IL-21 is a major negative regulator of IRF4-dependent lipolysis affecting T_{regs} in adipose tissue and systemic insulin sensitivity.

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word count 3977; number of tables (n=1); number of figures (7)

Diabetes Publish Ahead of Print, published online January 15, 2014

Abstract

Obesity elicits immune cells infiltration of adipose tissue provoking chronic low-grade inflammation. Regulatory T cells (T_{regs}) are specifically reduced in adipose tissue of obese animals. Since Interleukin 21 (IL-21) plays an important role in inducing and maintaining immune-mediated chronic inflammatory processes and negatively regulates T_{regs} differentiation/activity we hypothesized that it could play a role in obesity-induced insulin resistance.

We found IL-21 and IL-21R mRNA expression up-regulated in adipose tissue of high fat diet WT mice and in stromal-vascular fraction from human obese subjects in parallel to macrophage and inflammatory markers. Interestingly a larger infiltration of T_{reg} cells was seen in the adipose tissue of IL-21 knockout (IL-21 KO) mice compared to WT animals fed both ND and HFD.

In a context of diet-induced obesity, IL-21 KO mice, when compared to WT animals, exhibited lower body weight improved insulin sensitivity and decreased adipose and hepatic inflammation. This metabolic phenotype is accompanied by an higher induction of IRF4, a transcriptional regulator of fasting lipolysis in adipose tissue. Our data suggest that IL-21 exerts negative regulation on IRF4 and T_{regs} activity, developing and maintaining adipose tissue inflammation in the obesity state.

Introduction

Obesity-associated tissue inflammation is now recognized as a major cause of decreased insulin sensitivity (1,2). Obesity, insulin resistance and type 2 diabetes are closely associated with chronic inflammation characterized by abnormal cytokine production, increased acute-phase reactants and other mediators, and activation of a network of inflammatory signaling pathways (3,4). Excessive triglyceride accumulation within adipocytes leads to adipocyte hypertrophy and a dysregulation of adipokine secretory patterns. Adipocytes as well as cells of the stromal vascular fraction (SVF) that includes preadipocytes, fibroblasts, mesenchymal stem cells, and immune cells contribute to the production of proinflammatory cytokines in obesity (3-5), with a pivotal role played by macrophages and T lymphocytes (6-8). In lean adipose tissue, T-helper type 2 (Th2) cells produce anti-inflammatory cytokines such as interleukin (IL)-4, 10, and 13 which promote alternative activated M2 macrophage polarization (9). M2 polarization is also induced by regulatory T cells (T_{regs}) and eosinophils via IL-4. Conversely, in obese adipose tissue, it has been observed an increase in the number of Th1 type cytokines, M1 polarized macrophages, mast cells, B cells and CD8+ T cells which contribute to insulin resistance, promote macrophages M1 accumulation and proinflammatory gene expression (9-13). Factors orchestrating the switch between M1 and M2 are still undefined. Loss of Interferon Regulatory Factor 4 (IRF4) specifically in the myeloid cells evoked a constitutive M1 polarization in the adipose tissue, suggesting that IRF4 is a negative regulator of inflammation in diet-induced obesity, in part through regulation of macrophage polarization (14). Interestingly IRF4 expression is nutritionally regulated by the actions of insulin and FoxO1, playing a significant role in the transcriptional regulation of lipid handling in adipocytes, promoting lipolysis, at least in part by inducing the expression of the lipases ATGL (*Pnpla2*) and HSL (*Lipe*) (15).

Naturally occurring T_{reg} cells are a unique subpopulation of CD4+ T cell specifically adapted to the suppression of aberrant or excessive immune responses that are harmful to the host (16). T_{reg} cells are

abundant in visceral adipose tissue (VAT) and have a different T cell receptor repertoire compared with T_{reg} cells in other tissues, suggesting that they might be activated via the recognition of a fat tissue-specific antigen (13). A recent study reveals an important role for VAT-specific natural T_{reg} cells in the suppression of obesity-associated inflammation in adipose tissue and consequently in reducing insulin resistance (17). The number of VAT T_{reg} cells is strikingly and specifically reduced in insulin-resistant models of obesity, and are characterized by the expression of the transcription factor Foxp3 and the nuclear receptor peroxisome proliferator-activated receptor (PPAR)-γ (17).

IL-21 is a member of the type-I cytokine family and is synthesized by a range of CD4+ Th cells, including Th1 and Th17 cells, activated NKT cells, and T follicular helper cells (18-20). IL-21 biological functions are mediated via IL-21R and following activation of the JAK family protein tyrosine kinases JAK1 and JAK3 and, subsequently, to the activation of Stat1, Stat3, and to a lesser degree Stat4, Stat5, and Stat6 (21-23). IL-21 expression in T cells can be regulated by IL-21 via an autocrine positive feedback loop, involving the activation of STAT3 (24). This feedback loop is essential for the development of Th17 cells (25,26). IL-21-mediated T cell activation relies partly on its ability to inhibit the differentiation of inducible regulatory T cells, and to make T cells resistant to the T_{reg}-mediated immunosuppression (27,28).

Because IL-21 is known to exert negative effects on regulatory T cells activity we hypothesized that it could play a role in obesity-induced insulin resistance.

METHODS

Mouse models and metabolic analysis. WT and IL-21 KO (129S5-*Il21*^{tm1Lex}) male mice, both on the same genetic background (C57BL/6J), were purchased from Lexicon Genetics Inc. IL-21 KO mice are viable and do not exhibit any phenotype. Mice were maintained in standard animal cages under specific pathogen–free conditions in the animal facility at the University of Rome Tor Vergata. Mice were maintained under a strict 12-h light cycle (lights on at 7:00 am and off at 7:00 pm), genotyped and divided in separate cages at the beginning of each experiment. For Diet Induced Obesity model, individually caged mice from all groups were fed a High Fat Diet (HFD) (60% of calories from fat; Research Diets, New Brunswick, NJ) or normal diet (ND, 10% calories from fat; GLP Mucedola Srl, Settimo Milanese, Italy) for 18 weeks after weaning as indicated. Metabolic testing procedures were performed as previously described (29,30).

Hormone and metabolite levels were measured using commercial kits: insulin (Mercodia), NEFA (Wako),glycerol (Sigma-Aldrich, St.Louis, MO), and Glucagon (Uscn Life science Inc).

Evaluation of peripheral insulin sensitivity (clamp). After 12 weeks of HFD, we evaluated peripheral insulin sensitivity by the euglycemic-hyperinsulinemic clamp technique. Surgery for the positioning of catheters was performed 3 to 5 days prior to the insulin clamp procedure as previously described (31,32) and then mice were housed in individual cages. The euglycemic-hyperinsulinemic clamp was performed in the awake state after 6 hour-fast. At time zero, a primed continuous (18.0 $mU \cdot kg^{-1} \cdot min^{-1}$, Actrapid 100U, Novo Nordisk, Copenhagen, Denmark) infusion of human insulin was started simultaneously with a variable infusion of 20% dextrose in order to maintain the plasma glucose concentration constant at its basal level (80–100 mg/dl). Fasting Plasma Glucose (FPG) was measured at time 0. Subsequently, blood samples (~2 μl) were taken from the tail vein at 10 min intervals for at

least 2 hours to measure glucose concentration and adjust dextrose infusion rates. Insulin sensitivity (rate of peripheral glucose uptake, $mg \cdot kg^{-1} \cdot min^{-1}$) was calculated from average glucose concentrations and dextrose infusion rates during the last 30 min of the steady-state clamp period.

Analysis of adipose and hepatic tissue. Epigonadal fat and liver were obtained from WT and IL-21 KO mice; specimens were fixed in 10% paraformaldehyde and embedded in paraffin. Ten-micrometer consecutive sections were then mounted on slides and stained with hematoxylin and eosin. Adipose cell size and density were calculated as previously described (33).

Isolation of adipocytes and stromal vascular fraction (SVF). VAT was subjected to collagenase digestion (1 mg/mL collagenase type 1; Sigma-Aldrich) in Krebs-Ringer buffer, with shaking at 180 rpm for 30 min at 37° C. After digestion, adipocytes were allowed to separate by flotation and the infranatant solution was centrifuged for 5 min at 300g to pellet the SVF. The adipocyte fraction was washed three times with the Krebs-Ringer buffer. Subsequently, RNA was isolated from adipocytes and the SVF fractions and analyzed by real time PCR. The profile of adiponectin mRNA expression was used to test the purity of the isolated fractions. The SVF was analyzed by flow cytometry techniques.

Cell culture. 3T3-L1 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% Bovine Calf Serum (Invitrogen) in 5% CO_2. Two days post confluence, cells were exposed to DMEM 10% Fetal Bovine Serum (FBS; Invitrogen) with 1 μ M dexamethasone (Sigma), 5 μ g/ml insulin (Sigma), and 0.5mM isobutylmethylxanthine (Sigma). After 2 days, cells were maintained in medium containing FBS only. For IRF4 regulation experiments, fully differentiated 3T3-L1 adipocytes were incubated in serum-free DMEM containing 1% fatty acid-

free BSA (Sigma) with isoproterenol (10μM, (Sigma) and IL-21 (100 ng/ml) (R&D) at the doses and times indicated.

Western blot. Preparation of tissue lysates, quantification and immunoblot analysis were performed as previously described (33). Antibodies to IRF4, actin and total FoxO1 (Santa Cruz), phospho-Ser473 Akt ,total Akt, phospho-Ser256 FoxO1 (Cell Signaling Technology) were used.

