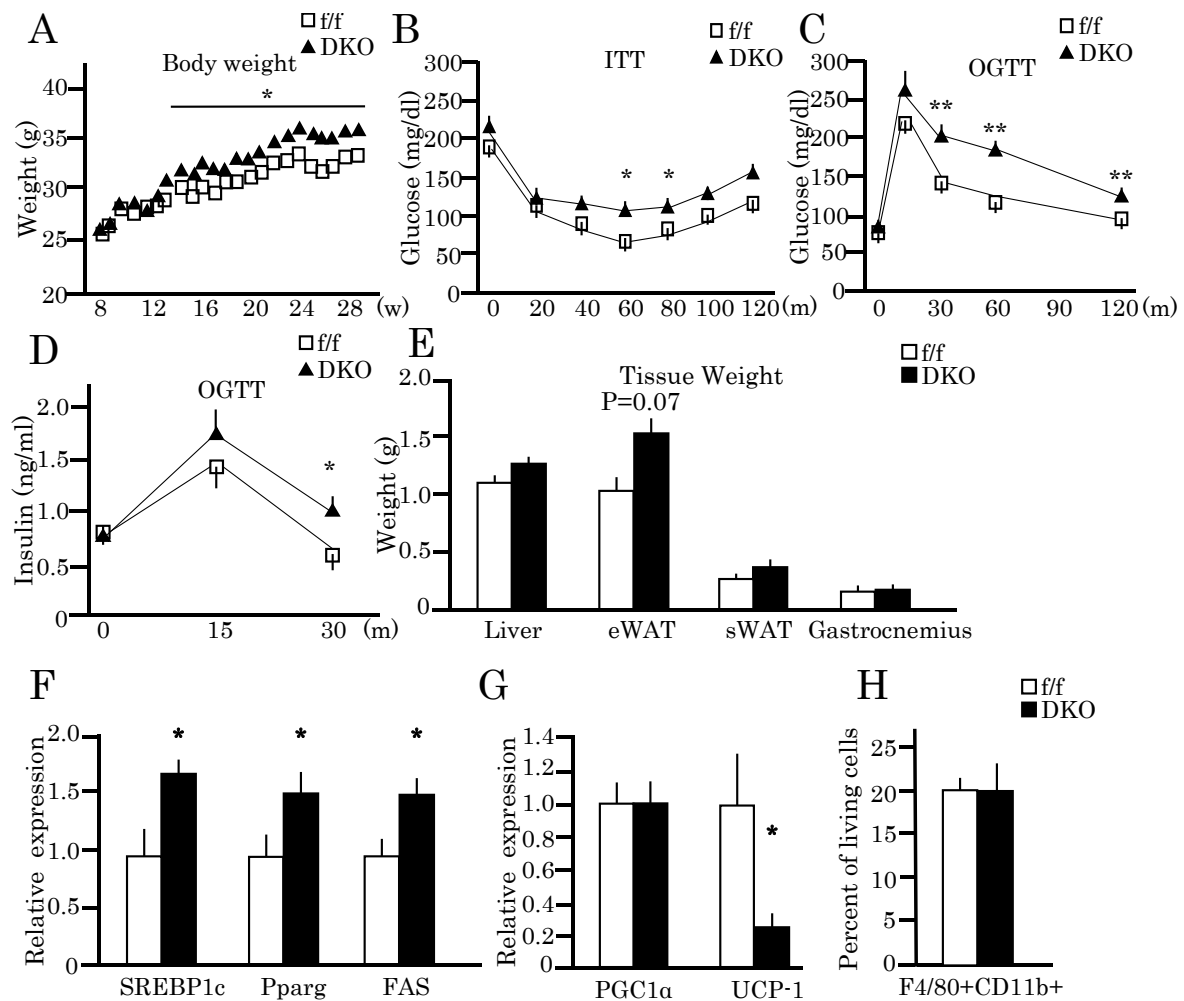


Figure 3: The metabolic phenotype of LysMDKO mice on a normal chow diet. A: Body weight of normal chow fed mice ($P < 0.05$ after 14 weeks of age). B: Plasma glucose values in an insulin tolerance test (1.25 U/kgBW of regular insulin was administered intraperitoneally). C: Plasma glucose concentrations in an oral glucose tolerance test (1.25 g/kgBW of glucose administered p.o. after 16 hour fast). D: Serum insulin levels during OGTT, assayed by ELISA. E: Tissue mass of LysM DKO mice and control mice (A-E $n = 6-9$ mice per condition at 25-30 weeks of age). F: Gene expression in epididymal WAT of LysMDKO mice, assessed by RT-PCR using whole RNA extracts. G: Gene expression in subcutaneous WAT as in A (6-9 mice per group, at 25-30 weeks. Tissue was sampled after 16 hour fast). H: The ratio of F4/80 CD11b double positive cells among living cells in SVF cells from eWAT, counted by FACS (3 mice per group at 30 weeks of age). *: $P < 0.05$ **: $P < 0.01$

weeks of age (Figure 3A). Plasma glucose values were higher in LysMDKO mice in insulin tolerance tests and glucose tolerance tests at multiple time points, and plasma insulin concentrations were higher after administration of glucose in glucose tolerance tests, indicating the presence of insulin resistance and impaired glucose tolerance in LysMDKO mice (Figure 3B-D). Epididymal fat pads in LysMDKO mice fed on a normal chow diet tended to weigh

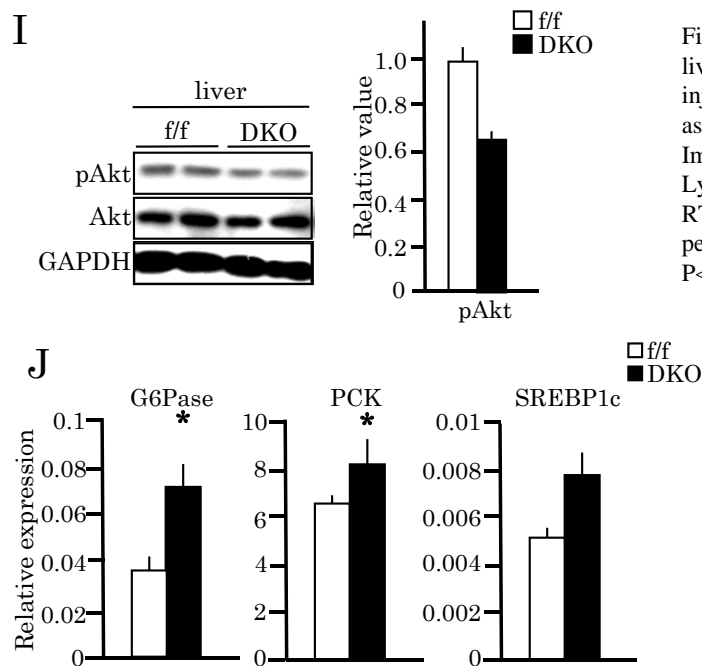


Figure 3, continued: I: Akt phosphorylation in liver of LysMDKO mice 5 minutes after injection of 5 units of regular insulin per mouse, assessed by western blot, and quantified using Image J software. J: Gene expression in livers of LysMDKO mice and control mice, assessed by RT-PCR using total RNA extracts (n=7-8 mice per group, fasted 16 hours at 30 weeks). *: $P < 0.05$ **: $P < 0.01$

more than those of control mice, and exhibited higher expression of adipogenic genes, and lower expression of UCP-1 which is a gene associated with browning of white adipose tissue (Figure 3E-G). No differences in the number of macrophages in the stromal vascular fraction of epididymal white adipose tissue (WAT) was detected (Figure 3H). When 5 units per body of regular insulin was injected, Akt phosphorylation was markedly decreased in livers of LysMDKO mice, but the difference was obscure in white adipose tissue and skeletal muscle, suggesting the presence of tissue specific insulin resistance in the liver (Figure 3I). The expression of glucose 6-phosphatase (G6-Pase) and phosphoenol pyruvate carboxykinase (PCK) was elevated in the livers of LysMDKO mice (Figure 3J). Moreover, the expression of SREBP1c, which is the master regulator of fatty acid synthesis and is often elevated in liver steatosis^{15,59,60}, was higher in the liver of LysMDKO mice (Figure 3J), although this difference

did not reach a statistical significance, and difference in steatosis could not be discerned in liver sections. Alterations were not detected in the metabolic profile of LysMCre Akt1 floxed mice and LysMCre Akt2 floxed mice, indicating both Akt1 and Akt2 are required in macrophages to maintain metabolic homeostasis.

