

Paradoxical resistance to high fat diet-induced obesity and altered macrophage polarization in mineralocorticoid receptor overexpressing mice.

Emmanuelle Kuhn^{1,2}, Christine Bourgeois³, Vixra Keo^{1,2}, Say Viengchareun^{1,2}, Adeline Muscat⁴, Geri Meduri^{1,2}, Damien Le Menuet^{1,2}, Bruno Fève^{4,5}, Marc Lombès^{1,2,6}

¹ Inserm U693, Le Kremlin-Bicêtre, F-94276, France;

²Univ Paris-Sud, Faculté de Médecine Paris-Sud, UMR-S693, Le Kremlin-Bicêtre, F-94276, France ;

³ Inserm U1012, Le Kremlin-Bicêtre, F-94276, France ;

⁴ UMR-S938, Faculté de Médecine, Univ Pierre et Marie Curie, Paris F-75012, France;

⁵Assistance Publique-Hôpitaux de Paris, Hôpital Saint Antoine, Service d'Endocrinologie, Diabétologie et d'Endocrinologie de la Reproduction, Paris F-75012, France;

⁶ Assistance Publique-Hôpitaux de Paris, Hôpital de Bicêtre, Service d'Endocrinologie et des Maladies de la Reproduction, Le Kremlin Bicêtre F-94275, France

*Corresponding author: Dr Marc LOMBES, MD, PhD

Tel: 33 1 49 59 67 02

E-mail address: marc.lombes@u-psud.fr

Running title: MR overexpression and adipocyte differentiation

ABSTRACT

The mineralocorticoid receptor (MR) exerts pro-adipogenic and anti-thermogenic effects *in vitro*, yet its *in vivo* metabolic impact remains elusive. Wild type (WT) and transgenic (Tg) mice overexpressing human MR were subjected to standard chow (SC) or high fat diet (HFD) for 16 wks. Tg mice had a lower body weight gain than WT animals, and exhibited a relative resistance to HFD-induced obesity. This was associated with a decrease of fat mass, an increased population of smaller adipocytes, and an improved glucose tolerance compared to WT animals. Quantitative RT-PCR studies revealed decreased expression of PPAR γ 2, a master adipogenic gene, and of glucocorticoid receptor and 11 β -hydroxysteroid dehydrogenase type1, consistent with an impaired local glucocorticoid signaling in adipose tissues (AT). This paradoxical resistance to HFD-induced obesity was not related to an adipogenesis defect since differentiation capacity of Tgpreadipocytes isolated from stroma-vascular fractions was unaltered, suggesting that other non-adipocyte factors might compromise AT development. While AT macrophage infiltration was not different between genotypes, Tg mice exhibited a distinct macrophage polarization as revealed by FACS analysis and CD11c/CD206 expression studies. We further demonstrated that Tg macrophage-conditioned medium partially impaired preadipocyte differentiation. We propose that the modification of M1/M2 polarization of MR overexpressing macrophages could account in part for the metabolic phenotype of Tg mice. Collectively, our results provide evidence that MR exerts a pivotal immunometabolic role by directly controlling adipocyte differentiation processes but also indirectly through macrophage polarization regulation. Our findings should be taken into account for the pharmacological treatment of metabolic disorders.

Key words: adipocyte, glucocorticoid signaling, energy homeostasis, immunometabolism, mineralocorticoid receptor

INTRODUCTION

The obesity pandemic of our industrialized countries is related to dramatic changes in nutritional and physical activities during the last decades. More recently, it was reported that obesity is a pro-inflammatory state characterized by adipose tissue (AT) inflammation, including AT infiltration by macrophages and lymphocytes, and local and systemic overproduction of cytokines, chemokines, and prothrombotic factors **(3, 11, 16, 25, 27, 35, 36, 63, 64, 72, 73)**. There is likely a causal relationship between the obesity-associated remodeling of AT and the development of insulin resistance, type 2 diabetes, and cardiovascular diseases **(14, 36, 74)**. Conversely, weight loss in obese patients decreases the magnitude of adipose tissue inflammation **(7, 12, 15, 18, 21, 62)**.

The mineralocorticoid receptor (MR), a steroid receptor belonging to the nuclear receptor superfamily of transcription factors, plays a key role in hydroelectrolytic homeostasis in epithelial tissues, such as kidney and colon. Due to the similar affinity of the MR for aldosterone and cortisol **(1, 24)**, the tissue-selectivity of aldosterone effects is ensured by the presence of the 11 β -hydroxysteroid dehydrogenase type 2 (11HSD2), an enzyme that converts cortisol (corticosterone in rodents) into its inactive metabolites unable to bind MR **(30)**. By contrast, in non-epithelial tissues lacking 11HSD2, and because plasma glucocorticoid concentration is 100-1000 times higher than that of aldosterone, it is generally considered that glucocorticoids are the physiological MR ligands **(70)**. Moreover, in AT, the local effect of glucocorticoids is even reinforced by the high local expression of 11 β -hydroxysteroid dehydrogenase type 1 (11HSD1) that regenerates active cortisol (corticosterone in rodents) from its inactive metabolites **(19, 54)**.