Gene expression analysis by qRT-PCR. Total RNA was isolated and gene expression analysis were performed as previously described (34).

Flow cytometry analysis. Cells from the SVF of adipose tissue were stained for surface antigens CD4, and CD25 and for the intracellular $Foxp3^+$ transcription factor by using a mouse Regulatory T-Cell Detection Kit, as directed by the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). For intracellular lipids, cells were stained with Nile red (Sigma-Aldrich) (1μg/ml). Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) and FlowJo software.

Human study. 207 adipose tissue samples (112 visceral and 95 subcutaneous) were collected at the Endocrinology Service of the Hospital Universitari Dr. Josep Trueta (Girona, Spain) from a group of Caucasian subjects with body mass index (BMI) between 20 and 58 kg/m^2. All subjects reviewed that their body weight had been stable for at least 3 months before the study and gave written informed consent after the purpose, nature and potential risks of the study were explained to them.

Adipose tissue samples were obtained from subcutaneous and visceral depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia, and gastric by-pass surgery), washed,

fragmented and immediately flash-frozen in liquid nitrogen before be stored at -80ºC and used for gene expression analysis.

Statistical analysis. Results of the experimental studies are expressed as means ± SD. Statistical analyses were performed using the unpaired Student *t* test as indicated. Values of $P< 0.05$ were considered statistically significant.

Results

IL-21/IL-21R and T_{reg} cells are increased in obese adipose tissue.

Recent study demonstrated that T_{reg} cells with a unique phenotype were highly enriched in the abdominal fat of lean mice, but were strikingly and specifically reduced at this site in insulin-resistant models of obesity (13). It is also reported that IL-21 can counteract the immune-suppressive properties of T_{regs} in several types of tissues both *in vitro* and *in vivo* (27,35). In order to determine whether this IL-21 effect on T_{reg} cells could be extended to those residing in the adipose tissue, we firstly studied the amount of regulatory T cells, marked as CD4+CD25+Foxp3+, in the stromal vascular fraction of IL-21 KO mice finding a significant increase when compared to WT littermates (Figure 1A). Next, in visceral (epigonadal) adipose tissue of WT mice fed a HFD for 16 weeks compared to WT fed a normal diet we found a significant increase of both IL-21 and IL-21R mRNA expression (Figure 1B). More in detail we observed an augment of IL-21 and IL-21R expression both in adipocyte and stromal vascular fractions although not significant (Figure 1C). We also found IL-21 and IL-21R gene expression in fully differentiated 3T3-L1 adipocytes (Figure 1D). This suggested an association between increased IL-21/IL-21R signaling and the progression of obesity.

Metabolic effect of diet-induced obesity on IL-21 KO mice.

Next, in order to understand the effects of this cytokine on diet induced obesity, we conducted a complete metabolic characterization of IL-21 KO mice under ND and HFD conditions in order to understand the effect of this cytokine on the diet induced obesity condition. IL-21 KO mice fed normal diet did not show differences in body weight and fasting plasma glucose levels when compared to WT mice (Figure 2A). We fed 6- to 7-wk-old WT and IL-21 KO mice in a context of high fat diet for 18 weeks. Fasting and fed body weight and fasting glycemia were comparable at the beginning of treatment but their curves significantly diverged from week 5 to the end of our observation at week 18

(Figure 2B). Intraperitoneal glucose tolerance test (IPGTT), intraperitoneal insulin tolerance test (IPITT) and serum insulin levels suggested that metabolic control was improved, on an HFD, by IL-21 deficiency (Figure 2C-D). The relief from diet-induced insulin resistance was finally confirmed through the measurement of peripheral (skeletal muscle) insulin sensitivity by the euglycemic hyperinsulinemic clamp (Figure 2E).

Effect of IL-21 knockout on adipose tissue morphology and function during diet induced obesity.

Afterwards, we conducted a morphological and molecular characterization of adipose tissue in order to better understand the mechanism by which IL-21 deficiency protect from metabolic injury caused by diet induced obesity. IL-21 KO mice fed a normal diet did not show significant differences in adipose tissue morphometry compared to WT mice littermates (Supplementary Figure 1A). On the other hand the IL-21 KO reduced body weight, observed with HFD, was characterized by lower adiposity associated with decreased fat pad mass (Figure 3A), reduced adipocyte size and a higher density of smaller adipocytes (Figure 3B).

Gene expression analysis of adipose tissue revealed significantly reduced levels of macrophage markers such as F4/80 and CD68 in IL-21 KO mice. This may indicate a low grade of infiltration of proinflammatory macrophages. On the other hand, we have found increased levels of YM1 and Mgl2 mRNA, suggesting a high presence of alternatively activated macrophages M2 (Figure 3C top). Next, we analyzed genes involved in the regulation of glucose/lipid metabolism and mitochondrial function finding significantly increased levels of adiponectin, FoxO1, SOCS3, Sirt1, ERRα and Nrf1 (Figure 3C bottom). The improved metabolic state of IL-21 KO mice was also supported by increased phosphorylation of Ser473Akt in the refeeding condition (Figure 3D).

Expression of IRF4 in adipose tissue from IL-21 KO and WT mice.

In adipose tissue, fasting induces IRF4-dependent lypolisis and insulin, during refeeding, inhibits its expression via AKT/FoxO1. In adipose tissue of IL-21 KO mice, despite higher AKT phosphorylation (Figure 3D) we observed significantly increased expression of IRF4 in the refeeding state at both mRNA and protein levels (Figure 4A). In the fasting state, we found increased expression of IRF4 only in adipose tissue from IL-21 KO mice fed a HFD compared to WT. Expression of IRF4 targets *pnpla2* and *lipe* confirmed increased expression of both lipolytic genes particularly in the refeeding state (Figure 4A). To control that this nutritional effect was specific for adipose tissue we measured IRF4 expression in spleen from the same mice finding no differences (Figure 4C). Analysis of NEFA and glycerol in fasting sera confirmed a trend to increased lipolysis in IL-21 KO compared with WT littermates during both ND and HFD (Figure 4D).

Effect of IL-21 on IRF4 expression in 3T3-L1 adipocytes and in SVFs.

IRF4 mRNA expression rose significantly in 3T3-L1 adipocytes treated with isoproterenol for two hours and decreased when adipocytes were pretreated with IL-21. Accordingly we found decreased mRNA levels of IRF4 targets when the treatment was extended to 4 hours (Figure 5A).

A recent study demonstrates that IRF4 promotes M2 polarization of adipose tissue macrophages (14). In SVFs from IL-21 KO adipose tissue we found significant increased IRF4 and M2 markers mRNA expression (Figure 5B). M2 macrophages and T_{reg} cells are known to prevalently use fatty acids for ATP generation to maintain their functions (36). Recently, PPAR-γ was highlighted as a crucial molecular orchestrator of VAT T_{regs} and M2 macrophages accumulation, phenotypes and functions (17). We found increased levels of PPAR-γ mRNA in SVF of IL-21 KO HFD compared to WT. The profile of adiponectin mRNA expression provides evidence of the purity of the fraction preparations (Figure 5B).

Effect of IL-21 deficiency on VAT T_{regs} during HFD.

To determine whether the degree of infiltration of regulatory T cells in the adipose tissue in our knockout model could be influenced by a treatment inducing obesity treatment, we quantified by flow cytometry the number of T_{reg} cells in SVFs of the two experimental groups at the end of 18 weeks of HFD. As well as for the animal knockout fed a normal diet, the amount of regulatory T cells present in the adipose tissue of HFD IL-21 KO mice was significantly higher than the equivalent WT animals (Figure 6A). Comparative analysis of T_{regs} in SVF of IL-21 KO and WT fed a normal or a HFD confirmed that obesity condition reduced T_{regs} in SVF of WT mice. It is interesting to note that IL-21 KO animals fed HFD maintained a high number of T_{regs} comparable to that of WT fed normal diet (Figure 6B). Noteworthy, loss of IL-21 is associated to increased lipolysis, increased T_{regs} and M2 macrophage markers suggesting that a state in which IL-21 is reduced is possibly associated to increased lipids uptake from anti-inflammatory cells such T_{regs} and M2 macrophages. Interestingly, we found increased lipid content in T_{reg} cells from IL-21 KO compared to WT (Figure 6C).

Reduced liver steatosis in IL-21 KO subjected to obesity challenge.

The overall improvement of glucose tolerance was associated to absence of liver steatosis in IL-21 KO compared with WT mice during high fat diet (Figure 7A), that was associated to reduced inflammatory markers such as F4/80, CD68 and an unexpected mild increase in gluconeogenic enzymes such as *Pck1* and *G6pc* (Figure 7B). Consistently we found reduced Ser256 phosphorlation of FoxO1 in the fasting IL-21 KO liver (Figure 7C). IL-21 KO mice during HFD revealed a marked tendency to lower fasting glucose compared to WT littermates, with no differences in glucagon levels (Supplementary Figure 2A). This suggests that the slight increase in gluconeogenic enzymes is a reactive response to maintain glucose at physiological levels. The concept of reactive response is also supported by the

intraperitoneal pyruvate tolerance test showing increased glucose levels in IL-21 KO mice fed HFD (Fig. 7D). Furthermore, we analyzed *Pck1* and *G6pc* expression also in livers from HFD re-fed mice and at the end of euglycemic hyperinsulinemic clamp (Supplementary Figure 2B); overall the data suggest that during fasting or intense glucose uptake from the muscle the absence of IL-21 increases *Pck1* expression possibly to compensate lower peripheral glucose level.