Lack of altered metabolism in LysMDKO mice on HFD

As inflammation has been shown to be important in the pathogenesis of diet induced obesity and type 2 diabetes, we assessed the effect of HFD in LysMDKO mice. Interestingly, LysMDKO mice did not exhibit differences in body weight, insulin resistance, glucose tolerance, or tissue weight after being fed a HFD (Figure 4A-E). They also did not show differences in the expression of genes associated with adipogenesis in white adipose tissue, or gluconeogenesis and steatosis in the liver compared to control mice on a high fat diet (Figure 4F-G and Figure 10B). The ratio of macrophages in SVF derived from WAT was unaltered (Figure 4H) to the control mice on HFD, and the ratio of IL-10 positive macrophages in adipose tissue was unaltered between LysMDKO mice and control mice on HFD, and lower than in mice on a normal chow diet. (Figure 4I-J). As crown like structures (CLSs), consisting of adipose cells surrounded by macrophages, are considered to be a hallmark of adipose tissue inflammation in DIO, we assessed the formation of these structures in LysMDKO mice on

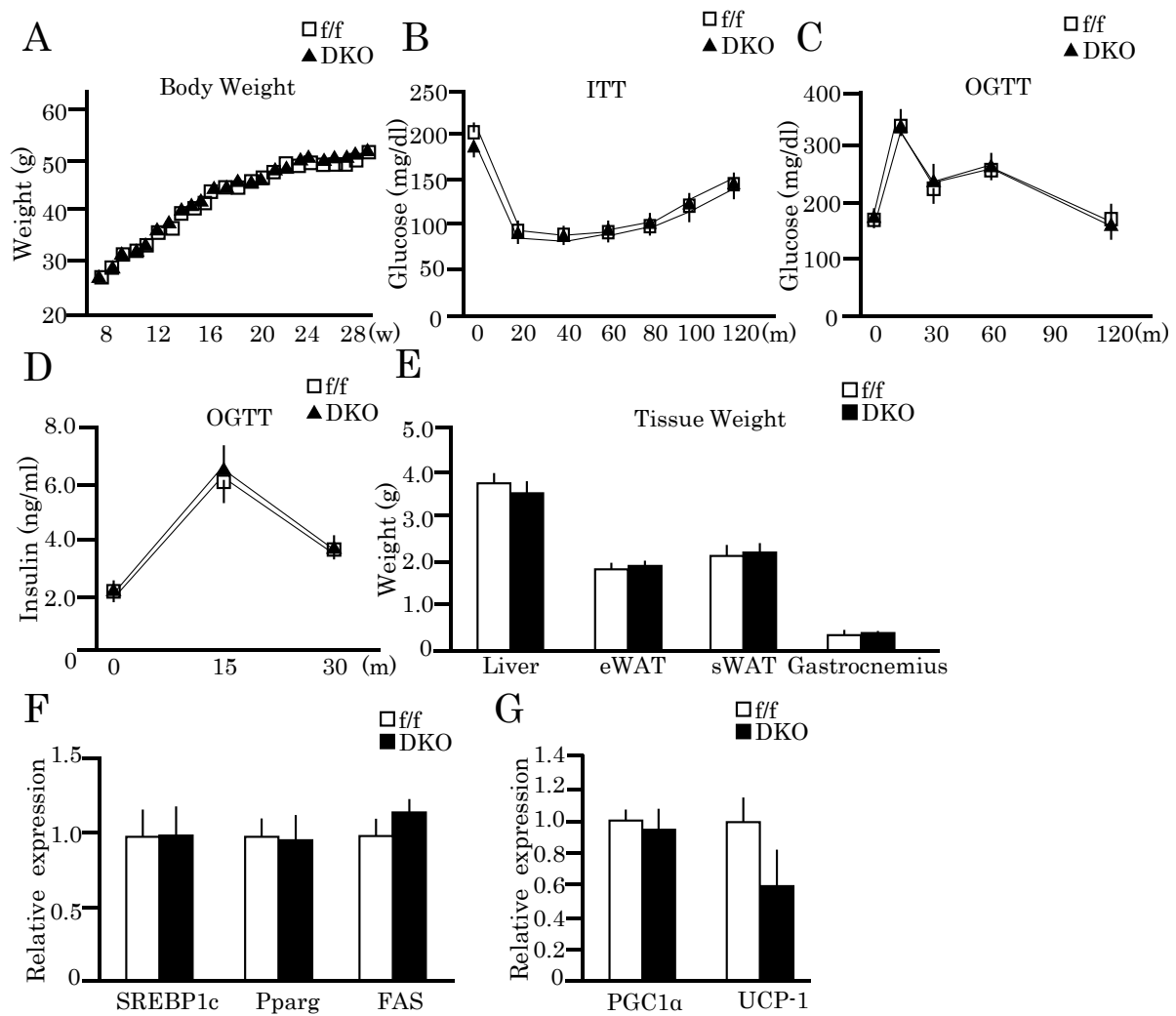


Figure 4: Phenotype of LysM DKO mice fed on a high fat diet. A: Body weight measurements of high fat diet fed mice. B: Plasma glucose measurements during ITT (2.0 U/kgBW of regular insulin was administered intraperitoneally). C: Plasma glucose measurements during OGTT (1.0 g/kgBW glucose was administered p.o. after 16 hour fast). D: Serum insulin levels during OGTT, assayed by ELISA. E: Tissue mass of LysMDKO mice and control mice (A-E: n=8 mice per group, at 25-30 weeks). F-G: Gene expression in WAT, assessed by RT-PCR using RNA extracts from tissue (F: epididymal WAT, G: subcutaneous WAT. n=8 mice per group, at 25-30 weeks).

HFD^{33,54}. Of interest, such structures were rather fewer in LysMDKO mice (Figure 4K), suggesting attenuation of this aspect of adipose tissue inflammatory response under impaired Akt signaling in macrophages. As increments in Akt phosphorylation by TLR4 signaling were nonexistent in adipose tissue macrophages from mice on a high fat diet and LysMDKO mice alike, both of which have obese traits, and from observations that Akt deficiency in

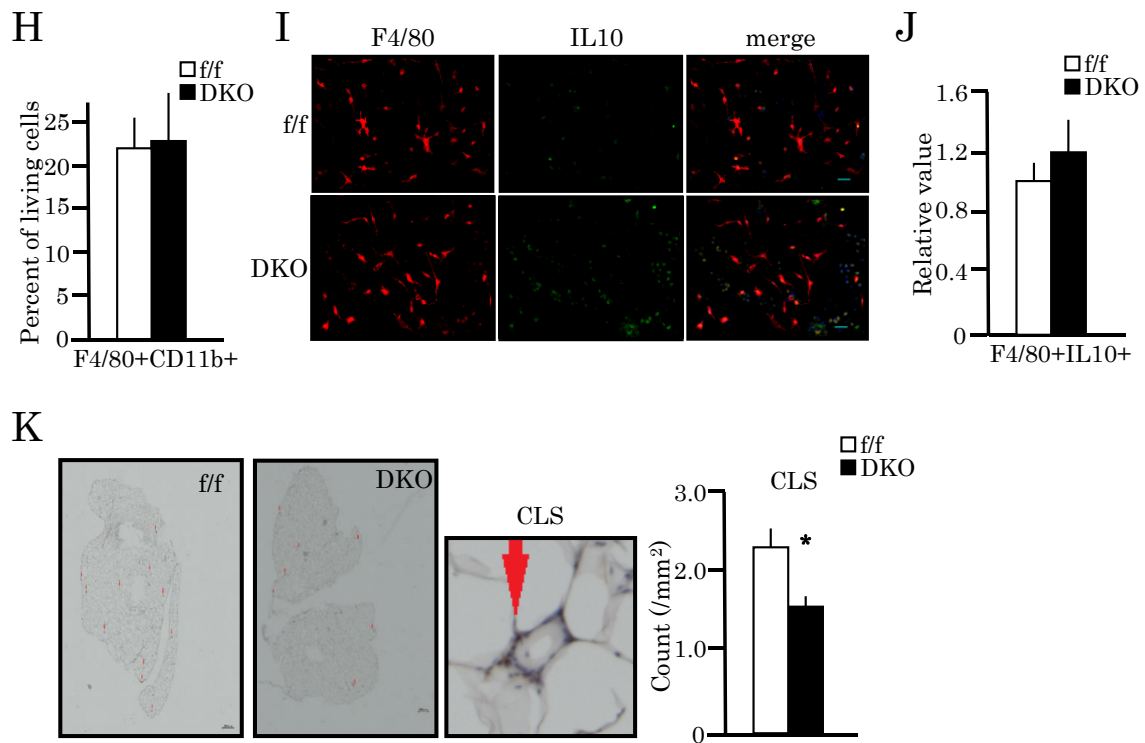


Figure 4, continued. H: Quantification of macrophages in the SVF of epididymal WAT, counted in FACS analysis of F4/80 CD11b positive cells, and plotted as percentage of living cells (n=3 mice per group at 30 weeks). I: Production of IL-10 in macrophages in the SVF of WAT from LysMDKO mice, assessed by immunofluorescence of F4/80 (red) and IL-10 (green). J: Quantification of IL10 F4/80 double positive cells in I (n=10 fields per condition. Number of double positive areas normalized to F4/80 positive areas. Results are shown as relative values to unstimulated control mice). K: Crown like structure formation in LysMDKO mice assessed by immunohistochemistry of F4/80 in sections of epididymal WAT. Crown like structures counted and normalized to area of sections analyzed by Image J software (n=6 mice per condition). *: P<0.05

macrophages do not add to obese traits of mice with DIO, we considered these data supportive of a model in which macrophages defective in Akt phosphorylation from extracellular signals contribute to obesity.