IL-21R expression in adipose tissue from human subjects with obesity and glucose intolerance.

To explore the involvement of IL-21 effects on human adipose tissue inflammation we analyzed its expression in adipose tissue biopsies from patients with different degrees of obesity (Supplementary Table 1). We found a significant negative correlation between IL-21R and the CD206/CD68 ratio expression; this ratio is known to be higher in subjects with less body fat and lower fasting glucose concentrations (37). Moreover PPAR-γ was also found to be significantly and negatively correlated to IL-21R expression in subcutaneous adipose tissue of obese subjects (Table 1). On the other hand we found a significant positive correlation between IL-21R and TNFα both in visceral and subcutaneous adipose tissue (Table 1) indicating that exists an involvement of IL-21 signaling in the development or persistence of adipose tissue inflammation.

Discussion

IRF4 expression is highly restricted to immune cells and adipose tissue and is more abundant in mature adipocytes (38). IRF4 is nutritionally regulated by the action of insulin and FoxO1, and plays a significant role in the transcriptional regulation of lipid handling in adipocytes, promoting lipolysis. Interestingly we found a strong relationship between IRF4 and ADRP gene expression, a lipolytic gene, in human visceral adipose tissue ($r = 0.47$, $p<0.0001$, data not shown). During fasting, in adipocytes, mRNA and protein levels of IRF4 raise dramatically with subsequent down regulation after refeeding. IRF4 promotes lipolysis at least in part by inducing the expression of the lipases ATGL and HSL (15). We measured in adipose tissue of IL-21 KO mice fed a ND high levels of expression of the transcription factor IRF4 and its targets *lipe* (HSL) and *pnpla2* (ATGL), particularly in the refeeding conditions. Interestingly IRF4 expression is repressed in whole VAT of three different rodent models of obesity, an hyperinsulinemic state (15). This may seem paradoxical given that obesity is associated with insulin resistance, and mice lacking insulin receptors in fat display elevated *Irf4* expression (15). We found large induction of IRF4 and its targets also in HFD IL-21 KO adipose tissue both in fasting and in refeeding state. Therefore, it remains possible that other factors, such as IL-21, dominate the control of *Irf4* gene expression in the context of obesity. Indeed our *in vitro* studies confirm a role for IL-21 in reducing IRF4 and its targets levels during lipolysis. Despite the high levels of IRF4, we unexpectedly found only mildly elevated NEFA levels in fasting sera of IL-21 KO animals. The reason could be attributed to the abundance of M2 macrophages and regulatory T cells residing in the adipose tissue of these animals, immune populations with a strong ability to capture and oxidize fatty acids released by adipocytes (17,36). IRF4 is a well-known player in a variety of immune activities, including regulatory T cell function and the development of inflammatory Th17 cells and is absolutely required for the autocrine production of IL-21 in Th17 cells (39). Recently, IL-21 has emerged as a key cytokine for the maintenance of the mucosal immune system homeostasis by modulating the balance

between T_{reg} cells and pro-inflammatory Th17 cells (28). Interestingly, the frequencies of T_{regs} and Th17 cells often show an inverse relationship, as their differentiation processes are also counterbalanced (40). It is worth to note that the *in vivo* presence of Th17 T cells in adipose tissue under normal chow conditions or a HFD has not yet been extensively reported. Few recent observations demonstrate that diet induced obesity predisposes to IL-6-dependent Th17 expansion in adipose tissue (41). Given that IL-21 induces and amplifies Th17 development independently of IL-6 (39) it is possible that IL-21 is secreted in specific phases of adipose tissue expansion eventually exacerbating early disease progression.

T_{reg} cells with a unique phenotype were highly enriched in the abdominal fat of lean mice, but their numbers were strikingly and specifically reduced at this site in insulin-resistant models of obesity (11). Recent studies reveal an important role for VAT-specific natural T_{reg} cells in the suppression of obesity-associated inflammation in VAT and consequently in reducing insulin resistance .The number of VAT T_{reg} cells decreases with obesity and a boost in the number of these cells in obese mice can improve insulin sensitivity (11,13). T_{reg} cells expressing Foxp3 can secrete anti-inflammatory signals such as IL-10 and TGFβ, inhibit macrophage migration and induce M2-like macrophage differentiation (11). IL-21-mediated T cell activation relies partly on its ability to inhibit the differentiation of regulatory T cells, and to make T cells resistant to the T_{reg}-mediated immunosuppression (27,28). In SVF of IL-21 knockout adipose tissue we found a significant increase in the number of resident regulatory T cells in both mice fed a normal diet than in those receiving high-fat diet. This may suggest the possibility that IL-21 regulates T_{regs} number and differentiation even in the adipose tissue. What causes the decrease in T_{regs} fraction in abdominal adipose tissue during obesity is still undefined, nevertheless recent reports indicate a possible role for the hyperleptinemic state characterizing obesity. to modulate T_{regs} number and activity. (42,43). Thus an hypothesis that needs further exploitation in appropriate models is that leptin and IL-21 share some biological function and cooperate in causing

T_{regs} dramatic reduction in adipose tissue during obesity development. Since the adipose tissue of IL-21 KO mice is characterized by a lower degree of macrophage infiltration and increased expression of M2 polarization antigens (YM1, Mgl2), it is intriguing to hypothesize that IL-21/T_{regs} axis might regulate the balance between macrophage M2 polarization and M1 infiltration in the context of obese adipose tissue. A recent study shows that VAT-resident T_{regs} and M2 macrophages specifically express the peroxisome proliferator-activated receptor (PPAR-γ), an important factor controlling their accumulation, phenotype and function (17,36). Consistently, we measured significant increased levels of PPAR-γ and M2 markers in SVF of IL-21 KO mice. In this context, in humans we found a negative association between IL-21R and PPAR-γ gene expression in SAT suggesting that IL21 signalling runs in parallel to PPAR-γ. A newly discovered property was that in VAT, but not in lymphoid tissue, T_{regs} can take up lipids, an ability not shared by conventional T cells residing at the same site (17). It is also known that M2 macrophages and T_{reg} cells prevalently use fatty acids for ATP generation to maintain their functions (36). In IL-21 KO mice we observed an high induction of IRF4 related lipolysis and at the same time increased lipid uptake by T_{regs}. This highlights the possibility that IL-21 regulates T_{regs} activity in adipose tissue.

In conclusion we relate for the first time the IL-21/IL-21R dyad to IRF4 dependent regulation of lipolysis and reduction of T_{reg} cells in adipose tissue. We hypothesize that IL-21 is a crucial player in this context since we found an increase in mRNA levels of IL-21 and its receptor (IL-21R) in adipose tissue of obese animals and obese human subjects compared to their lean controls.

Our data suggest that preventing IL-21 signaling might counteract obesity and the consequent metabolic defects in experimental model, a finding with potential therapeutic implications in human subjects with metabolic syndrome and Type 2 Diabetes Mellitus.

Acknowledgements

This manuscript was funded in part by Fondazione Roma 2008, ESFD/Lilly 2012, AIRC 2012 Project IG 13163, Fondazione Roma 2008, FP7-Health-241913 FLORINASH, FP-7 Health-EURHYTHDIA, and PRIN 2012 (all to M.F.). A.G. has received support by grants from Università Cattolica del Sacro Cuore (Fondi Ateneo Linea D.3.2 Sindrome Metabolica), from the Italian Ministry of Education, University and Research (PRIN 2010JS3PMZ_011) and from Fondazione Don Gnocchi, Milan,Italy. TM is the recipient of the Albert Reynolds Travel Fellowship by European Association for the Study of Diabetes and Fellowship Prize by the Società Italiana di Diabetologia. GPS is the recipient of fellowship from Laboratori Guidotti, Italy.

M. Fabrizi performed experiments, analyzed data, drafted the manuscript and wrote the final version of the manuscript. V.M. performed experiments, analyzed data and reviewed the manuscript. M.M, A.M., V.C. M.C., T.M., G.P.S. and A.G. performed experiments and analyzed data. J.M.M.N. performed experiments. L.F. and RM contributed to the discussion and drafted the manuscript. G.M. and R.L. contributed to the discussion and edited the manuscript. J.M.F.R, analyzed data and reviewed the manuscript. M. Federici drafted the manuscript, wrote the final version of the manuscript and is the guarantor of this work and such as, had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of data analysis.

Authors do not have potential conflict of interest relevant to the content of this manuscript.

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Figures and legends

Figure 1.*T_{reg} cells are increased in SVF of IL-21 KO mice and IL-21/IL-21R genes are increased in obese adipose tissue.* (A) Cells from epigonadal fat stromal vascular fraction (SVF) were stained and analyzed by flow cytometry. Tregs are defined as CD25+CD4+Foxp3+. Tregs from WT and IL-21 KO mice. Left, representative dot plots; right, summary data. Numbers on dot plots indicate the percentage of cells in that gate for that particular experiment. (n= 4 per group, *p<0.05, Student *t* test. Error bars represent the mean ± SD). (B) mRNA expression of IL-21 and IL-21R in epigonadal adipose tissue of WT mice fed ND or HFD. (C) mRNA expression of Adiponectin, (considered adipocyte markers) IL-21 and IL-21R in adipocyte and stromal vascular fraction from epigonadal adipose tissue of WT mice fed ND or HFD (n= 6 per group, *p <0.05, Student *t* test. Error bars represent the mean ± SD). (D) mRNA expression of IL-21 and IL-21R in 3T3-L1 cells under basal and starving condition (n=3 per group, *p <0.05, Student *t* test. Error bars represent the mean ± SD).

Figure 2. *Metabolic effect of IL-21 deficiency on diet induced obesity.* At 6 weeks of age, WT and IL-21 KO mice were fed a ND or HFD for 18 weeks. (A) Body weight curves and blood glucose level of WT and IL-21 KO mice fed ND in the fasting conditions. (B) Fasting body weight curves and fasting glucose levels during HFD. (C) Intraperitoneal glucose and insulin tolerance tests and relative AUC(n= 7 per group, *p<0.05, **p<0.01, ***p<0.001 Student *t* test (D) Insulin levels measured during IPGTT (n= 5 per group, ***p<0.001 Student *t* test. Error bars represent the mean ± SD) (E) Glucose uptake in WT and IL-21 KO mice fed HFD. Glucose infusion rates (GIR) during the euglycemic hyperinsulinemc clamp (n= 4 WT vs 6 KO, (**p<0.0025 Student *t* test. Error bars represent the mean ± SD).

Figure 3. *Adipose tissue structure and molecular characterization in IL-21 knockout during diet induced obesity.* Epigonadal adipose tissue of IL-21 KO and WT mice after 18 weeks of HFD. (A) Representative images of WT and IL-21 KO mice and respective fat pad after 18 weeks of HFD. (B) Representative sections of adipose tissue stained with hematoxylin-eosin (H/E), mean adipocyte area, frequency distribution of adipocyte area in IL-21 KO mice vs WT littermates. (n= 6 per group; ***p< 0.001, Student *t* test. Data are means ± SD). (C) WAT expression of genes involved in inflammation, metabolism and mitochondrial biogenesis. Expression of mRNA was determined by real-time PCR and normalized to b-actin. (n= 6 per group; *p<0.05 and **p<0.01, *** p<0.001, Student *t* test. Error bars

represent the mean ± SD). (D) Akt phosphorylation (p) in refeeding condition was analyzed by Western blot. (n= 6 per group; *p<0.05, Student t test. Data are means ± SD.) A representative image of four mice per group is shown. a.u., arbitrary unit.

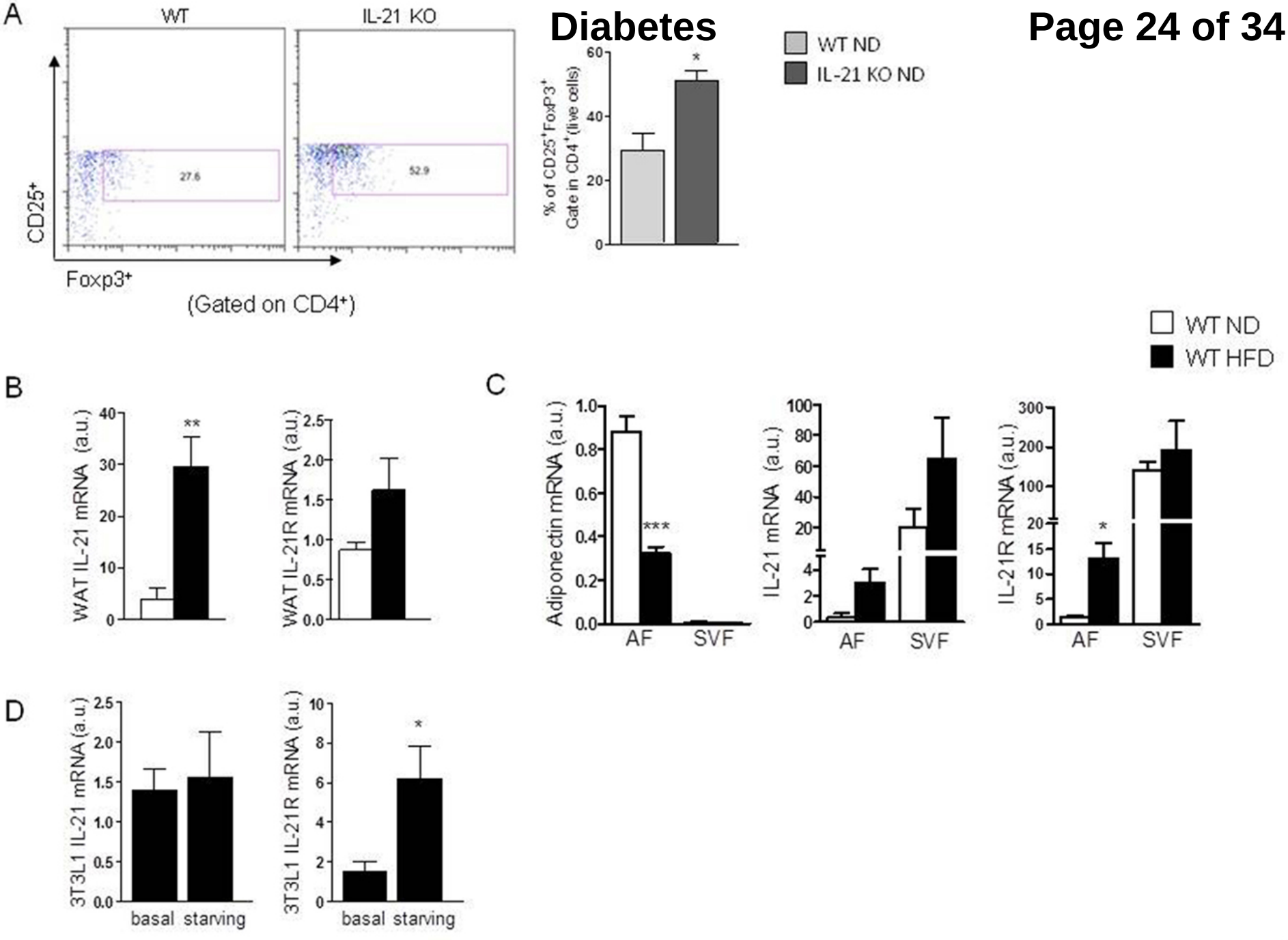
Figure 4. *Lipolysis regulation in IL-21 KO adipose tissue*. (A) Expression of IRF4, *Lipe* and *Pnpla2* mRNA in fasted and refed WT and IL-21 fed ND or HFD (*p<0.05, n= 6 per group). (B) Protein expression of IRF4 in IL-21 KO in both fasting and refeeding conditions in ND and HFD mice (*p<0.05) (C) Spleen mRNA expression of IRF4 in ND and HFD mice (n= 6 per group). (D) Fasting NEFA and glycerol serum levels (*p<0.05 n= 6 per group).

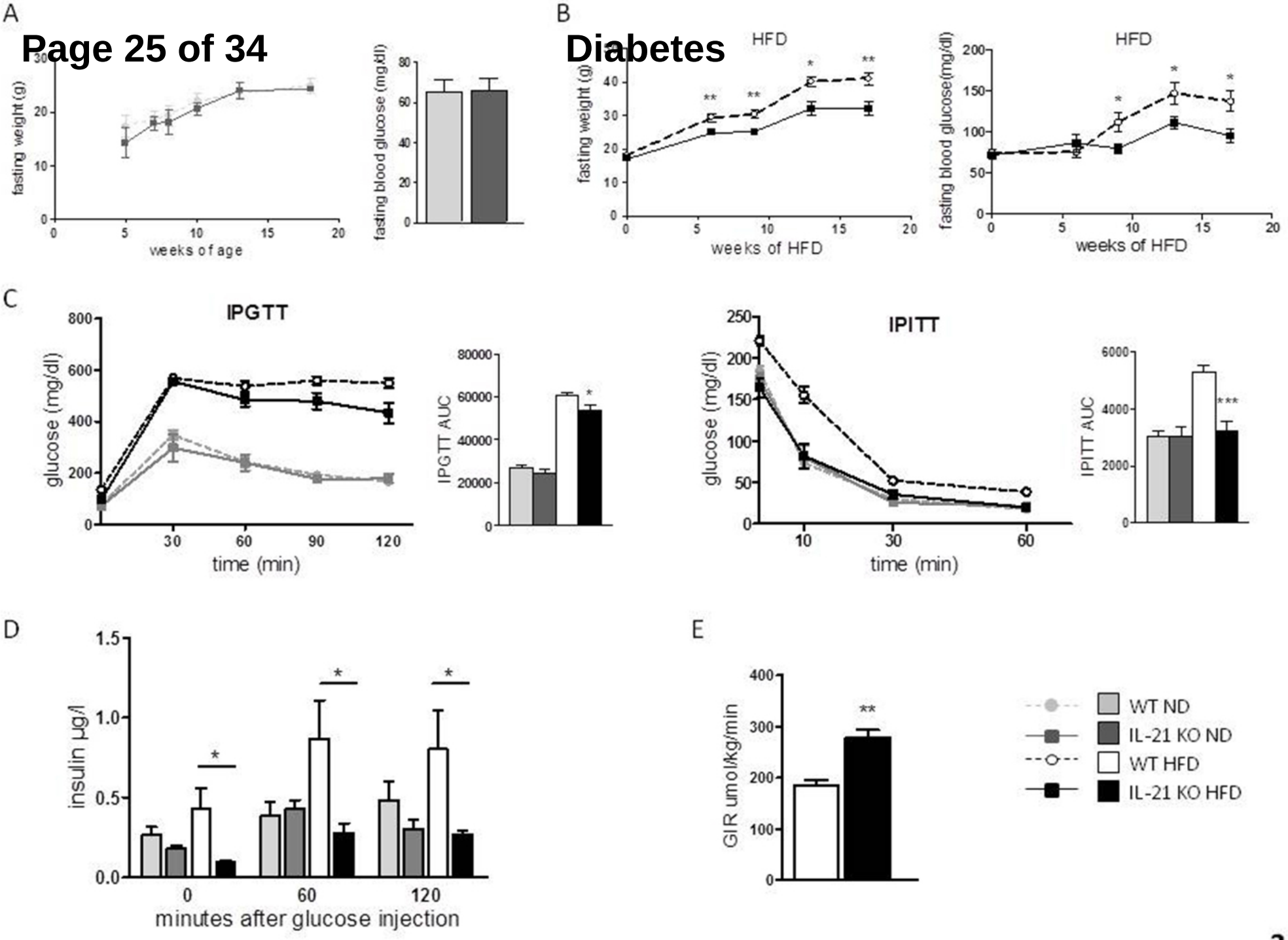
Figure 5. *Regulation of IRF4 in 3T3-L1 adipocytes, adipose and stromal vascular fractions.* 3T3-L1 adipocytes were incubated in serum free DMEM with isoproterenol and IL-21 as indicated. (A) IRF4, Lipe and Pnpla2 mRNA expression after 2 or 4 hours of treatments (^p<0.05; ^^^p<0.001 isoproterenol vs control. *p<0.05; **p<0.01 isoproterenol vs isoproterenol + IL-21). (B) mRNA expression of IRF4, F4/80, YM1 Arg1 and Mgl2 PPAR-γ in SVF from WT and IL-21 KO mice; Adiponectin was used as a marker for AF. (n = 5 per group; *p< 0.05. Student *t* test. Error bars represent the mean ±).