Suppressed IL-10 production in Akt deficient macrophages

Since LysMDKO mice on a normal chow diet exhibited insulin resistance without alterations in the number of tissue macrophages, we speculated that deletion of Akt in macrophages changed the expression or secretion of factors that affect body weight and insulin

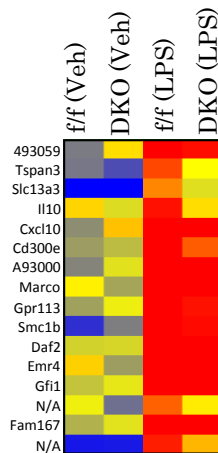


Figure 5: Transcriptome analysis of BMDMs stimulated with 10 ng/ml LPS for 72 hours, assessed by a DNA microarray using RNA extracts.

sensitivity. As Akt stimulation was particularly suppressed when stimulated by LPS, we performed transcriptome analyses on BMDMs using a DNA microarray after chronic LPS stimulation. Among genes which were induced by twice or more than control cells, the only gene expressed less in Akt deficient macrophages with known extracellular functions was interleukin 10 (IL-10), which is a Th2 cytokine with anti-inflammatory functions⁶¹⁻⁶³(Figure 5).

To verify deficient IL-10 expression, we analyzed BMDMs by RT-PCR. Suppressed IL-10 expression was evident in BMDMs from LysMDKO mice both at baseline, and after 72 hours of LPS stimulation (Figure 6A), indicating Akt dependent induction of IL-10 by LPS stimulation through TLR4. Suppressed production of IL-10 was observed in cell supernatants under the same conditions as well (Figure 6B). When stimulated by intraperitoneal injection of LPS, serum IL-10 was lower in LysMDKO mice at multiple time points from immediately after stimulation, suggesting the innate immune response to LPS in macrophages elicits production of IL-10 through Akt both *in vivo* and *ex vivo* (Figure 6C). Moreover, fewer tissue macrophages

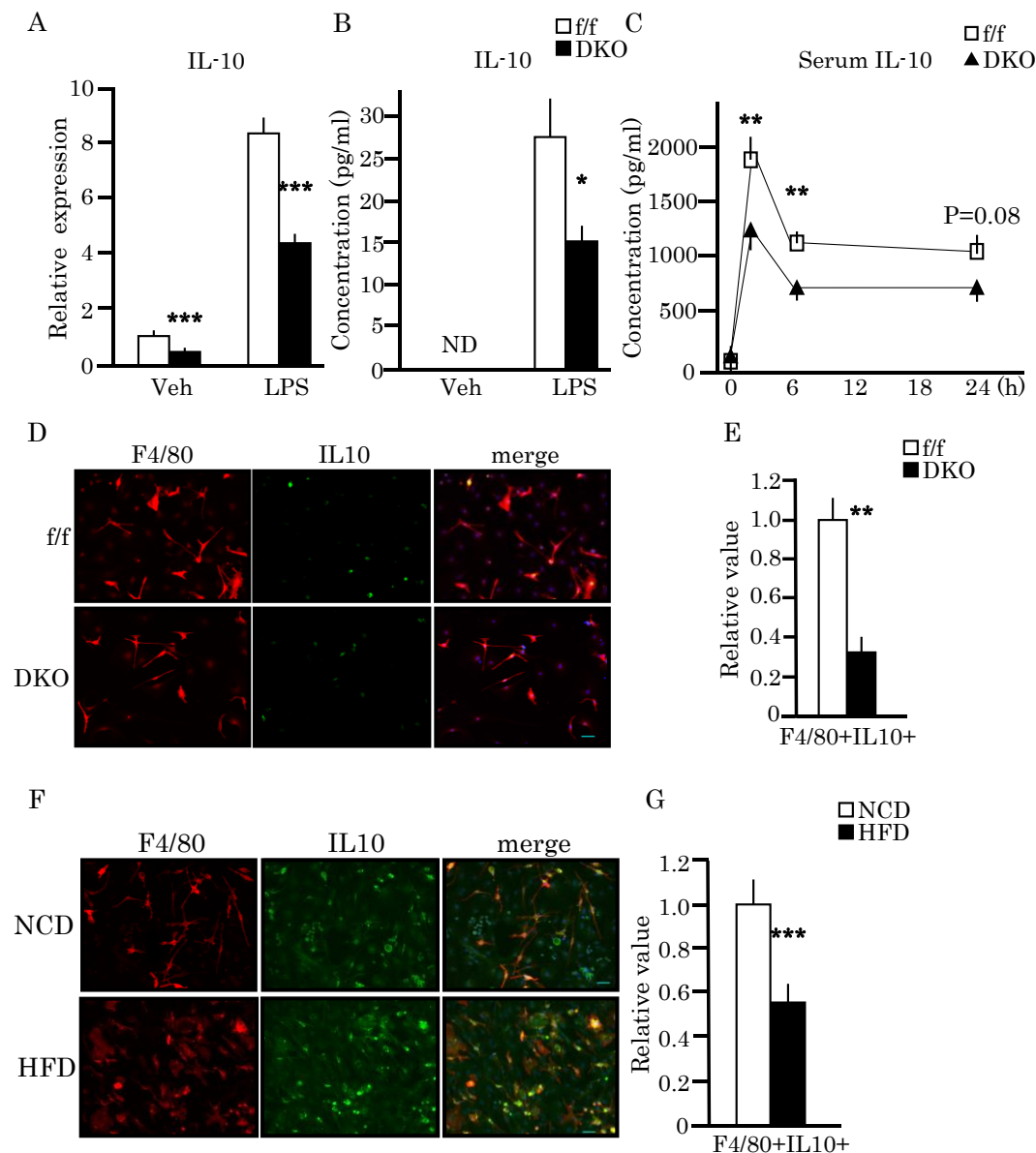


Figure 6: IL-10 expression in Akt deficient macrophages under stimulation by LPS, and in vivo. A: Expression of IL-10 in BMDMs after 72 hours of stimulation with 10 ng/ml LPS, assessed by RT-PCR using whole cell extracts (n=4wells per condition). B: Concentration of IL-10 in cell supernatants under the same conditions as A, assessed by ELISA. C: Serum IL-10 concentration in LysMDKO mice after intraperitoneal injection of 1 μ g/gBW LPS, assayed by ELISA (n=6 mice per group). D: IL-10 production in macrophages among SVF cells of LysMDKO mice, assessed by immunofluorescence of IL10 (green) and F4/80 (red). E: Quantification of IL-10 F4/80 double positive cells in K (n= 10 fields per condition). Quantity of double positive area are normalized to F4/80 positive area. Results are shown as relative values to unstimulated control mice). F-G: IL-10 positivity in tissue macrophages from wild type mice fed a NCD or HFD, analyzed by immunofluorescence of F4/80 and IL-10 and quantified using Hybrid cell count software (n=7-10 fields per condition. Number of double positive areas normalized to number of F4/80 positive areas. Results shown as relative values to SVF from NCD fed mice). *: P<0.05 **: P<0.01, ***: P<0.001.

were IL-10 positive in WAT of LysMDKO mice fed a normal chow diet, implying a similar response may be present in normal chow fed animals, without exogenous LPS stimulation

(Figure 6D-E). As IL-10 expression was not completely abolished in Akt deficient macrophages both *in vivo* and *ex vivo*, pathways dependent on Akt signaling and those independent were likely to regulate IL-10 expression.

As Akt was constitutively active and was unresponsive to stimulation by LPS in macrophages of mice on HFD, we assessed IL-10 production in tissue macrophages of mice with DIO. In wild type mice on HFD, there were fewer IL-10 positive macrophages than in those fed a normal chow diet, suggesting impaired induction of IL-10 by macrophages in mice with DIO as in Akt deficient macrophages (Figure 6F-G). These findings indicated defective Akt stimulation, IL-10 production by macrophages in adipose tissue as common factors in these two models with obese traits, implying their significance in the pathogenesis of obesity.

TLR4/PI3K/Akt/mTOR signaling induces IL-10 in macrophages

We went on to search for possible signaling events downstream of Akt in the induction of IL-10. In chronic LPS stimulation, expression of IL-10 was lowered in wild type BMDMs in the presence of a PI3 kinase inhibitor (Figure 7A), as well as the mTOR inhibitor rapamycin (Figure 7B). On the other hand, a GSK3 inhibitor did not induce changes compared to vehicle treated cells nor affect the changes in the rapamycin treated cells (Figure 7C-D).

Tuberous sclerosis complex 2 (TSC2) suppresses the activity of mTORC1, and the

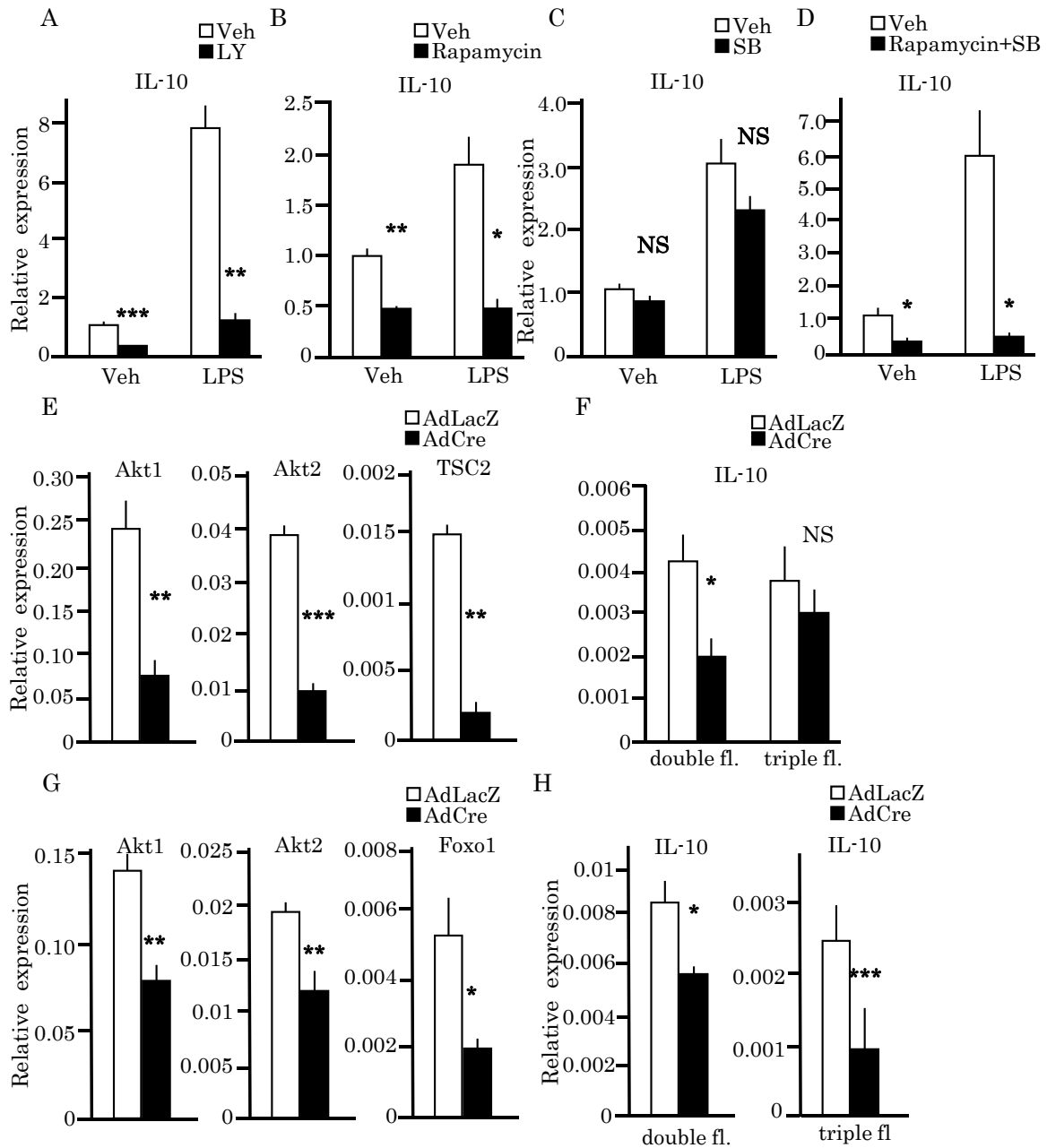


Figure 7: Assessment of signaling events in the induction of IL-10. A-D: Expression of IL-10 in wild type BMDMs assessed by RT-PCR of RNA extracts after stimulation by 10 ng/ml LPS, under inhibition with 1.4 μ M PI3K inhibitor LY294002(A), 10 ng/ml Rapamycin (B), 1 μ M GSK inhibitor SB216763 (C), and 10ng/ml Rapamycin and 1 μ M SB216763 simultaneously (D) (n=3 wells per condition). E: Akt1, Akt2, TSC2 expression in Akt1/Akt2/TSC2 floxed cells under overexpression of Cre recombinase or LacZ using adenovirus vectors. F: IL-10 expression at baseline in Akt1/Akt2 floxed cells (double fl.) and Akt1/Akt2/TSC2 floxed cells (triple fl.) under overexpression of Cre recombinase or LacZ using adenovirus vectors (E-F: n=6 wells per condition). G: Akt1, Akt2, Foxo1 expression in Akt1/Akt2/Foxo1 floxed cells under overexpression of Cre recombinase or LacZ using adenovirus vectors. H: IL-10 expression at baseline in Akt1/Akt2 floxed cells (double fl.) and Akt1/Akt2/Foxo1 floxed cells (triple fl.) under overexpression of Cre recombinase or LacZ using adenovirus vectors (G-H: n=6 wells per condition). *: P<0.05 **: P<0.01, ***: P<0.001

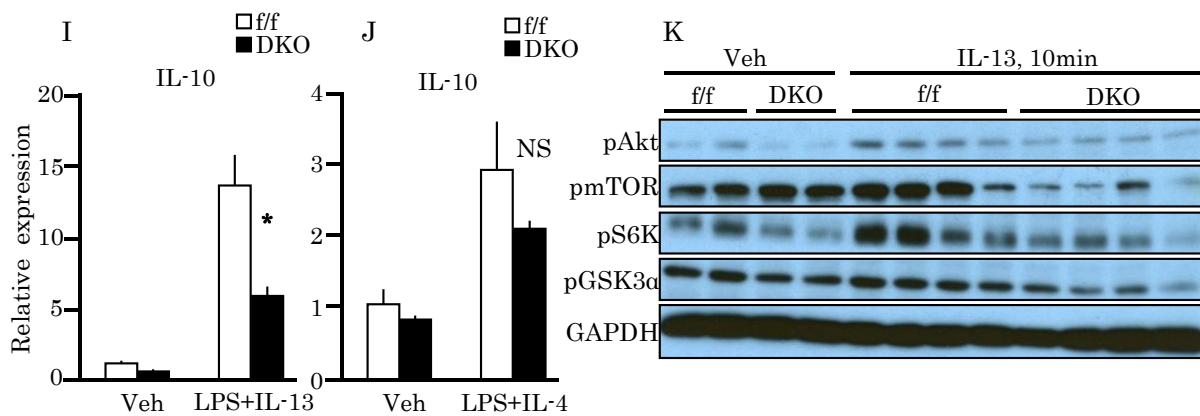


Figure 7, continued: I-J : Expression of IL-10 in BMDMs from LysMDKO mice treated overnight with 50 ng/ml IL-13 or IL-4 simultaneously with 10 ng/ml LPS, by RT-PCR analysis of RNA extracts (n=3wells per condition). K: Akt and Akt substrate phosphorylation assessed by western blot using whole protein samples from BMDM cells stimulated by 50 ng/ml IL-13 (n=4 wells per genotype). *: P<0.05 **: P<0.01, ***: P<0.001

activity of TSC2 is suppressed by Akt. To further elucidate the role of Akt/mTOR signaling in the expression of IL-10, we assessed the effect of Ad-Cre infection in macrophages from Akt1/Akt2/TSC2 floxed mice. In this model, Akt expression was suppressed to levels similar to macrophages in LysMDKO mice by infection of Ad-Cre, and TSC2 expression was suppressed to a similar degree (Figure 7E). In agreement with the gene expression profile under rapamycin treatment, IL-10 expression was similar in Ad Cre infected and control virus infected triple floxed cells, whereas expression was suppressed in double floxed cells, further supporting the contribution of TLR4/PI3K/Akt/mTOR signaling in the expression of IL-10 (Figure 7F).

Since decreased signaling to Foxo1 from PDK1 has been previously shown to be important in increased inflammation of adipose tissue and insulin resistance while on a high fat diet⁵⁴, we assessed the possibility that decreased signaling to Foxo1 would suppress expression of IL-10. Foxo1 expression was suppressed in macrophages from Akt1/Akt2/Foxo1 floxed

mice upon Ad-Cre infection (Figure 7G). Though suppression of the expression of Akt was milder than in macrophages from LysMDKO mice, suppressed expression of IL-10 could be verified in Akt1/Akt2 floxed cells at baseline, and when infected with Ad-Cre, IL-10 expression was rather further suppressed relative to Ad-LacZ infection in Akt1/Akt2/Foxo1 floxed cells compared to Akt1/Akt2 floxed cells, results that would not be anticipated in such a model (Figure 7H). These results supported the regulatory role of TLR4/Akt/mTOR signaling in the expression of IL-10.