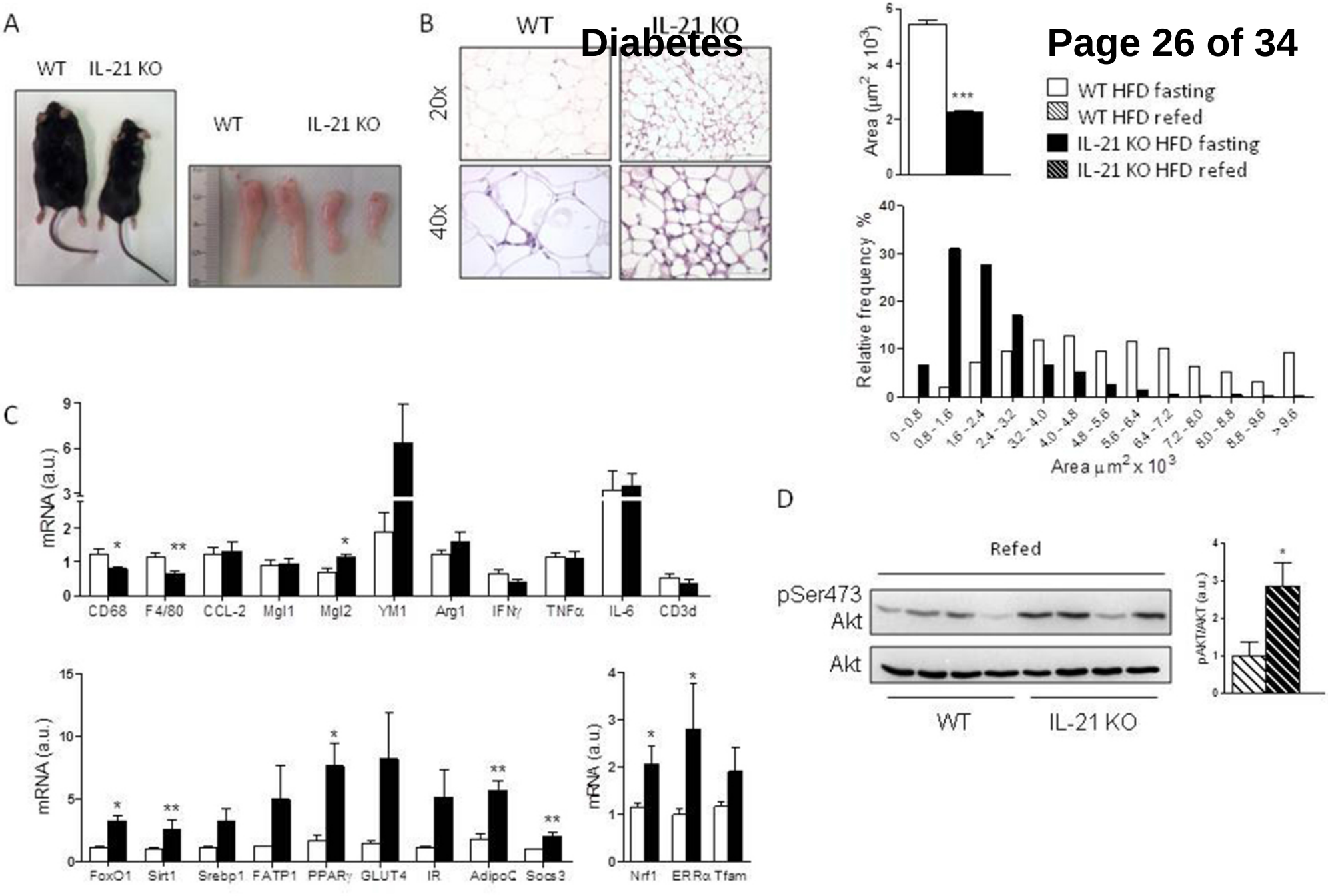
Figure 6. *Effect of IL-21 deficiency on VAT T_{regs} during diet induced obesity.* At 6 weeks of age, IL-21 KO mice and WT littermates were fed HFD for 18weeks. Cells from the epigonadal fat SVF were stained and analyzed by flow cytometry. T_{regs} are defined as CD25+CD4+Foxp3+. (A) Left, representative dot plots; right, summary data (for fraction of CD4+ live cells). Dot plot numbers indicate the percentage of cells in that gate for that particular experiment. (n=4 per group;*p<0.05, **p<0.01. Student *t* test. Error bars represent the mean ± SD).(B) Comparative analysis of T_{regs} in SVF from IL-21 KO and WT fed normal and a HFD for 18 weeks (*p<0.05). (C) Cells were isolated from epigonadal VAT SVFs of WT and IL-21 KO mice fed HFD and stained for CD4+, CD25+, Foxp3+ and Nile red. (n= 5 per group; *P < 0.05. Student *t* test. Error bars represent the mean ± SD).

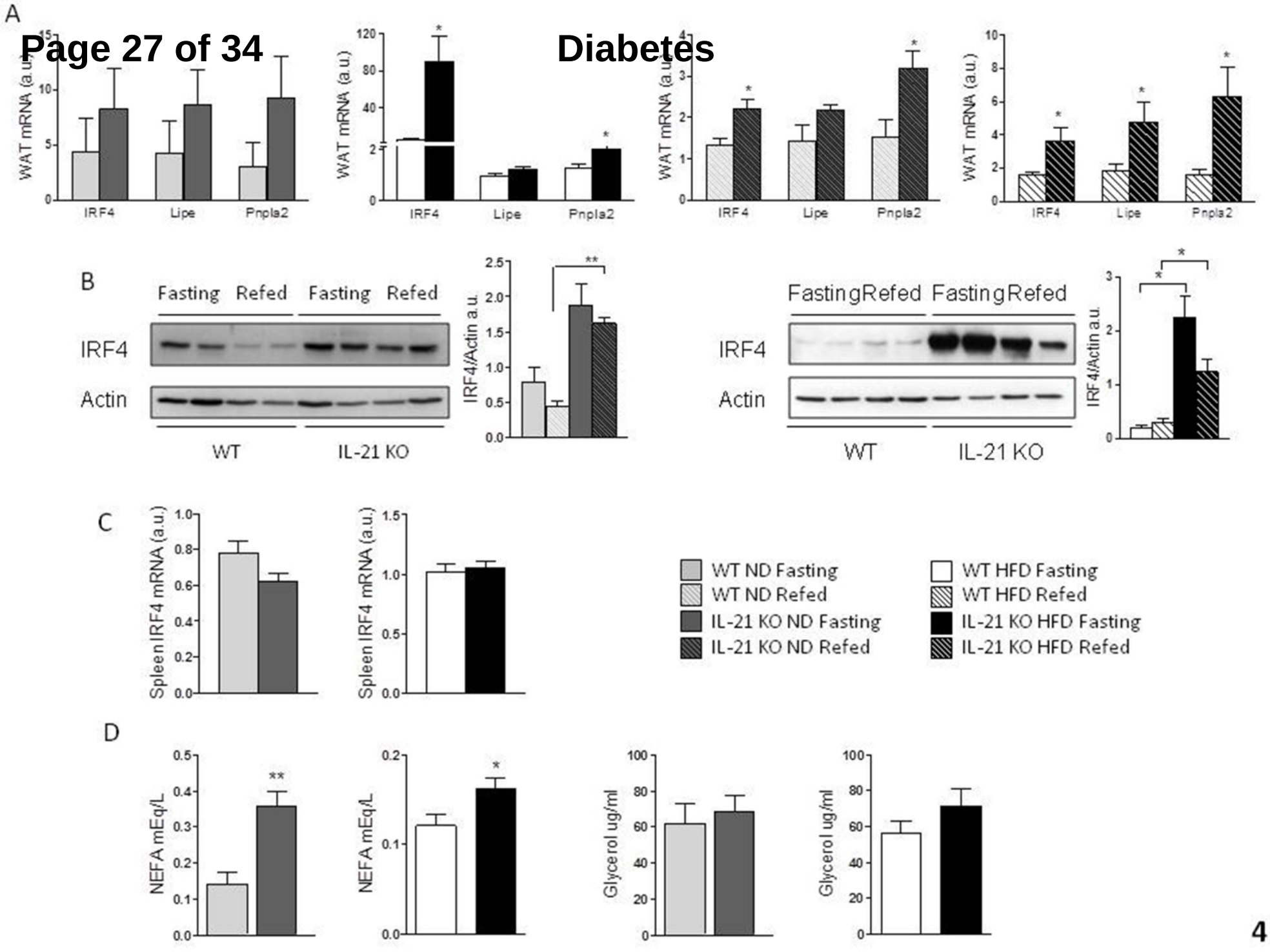
Figure 7. *IL-21 deficiency protects from hepatic steatosis during diet induced obesity*. (A) IL-21 KO do not show macrovescicular steatosis during diet induced obesity. (B) Expression of metabolic and inflammatory genes. Expression of mRNA was determined by real-time PCR and normalized to b-actin. (n= 6 per group; *p<0.05, Student *t* test. Error bars represent the mean ± SD). (C) Ser 473 Akt phosphorylation (p) in refeeding condition and Ser256 FoxO1 phosphorylation (p) in fasting condition