IL-13 signaling induces IL-10 through PI3K/Akt/mTOR

As TLR4 signaling also induces the expression of proinflammatory cytokines known to exacerbate insulin resistance, and from findings that IL-10 is induced by LPS *in vivo* from an earlier phase than macrophage cultures, we assessed the possible involvement of signals other than LPS in the induction of IL-10. Although the anti-inflammatory Th2 cytokines IL-4 and IL-13 did not induce IL-10 alone, costimulation with IL-4 and LPS, or IL-13 and LPS induced IL-10 overnight, in a shorter time span than chronic LPS stimulation in agreement with past studies ⁶⁴. Moreover, Akt deficient macrophages expressed lower levels of IL-10 after costimulation with IL-13, but not IL-4 (Figure 7H-I), suggesting an IL-13 dependent signal induced IL-10 in an Akt-mediated pathway. Phosphorylation of Akt and its substrates were

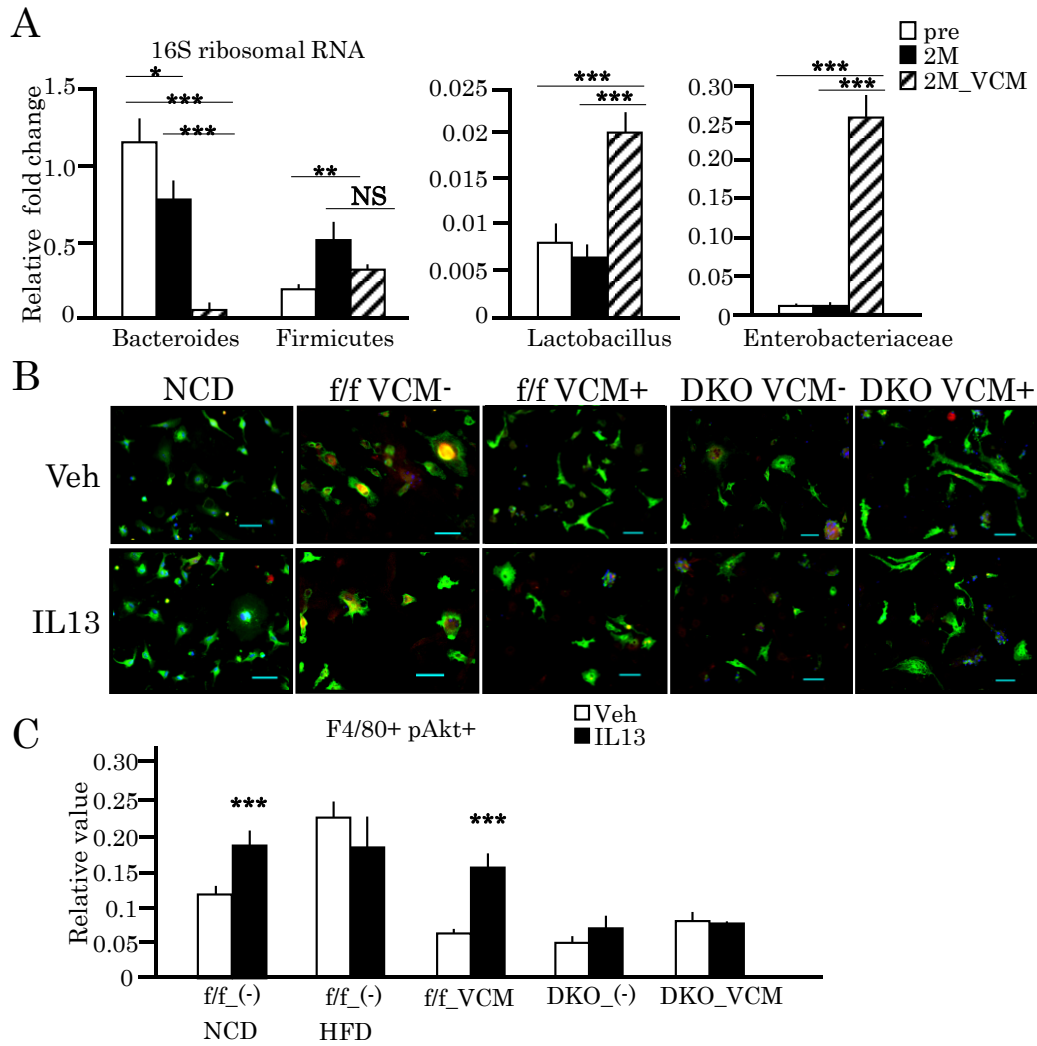


Figure 8: LysMDKO mice fed on a high fat diet and treated with vancomycin. A: Profile of intestinal flora assessed by quantitative PCR of 16S ribosomal RNA using total DNA derived from feces of mice after 2 months of high fat diet feeding (n=6-8 samples per group. Pre: before feeding high fat diet. 2M: after 2months of high fat feeding 2M_VCM: after 2 months of high fat diet feeding and vancomycin treatment). B-C: Akt signaling in tissue macrophages of LysMDKO mice assessed by immunofluorescence of phosphor-Akt (red) and F4/80 (green) in SVF cells derived from WAT of LysMDKO mice and control mice, and quantification of phosphor-Akt F4/80 double positive cells (n= 10-12 fields per condition. Number of double positive area is normalized to F4/80 positive area). D-E: IL-10 production in macrophages assessed by immunofluorescence of SVF cells derived from WAT of LysMDKO mice and control mice, and quantification of IL10 F4/80 double positive cells (n=10-12 fields per condition. Number of double positive area is normalized to F4/80 positive area). *: P<0.05, ***: P<0.001

verified in BMDM cells after IL-13 stimulation in a much shorter timespan than in LPS stimulation. Among downstream targets of Akt signaling, decreased phosphorylation of mTOR and its target p70 S6K (Figure 7J) were particularly evident in Akt deficient macrophages, supporting the involvement of mTOR and S6K in the Akt dependent induction of IL-10 by IL-

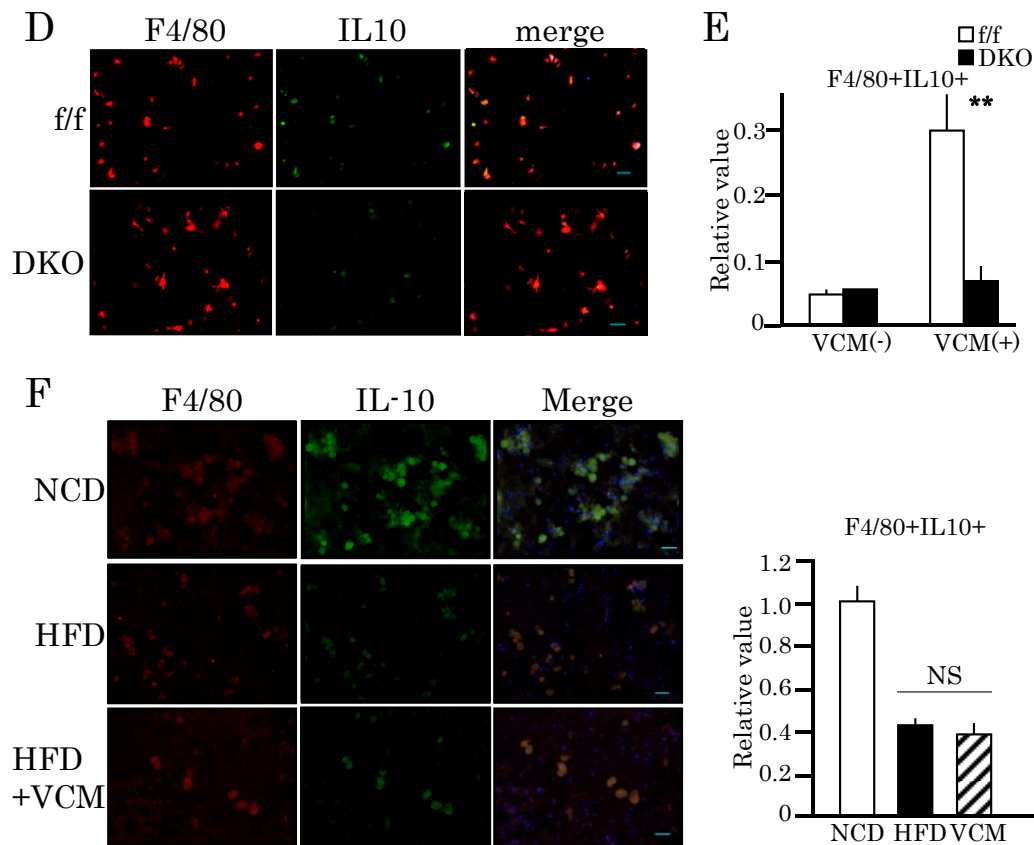


Figure 8, continued. D-E: IL-10 production in macrophages assessed by immunofluorescence of SVF cells derived from WAT of LysMDKO mice and control mice, and quantification of IL10 F4/80 double positive cells (n=10-12 fields per condition. Number of double positive area is normalized to F4/80 positive area). F: IL-10 production in macrophages assessed by immunofluorescence of F4/80 and IL-10 using primary cells derived from liver of control mice, and quantification of IL10 F4/80 double positive cells (n=10-12 fields per condition. Number of double positive area is normalized to F4/80 positive area) **: P<0.01

13 as well. As IL-4 and IL-13 expression alike were hardly detected in BMDM cells as assessed by RT-PCR at baseline and after LPS stimulation, these signals likely originated in extracellular sources *in vivo*. Taken together, LPS signaling through TLR4 and signaling by extracellular IL-13 could be important in the induction of IL-10 through the PI3K/Akt/mTOR pathway.

Akt-dependent IL-10 production by macrophages in vancomycin treated mice on HFD

We went on to search for possible aspects originating in the environment that may

signal through Akt in macrophages to maintain homeostasis. As observed in previous studies, treatment of obese mice with oral vancomycin induced marked changes in intestinal microflora, most notably an increase in enterobacteriaceae, which includes LPS producing gram negative bacteria^{65,66} (Figure 8A). From *ex vivo* studies mentioned previously, we postulated LPS or LPS-like signals originating from such intestinal aspects may act to maintain homeostasis through IL-10 production in macrophages. When treated with vancomycin, phosphor-Akt responsiveness was increased in adipose tissue macrophages from control mice, but not in macrophages from LysMDKO mice (Figure 8B-C). Furthermore, IL-10 positive adipose tissue macrophages in control mice were significantly increased (Figure 8D-E) in vancomycin treated mice, but not in LysMDKO mice, suggesting vancomycin treatment Akt-dependently induced IL-10 production in macrophages. Of note, high fat diet feeding in control mice induced marked decreases in IL-10 positivity in macrophages derived from liver, and IL-10 positivity did not increase upon vancomycin treatment, suggesting this treatment does not affect macrophages in the liver (Figure 8F).

Insulin resistance in LysMDKO mice on HFD under vancomycin treatment

To further explore the possibility that macrophages respond to LPS or LPS-like substances in the intestine and maintain homeostasis through Akt-dependent IL-10 production,

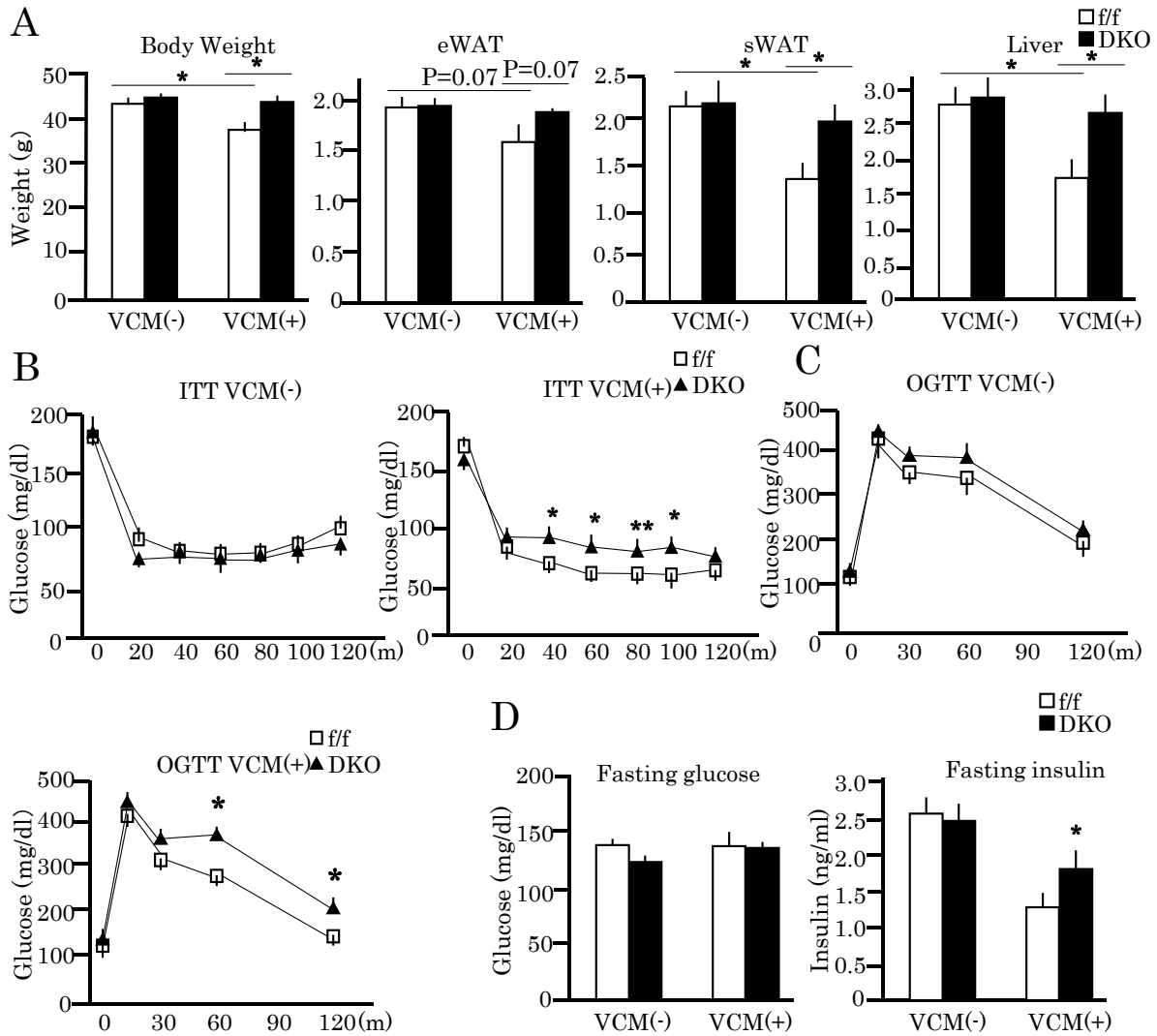
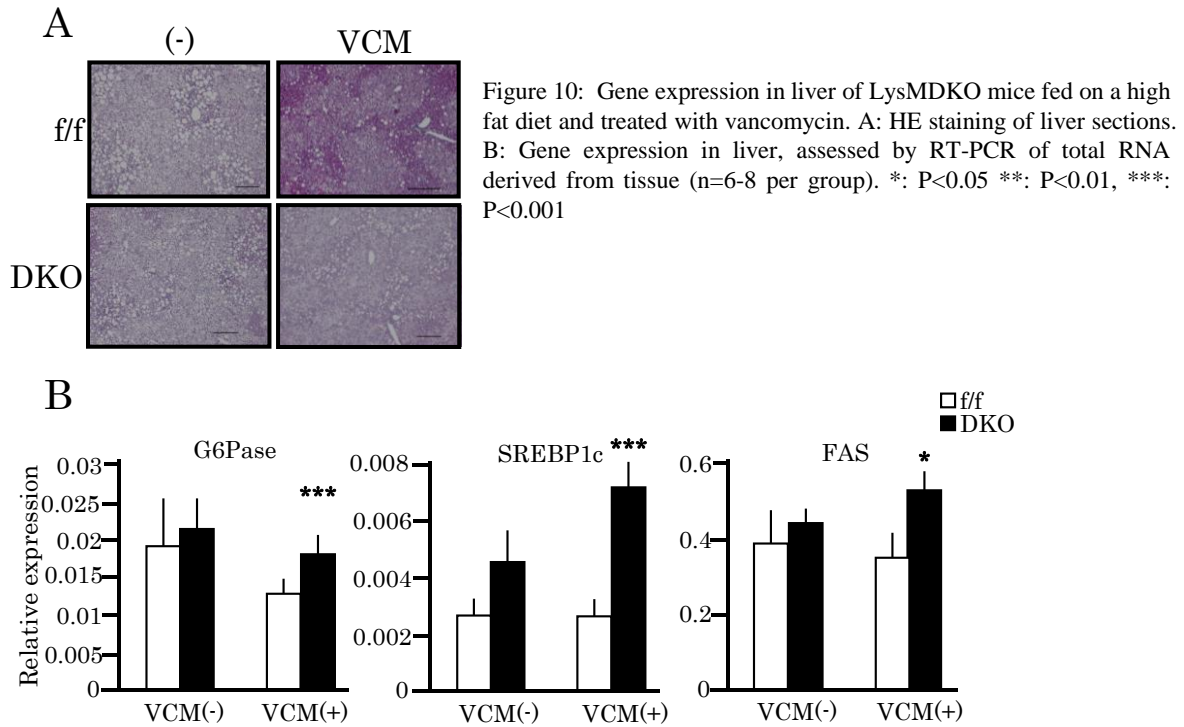