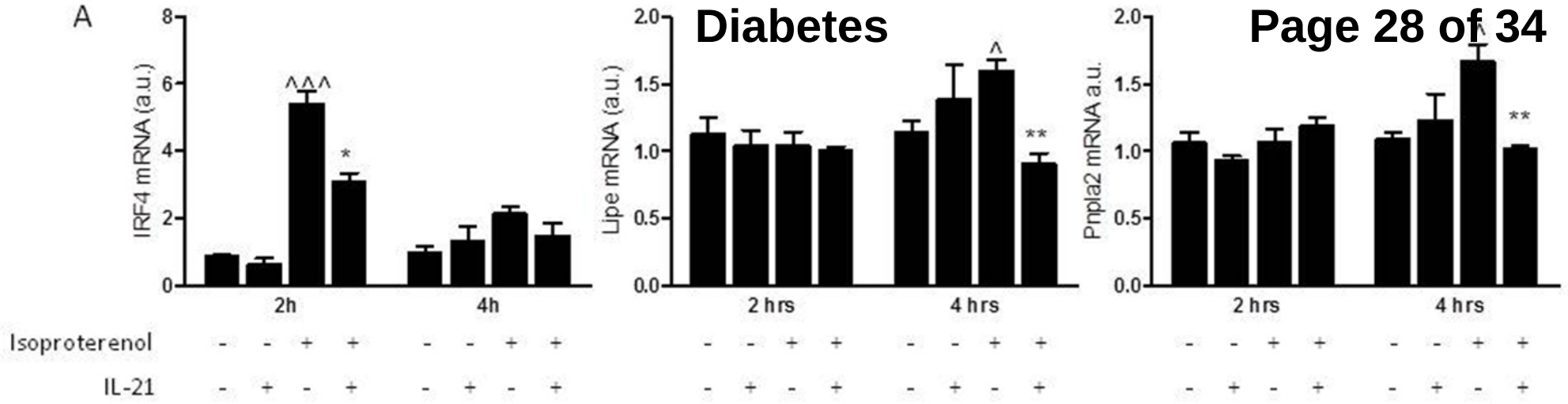
were analyzed by Western blot. (n= 6 per group; *p < 0.05, Student *t* test. Data are means ± SD.) A representative image of four and three mice per group is shown.(a.u., arbitrary unit). (D) intreaperitoneal pyruvate tolerance test (n= 6 per group).

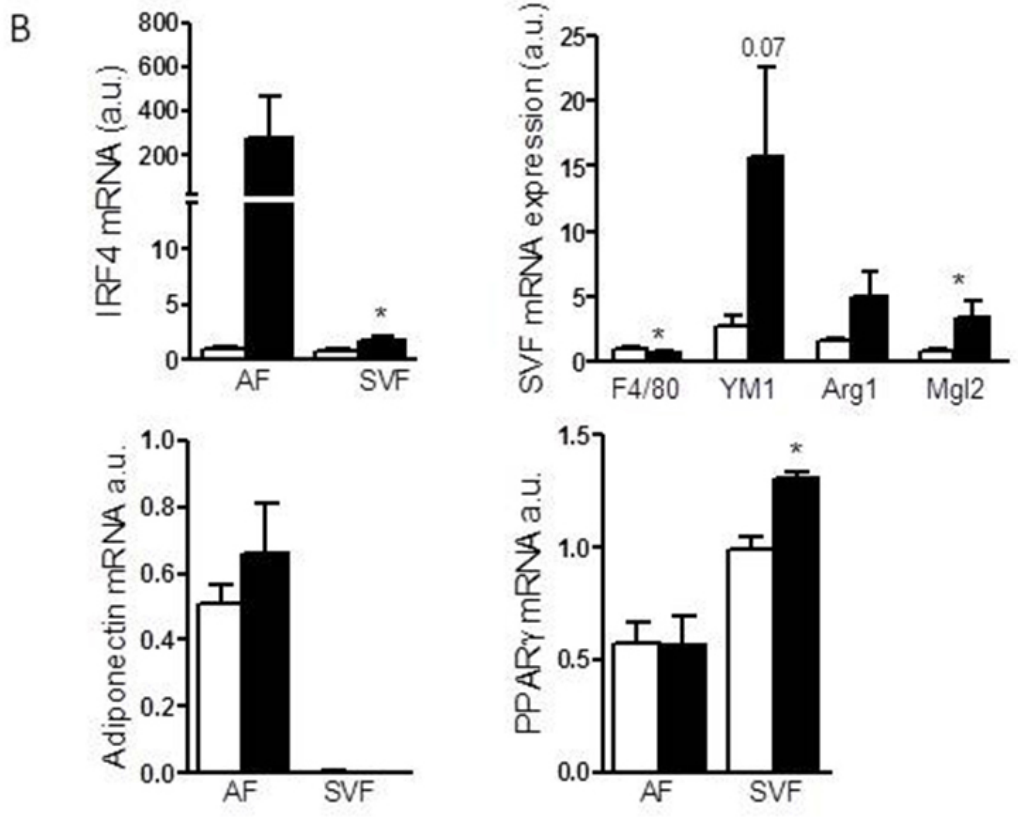

A
WT
IL-21 KO
CD25+
Foxp3+
(Gated on CD4+)
27.6
52.9
% of CD25+FoxP3+ Gate in CD4+ (live cells)
WT ND
IL-21 KO ND
B
WAT IL-21 mRNA (a.u.)
WAT IL-21R mRNA (a.u.)
C
WT ND
WT HFD
Adiponectin mRNA (a.u.)
IL-21 mRNA (a.u.)
IL-21R mRNA (a.u.)
AF
SVF
D
3T3L1 IL-21 mRNA (a.u.)
3T3L1 IL-21R mRNA (a.u.)
basal
starving

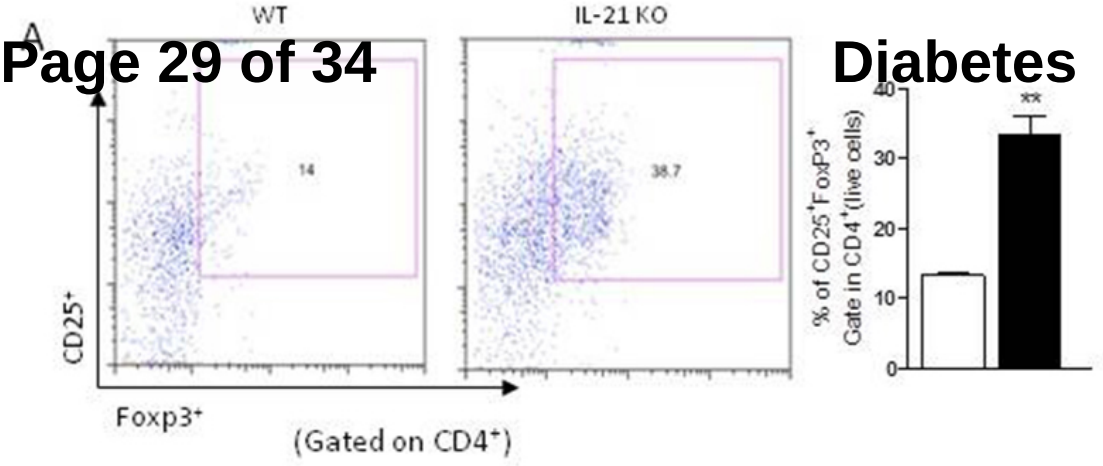

A
fasting weight (g)
weeks of age
fasting blood glucose (mg/dl)
B
HFD
fasting weight (g)
weeks of HFD
HFD
fasting blood glucose(mg/dl)
weeks of HFD
C
IPGTT
glucose (mg/dl)
time (min)
IPGTT AUC
IPITT
glucose (mg/dl)
time (min)
IPITT AUC
D
insulin µg/l
minutes after glucose injection
E
GIR umol/kg/min
WT ND
IL-21 KO ND
WT HFD
IL-21 KO HFD