Figure 9: Phenotype of LysMDKO mice fed on a high fat diet and treated with vancomycin. A: Body weight, weight of adipose tissue and liver after 2 months of high fat diet feeding (n=6-8 mice per group. VCM: vancomycin treatment). B: plasma glucose in ITT (1.75 U/KgBW of regular insulin was administered intraperitoneally (n=6-7 mice per group). C: Plasma glucose concentrations during OGTT (1.0 g/kgBW of glucose was administered after 16 hour fast. n=6-7 mice per group). D: Fasting plasma glucose and fasting plasma insulin in mice after 2 months on a high fat diet with or without antibiotics treatment (n=8-12 mice per group). *: P<0.05 **: P<0.01, ***: P<0.001

we then assessed the effect of vancomycin treatment on the metabolic profile of LysMDKO mice on HFD. Vancomycin treatment induced alleviation in the obese traits of control mice, such as reductions in body weight (p<0.05), epididymal fat pad mass (p=0.07), subcutaneous fat mass (p<0.05), and liver weight (p<0.05). Improvements were nonsignificant in LysMDKO



mice, resulting in alterations in the metabolic profile of LysMDKO mice to a similar extent to those observed as seen in normal chow fed mice, such as increased body weight, and increased adipose tissue mass (Figure 9A). Notably, liver weight of LysMDKO mice was not decreased by vancomycin treatment resulting in a marked difference to that of the control mice (Figure 10A). Insulin and glucose tolerance tests showed higher plasma glucose values in LysMDKO mice (Figure 9B-C) compared to control mice under vancomycin treatment. Fasting plasma insulin was markedly lowered in control mice under vancomycin treatment ($p < 0.05$), a difference not significant in LysMDKO mice ($p = 0.05$), resulting in significantly higher insulin levels in LysMDKO mice (Figure 9D), indicating insulin resistance.

In LysMDKO mice treated with vancomycin, exacerbated hepatic steatosis was observed as evidenced by the liver sections stained with hematoxylin and eosin (Figure 10A).

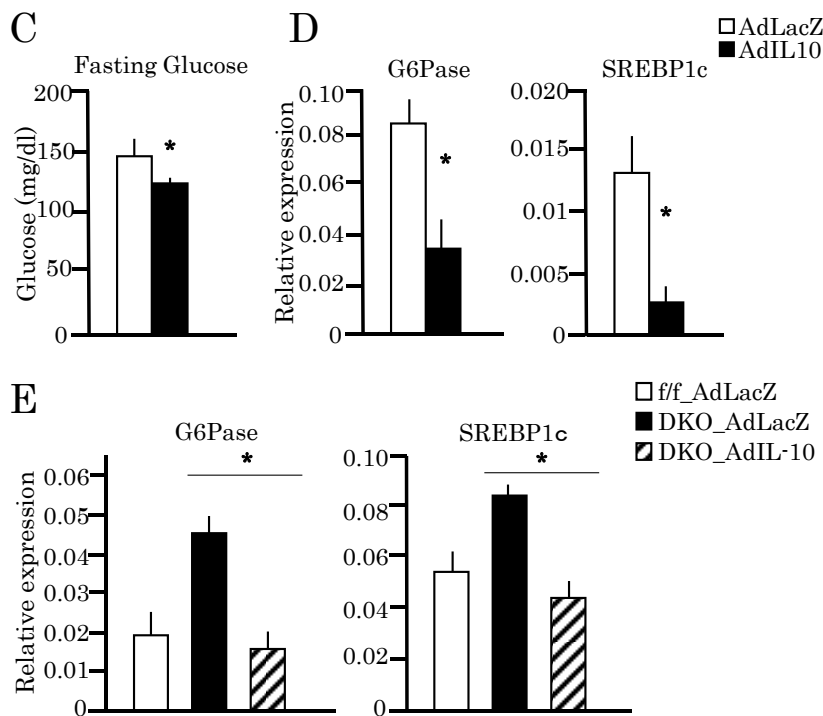


Figure 10, continued: Effect of IL-10 overexpression in the livers of LysMDKO mice fed on a high fat diet and treated with vancomycin. C: Fasting plasma glucose concentrations of wild type mice injected with adenoviruses. D: Gene expression analyses of liver by RT-PCR using whole liver RNA extracts. (n=4 per group). E: Gene expression in the liver of LysMDKO mice and control mice infused intravenously with adenovirus expressing human IL-10 or LacZ as control, by RT-PCR of tissue RNA extracts (n=4-6 per group). *: $P < 0.05$

In the liver of these mice, expression of G6Pase was elevated compared to control mice, as were genes associated with lipogenesis, SREBP1c and FAS (Figure 10B). As the *ex vivo* studies showed lower expression of IL-10 in the macrophages of LysMDKO mice when stimulated with LPS, we speculated LPS producing gram-negative bacteria in the intestinal microflora may have the potential of alleviating liver steatosis and insulin resistance through IL-10 production in macrophages, which was enhanced by vancomycin treatment.

Alleviation of insulin resistance by IL-10 in vancomycin treated LysMDKO mice on HFD

To further elucidate the role of IL-10 in the regulation of metabolic homeostasis in the

liver, we examined the effect of replenishing IL-10 in mice with apparently defective IL-10 production in macrophages by injecting an adenovirus vector expressing human IL-10 (Ad-IL10) under the CAG promoter intravenously at 10^8 pfu/mouse. In control mice with DIO, in which adipose tissue macrophages are IL-10 deficient as mentioned previously, injection of the adenovirus vector resulted in low but detectable levels of human IL-10 expression in the liver and serum in contrast to normal chow fed animals, mice with DIO, and obese mice treated with vancomycin, in which serum IL-10 is undetectable. Injection of the adenovirus vector led to slightly lower levels of plasma glucose, and suppressed SREBP1c and G6Pase expression compared to mice injected with control adenovirus expressing LacZ (Ad-LacZ) (Figure 10C-D). When fed on HFD for 2 months with or without administration of vancomycin, LysMDKO mice injected with Ad-IL10 showed expression of G6Pase and SREBP1c in liver similar to control mice injected with Ad-LacZ, and lower than those injected with control adenovirus in LysMDKO mice (Figure 10E). These data suggest that IL-10 produced by adipose tissue macrophages is involved in the regulation of gene expression associated with liver metabolism under stimulation by intestinal microflora through an Akt-dependent pathway.

4. Discussion

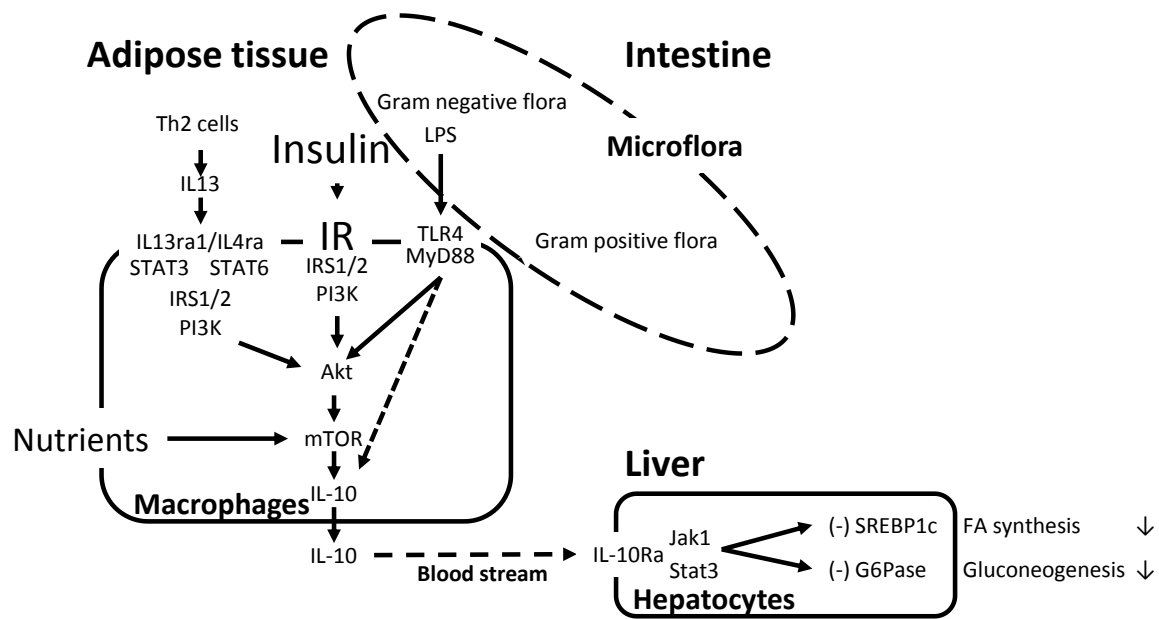


Figure 11: Schematic illustration of the response of macrophages to extracellular stimuli, and the regulation of liver homeostasis by macrophages through the production of IL-10.