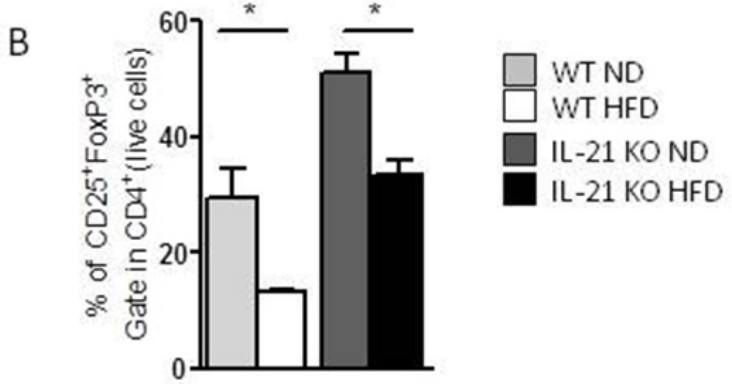

A
WT
IL-21 KO
WT
IL-21 KO
B
WT
IL-21 KO
20x
40x
Area (μm² x 10³)
***
WT HFD fasting
WT HFD refed
IL-21 KO HFD fasting
IL-21 KO HFD refed
Relative frequency %
0 - 0.8
0.8 - 1.6
1.6 - 2.4
2.4 - 3.2
3.2 - 4.0
4.0 - 4.8
4.8 - 5.6
5.6 - 6.4
6.4 - 7.2
7.2 - 8.0
8.0 - 8.8
8.8 - 9.6
>9.6
Area μm² x 10³
C
mRNA (a.u.)
CD68
F4/80
CCL-2
Mgl1
Mgl2
YM1
Arg1
IFNγ
TNFα
IL-6
CD3d
FoxO1
Sirt1
Srebp1
FATP1
PPARγ
GLUT4
IR
AdipoC
Socs3
Nrf1
ERRα
Tfam
D
Refed
pSer473 Akt
Akt
WT
IL-21 KO
pAKT/AKT (a.u.)

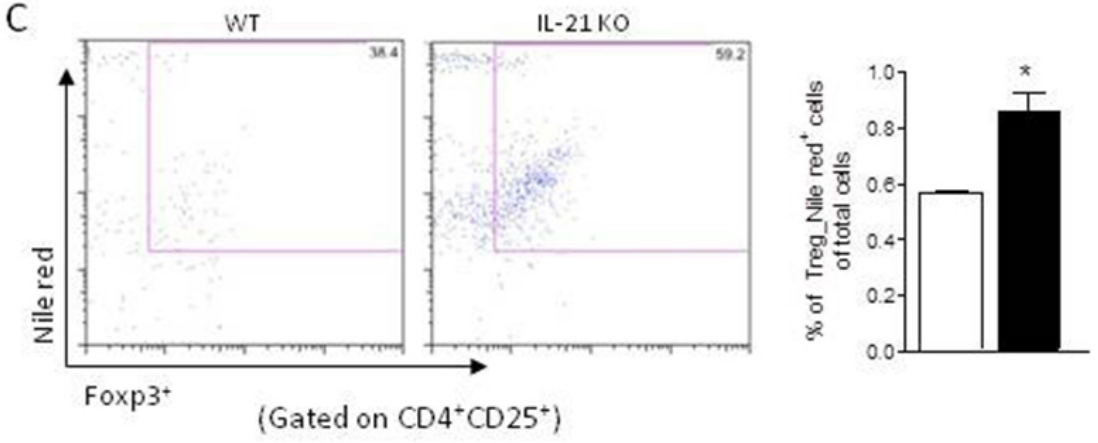

A
WAT mRNA (a.u.)
IRF4
Lipe
Pnpla2
B
Fasting
Refed
Fasting
Refed
IRF4
Actin
WT
IL-21 KO
IRF4/Actin a.u.
C
Spleen IRF4 mRNA (a.u.)
WT ND Fasting
WT ND Refed
IL-21 KO ND Fasting
IL-21 KO ND Refed
WT HFD Fasting
WT HFD Refed
IL-21 KO HFD Fasting
IL-21 KO HFD Refed
D
NEFA mEq/L
Glycerol ug/ml

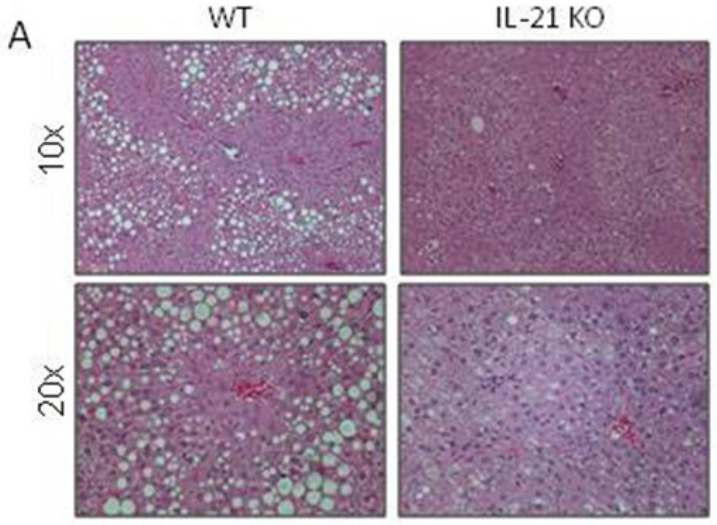

A
IRF4 mRNA (a.u.)
Lipe mRNA (a.u.)
Pnpla2 mRNA a.u.
2h
4h
2 hrs
4 hrs
Isoproterenol
IL-21

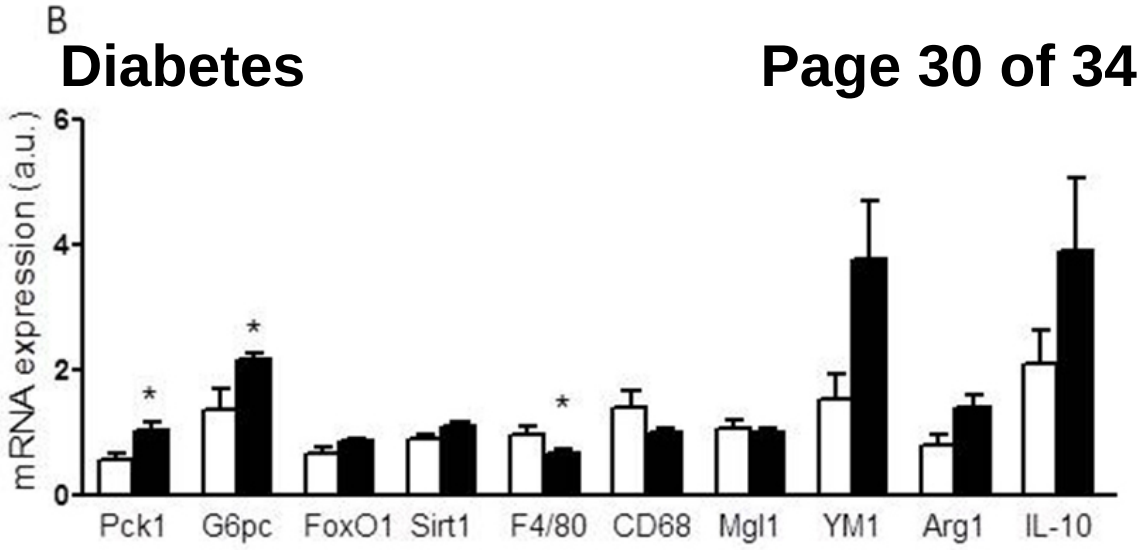

B
IRF4 mRNA (a.u.)
AF
SVF
SVF mRNA expression (a.u.)
0.07
F4/80
YM1
Arg1
Mgl2
Adiponectin mRNA a.u.
PPARγ mRNA a.u.