In the present study, we propose a novel paradigm in which Akt in macrophages contributes to maintaining body weight and glucose homeostasis on a normal chow diet in collaboration with the intestinal microflora, possibly by responding to stimuli such as feeding that act through insulin signaling, Th2 signals, the intestinal microflora, or other factors that phosphorylate Akt. (Figure 11). In this model, pathological conditions such as a diet induced obese state suppress Akt stimulation in tissue macrophages by chronic phosphorylation, and nullifies stimulation from the environment that act to maintain homeostasis in a normal state.

Akt in adipose tissue macrophages in diet induced obese mice is in a state of chronic phosphorylation, where stimulation by LPS and IL-13 are not observed in contrast to lean mice. As transient Akt stimulation is recovered in diet induced obese mice treated with vancomycin, where hyperinsulinemia is alleviated and vancomycin sensitive flora are eradicated, we

speculate these factors may be important in chronic Akt phosphorylation. Moreover, as feedback mechanisms are known to suppress Akt signaling via mTORC1 and suppressed IRS expression⁶⁷, additional intracellular mechanisms possibly contribute to chronic Akt phosphorylation in obesity.

Akt deficient macrophages are unresponsive to stimulation by insulin, LPS, and IL-13 at the cellular level, and displayed traits resembling DIO while feeding on a normal chow diet, with increased adiposity and liver insulin resistance. This observation led us to speculate suppressed Akt stimulation by extracellular factors affected by environmental stimulus such as feeding is of importance in the pathogenesis of obesity. We postulated some macrophage derived factor is induced through Akt in macrophages and plays a role in maintaining metabolic homeostasis. Extracellular cellular factors such as insulin, alterations in the intestinal flora, or Th2 cytokines may stimulate Akt in processes such as feeding, although further accumulation of data is essential to define precise processes.

Upon stimulation by LPS, macrophages display an early induction of the inflammatory Th1 response, followed by induction of the anti-inflammatory Th2 response⁴². Induction of the Th2 cytokine IL-10 can be detected around 72 hours after LPS stimulation, and can be induced after stimulation by LPS. As Th2 responses have been proposed to function in maintenance of homeostasis in the past studies^{46,47}, and Akt signaling in Akt deficient macrophages is markedly

suppressed under LPS stimulation, we endeavored to search for the macrophage derived factor responsible in maintaining metabolic homeostasis in the response following 72 hours of LPS stimulation. We postulated such a gene would be induced by stimulation and its expression would be altered in Akt deficient macrophages.

In transcriptome analyses of gene expression after chronic LPS stimulation, IL-10 is the only gene with known extracellular functions that meet these conditions. IL-10 signals through Jak1 and STAT3 in the liver⁶⁸, and has been implicated to enhance liver insulin sensitivity in the past studies. However, IL-10 mRNA expression has also been reported to increase in a parallel course with inflammatory cytokines, speculated to be a physiological feedback mechanism in response to inflammation³³, although direct effects of IL-10 in the context of obesity has not been researched as thoroughly. Numerous components of the insulin cascade have been reported to influence IL-10 expression^{43,48,54,67,69}, but its function in the context of metabolism has thus remained controversial^{61,70}. Akt deficient macrophages express lower levels of IL-10 after 72 hours of LPS stimulation, and lower levels of IL-10 expression are also observed when mTOR signaling is inhibited by rapamycin, but GSK3 inhibitors or genetical knockout of Foxo1 by a Cre/loxP dependent mechanism does not affect suppressed IL-10 expression in Akt deficient macrophages. Moreover, under genetical knockout by this mechanism of TSC2, an inhibitor of mTORC1 that is suppressed by Akt signaling, IL-10

expression is no longer suppressed, further supporting the primary role of Akt signaling to mTOR in the expression of IL-10. From observations that IL-10 expression is not completely abolished in Akt deficient macrophages, Akt independent signals are likely involved in the induction of IL-10 as well, but this needs further affirmation.

Although TLR4 signaling through Akt and mTOR may maintain metabolic homeostasis by inducing IL-10, we considered this signal was unlikely to act alone in such a process, as TLR4 signaling also induces inflammatory cytokines, and has been shown to be important in the pathogenesis of obesity⁴⁵. We therefore thought other signals were likely involved in this process. IL-4 and IL-13 are Th2 cytokines which activate transcriptional cascades regulating anti-inflammatory gene transcription. The IL-13 receptor is a heterodimer of IL13ra1 and the IL-4 receptor component IL4ra, and signals through IRS1/IRS2/PI3K/Akt, JAK1, STAT3, and STAT6⁷¹. Although IL-4 and IL-13 both induce IL-10 overnight with costimulation with LPS as mentioned in previous studies⁶⁴, IL-10 expression is suppressed in Akt deficient macrophages under IL-13 costimulation, but not IL-4 costimulation. This may be due to signals via the IL-4 receptor which induce IL-10, but are not dependent on Akt signaling. Western blots reveal decreased signaling to mTOR after IL-13 stimulation, supporting the involvement of mTOR in the Akt dependent induction of IL-10 by IL-13 as well. Collectively, these results indicate that TLR4 and the IL-13 receptor signal through Akt, and that IL-10 is

induced by both in an Akt dependent pathway. Together with the observation that *in vivo* LPS stimulation induces IL-10 in a very short time course compared to *ex vivo* stimulation, this raises the possibility of TLR4 stimulators and extracellular IL-13 or other signals, of importance insulin that transiently phosphorylate Akt induce IL-10 *in vivo*, most notably in processes such as feeding although this requires further acquirement of data.

As anti-inflammatory Th2 responses seem to be impaired in LysMDKO mice, we initially thought diet induced obesity, where a chronic inflammatory state plays a key role in the dysregulation of metabolism, LysMDKO mice may present further dysregulated metabolic states. In contrast to the past studies⁵⁴, LysMDKO mice and control mice on a high fat diet are equally obese. As both diet induced obese control mice and LysMDKO mice are deficient in the stimulation of Akt in macrophages, we considered these results consistent with our model in which suppressed Akt stimulation in macrophages is important in the pathogenesis of obesity.

The interaction between the intestinal microflora and the host is becoming increasingly recognized as an important aspect in the regulation of metabolism, although precise mechanisms linking these two components are only starting to be understood^{45,66,72,73}. When mice with diet induced obesity are treated with orally administered vancomycin, marked changes in intestinal microflora as can be seen as decreases of gram positive bacteria and increases in gram negative bacteria (Figure 12). Additionally, improvements in obese metabolic

profile can be observed as decreased body weight and decreased adipose tissue and lipid content of the liver, and moreover alleviation of hyperinsulinemia. In such a state, Akt in tissue macrophages in control mice recover responsiveness, suggesting hyperinsulinemia, or an intestinal aspect that suppresses Akt responsiveness in DIO is decreased by vancomycin treatment. Such an intestinal aspect likely originates in vancomycin sensitive intestinal flora, although this needs further assessment. Macrophages from mice with DIO treated with vancomycin Akt dependently increase in IL-10 positivity, and LysMDKO mice on HFD do not exhibit alleviation of obesity observed in control mice, showing markedly exacerbated hepatic insulin resistance compared to control mice. From the increase in enterobacteriaceae and *ex vivo* studies mentioned above, we speculate gram negative bacteria producing LPS, or other Akt stimulating substances such as insulin and IL-13 may signal through Akt in macrophages in the vancomycin treated state to induce IL-10 productions in processes such as feeding, alleviating insulin resistance in the liver.

By attempting replenishment of IL-10 in LysMDKO mice on HFD treated with vancomycin by adenovirus-mediated gene transfer of human IL-10, expression of G6Pase and SREBP1c associated with liver insulin resistance and liver steatosis respectively^{15,60,74}, are suppressed to the levels similar to those of leaner control mice. These results suggest that IL-10, likely originating in macrophages, acts to alleviate insulin resistance and steatosis in the

liver, although as the distribution of IL-10 is likely to be different from physiological states in this context, further studies are required to elucidate the precise origin and magnitude of IL-10 function *in vivo*,

From the evidence presented in this report, we propose a model where macrophages maintain liver metabolic homeostasis through Akt/mTOR dependent IL-10 secretion that is presumably stimulated by extracellular stimuli induced by rhythmical physiological processes such as feeding, in collaboration with some secreted factor from the intestinal microflora, such as gram-negative bacteria (figure 12). To apply this mechanism to development of a novel therapeutic strategy for the treatment of obesity-induced diabetes, further attempts will be crucial in identifying the nature of these extracellular stimuli, and the intestinal aspects which act with them.

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