A
WT
IL-21 KO
14
38.7
CD25+
Foxp3+
(Gated on CD4+)
% of CD25+FoxP3+ Gate in CD4+(live cells)
**

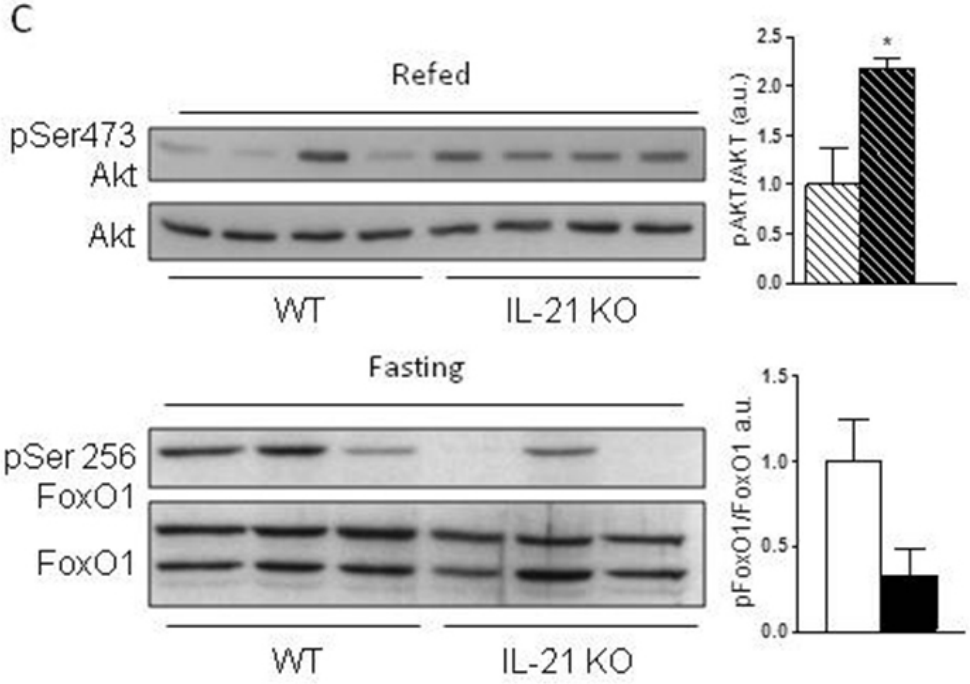

B
% of CD25+FoxP3+ Gate in CD4+(live cells)
*
*
WT ND
WT HFD
IL-21 KO ND
IL-21 KO HFD


C
WT
IL-21 KO
38.4
59.2
Nile red
Foxp3+
(Gated on CD4+CD25+)
% of Treg_Nile red+ cells of total cells
*


A
WT
IL-21 KO
10x
20x


B
mRNA expression (a.u.)
0
2
4
6
Pck1
G6pc
FoxO1
Sirt1
F4/80
CD68
Mgl1
YM1
Arg1
IL-10


WT HFD Fasting
IL-21 KO HFD Fasting
WT HFD Refed
IL-21 KO HFD Refed


C
Refed
pSer473 Akt
Akt
WT
IL-21 KO
pAKT/AKT (a.u.)
Fasting
pSer 256 FoxO1
FoxO1
WT
IL-21 KO
pFoxO1/FoxO1 a.u.

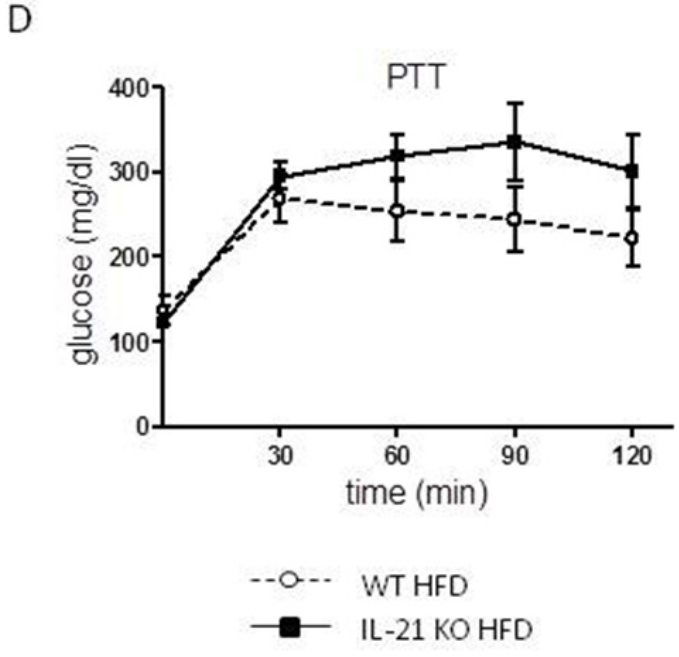
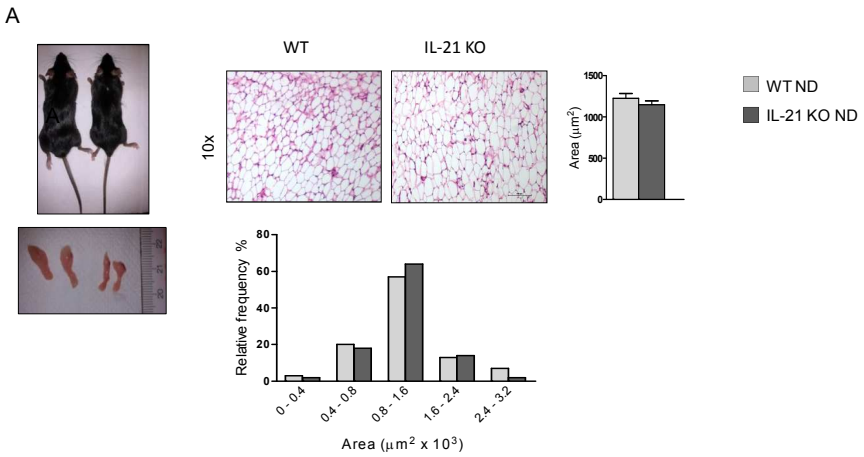

D
PTT
glucose (mg/dl)
time (min)
WT HFD
IL-21 KO HFD

Table 1. *IL-21R is positively correlated to inflammatory factors in human adipose tissue*. Bivariate correlation between IL-21R and anthropometric, clinical parameters as well as adipose tissue gene expression in human adipose tissue biopsies (n= 207). Expression of mRNA was determined by Real Time PCR and normalized to cyclophilin A (PPIA). Bivariate correlation was performed using non-parametric (Spearman) tests. VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; R.U., relative units of gene expression.

	VAT (n= 112)		SAT (n=95)	
	r	**p**	**r**	**p**
Age (years)	-0.11	0.2	-0.11	0.2
BMI (kg/m^2)	0.07	0.4	0.22	**0.03**
FAT MASS (%)	0.10	0.3	0.18	0.08
Fasting glucose (mg/dl)	0.08	0.4	-0.01	0.9
Total Cholesterol (mg/dl)	-0.021	0.8	-0.20	0.06
HDL-Cholesterol (mg/dl)	-0.07	0.4	-0.18	0.08
Fasting triglycerides (mg/dl)	0.20	**0.04**	0.5	0.6
***PPARγ* (R.U.)**	0.10	0.4	-0.31	**0.01**
***FASN* (R.U.)**	-0.25	**0.01**	-0.19	0.08
***ACC1* (R.U.)**	-0.16	0.08	-0.25	**0.02**
***TNFα* (R.U.)**	0.47	**<0.0001**	0.24	**0.02**
***CD206/CD68* (R.U.)**	-0.17	0.1	-0.26	**0.02**

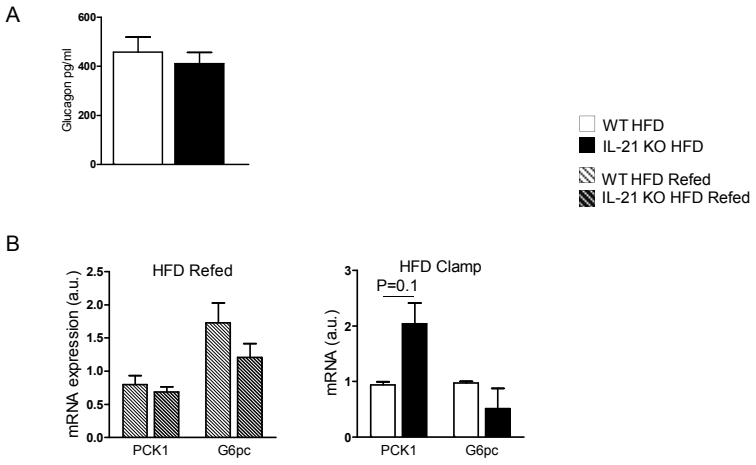
Supplementary Table 1. Anthropometrical and clinical parameters

N = 207	Non-obese	Obese	p
Age (years)	52.9 ± 13.4	44.2 ± 10.1*	0.001
BMI (kg/m^2)	26.1 ± 2.5	44.6 ± 6.9*	<0.0001
Fat mass (%)	32.6 ± 5.8	55.8 ± 10.2*	<0.0001
Fasting glucose (mg/dl)	92.5 ± 10.9	114.8 ± 50.3	<0.0001
Total Cholesterol (mg/dl)	213.1 ± 40.9	191.1 ± 32.3*	0.001
HDL-Cholesterol (mg/dl)	58.6 ± 17.5	57.6 ± 34.2	0.8
Fasting triglycerides (mg/dl)	121.8 ± 62.9	127.4 ± 88.2	0.8



S1

Supplementary Figure 1.
Epigonadal adipose tissue of IL-21 KO and WT mice fed normal diet (A) Representative images of WT and IL-21 KO mice and respective fat pad. Representative sections of adipose tissue stained with hematoxylin-eosin (H/E), mean adipocyte area, frequency distribution of adipocyte area in IL-21 KO mice vs WT littermates. (n= 6 per group; Student *t* test. Data are means ± SD).



Supplementary Figure 2.
(A) Fasting glucagon levels measured after 18 weeks of HFD in IL-21 KO and WT mice. (B) mRNA liver expression of *PCK1* and *G6pc* at the end of 18 weeks of HFD, in refeeding condition and at the end of euglycemic hyperinsulinemic clamp. (n=4 per group. Student *t* test. Error bars represent the mean ± SD)