Similarities between the metabolic syndrome (MetS) and the phenotype observed in patients with glucocorticoid excess (Cushing's syndrome) have suggested that altered corticosteroid AT metabolism could contribute to obesity and its related metabolic and cardiovascular comorbidities. For instance, mice with targeted overexpression of 11HSD1 in AT exhibit the prototypical features of the MetS with visceral obesity, insulin resistance, diabetes, dyslipidemia, and hypertension **(46, 47)**. On the opposite, selective inactivation of the glucocorticoid biological effect in AT protects the mice from diet-induced obesity and insulin resistance **(38, 53)**. Clinical

investigations have also suggested that local AT production of glucocorticoids is exacerbated in human obesity, and could contribute to the metabolic phenotype **(54)**. Although it has been considered for a long time that glucocorticoid action in AT was mediated exclusively by the glucocorticoid receptor (GR), more recent works have shown that MR is expressed in white and brown AT (WAT and BAT, respectively) **(61, 76)**, and could mediate pleiotropic effects in these tissues. Thus, it has been reported that MR activation promotes white and brown adipogenesis**(8, 9, 45, 57, 76)**, modulates adipokine secretion**(6, 34)**, and inhibits thermogenesis **(71)**. Moreover, recent studies demonstrate that MR could mediate pro-inflammatory events in AT **(32–34)**. MR was overexpressed in AT from obese mice **(33)**, and treatment of genetically obese *db/db* or *ob/ob* mice with the selective MR antagonist eplerenone significantly reduced insulin resistance, suppressed macrophage infiltration and reactive oxygen species production in AT, and normalized mRNA levels of obesity-related genes **(32, 33)**. It was proposed that the adipocyte MR was directly involved in these pro-inflammatory properties **(32–34)**. Collectively, these results suggest that MR is a pro-adipogenic and pro-inflammatory effector that mediates glucocorticoid and mineralocorticoid hormones effects on adipocyte development and functions.

In order to further evaluate the metabolic functions of MR, this study aims at examining the *in vivo* metabolic consequences of human MR (hMR) overexpression in transgenic mice (Tg) previously generated in our laboratory **(49)**. We subjected male wild type (WT) and Tg mice to a standard chow (SC) or high fat diet (HFD). Unexpectedly, Tg mice displayed a reduction in body and fat pad weight, associated with an improved glucose tolerance, while insulin sensitivity remained unchanged. These alterations in AT development were not explained by an intrinsic loss of preadipocyte differentiation capacities, but rather likely involved the pro-inflammatory properties of the receptor. These results provide new insights into MR involvement in the regulation of energy homeostasis, and open new pharmacological strategies for human metabolic disorders with the potential use of selective MR modulators.

MATERIALS AND METHODS

Animals

Eight-wk-old wild type (WT) and transgenic (Tg) male mice overexpressing hMR, previously generated (49), were kept on a 12 h-12 h light-dark cycle in a temperature-controlled colony room with *ad libitum* access to food and tap water. Mice were challenged for 16 wks with a standard chow (SC; 17% fat, 23% protein, 60% carbohydrates; Harlan, Gannat, France) or high fat diet (HFD, 60% fat, 20% protein, 20% carbohydrates; Research Diets, Gentofte, Denmark). Body weight (BW) was measured weekly. Mice were sacrificed, blood samples were collected after intra-cardiac puncture on heparinized syringes and tissues were removed, weighted and used for subsequent gene/protein expression studies, histological and/or FACS analyses or primary cultures. Mice were bred according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). The animal facility was granted approval (N°C94-043-12), given by the French Administration. All procedures were approved by the local ethic committee CAPSud (N°2012-021).

Metabolic parameters exploration

Oral glucose tolerance test (OGTT) was performed, after 16 wks of diet, on animals that have been fasted overnight for 16 h. Animals were weighed and were orally fed using gavage feeding needles (Ref 18060-020, Phymep, Paris) with a 1 g/kg BW of a 20% glucose solution prepared with tap water. Glucose levels were determined with a glucometer (Accu-Chek Performa, Roche, Bâle, Switzerland) in blood collected from the tail at indicated times. In parallel, blood samples were also collected, when possible, at basal time for plasma insulin or corticosterone measurements. Collected blood samples were centrifuged at room temperature (RT) for 3-5 min at 200-600 g. Supernatants were collected and stored at -20°C before subsequent analyses. The area under the curve (AUC) was calculated using the trapezium method.

Insulin tolerance test (ITT) was performed on animals that have been fasted for 4 h. Animals were weighed and intraperitoneally injected with 0.75 U/kg of BW of insulin (Umuline Rapid, Lilly, insulin solution diluted in sterile NaCl solution). Glucose levels

were determined at indicated times as described above. Results are expressed as % of basal glucose levels.

Plasma measurements

Fasting plasma insulin in mice fasted for 16 h was measured using the Ultra Sensitive Insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL, USA). Plasma corticosterone was measured using the AssayMaxCorticosterone ELISA Kit (Assaypro, Saint Charles, MA, USA). Total cholesterol, HDL cholesterol, triglycerides and non-esterified fatty acid levels were determined by enzymatic colorimetric assays (Sobodia, Montbonnot-Saint-Martin, France). Leptin level was measured using Bio-Plex Pros Assays (Biorad, Marnes-la-Coquette, France).

Immunocytochemical and histomorphological procedures

Tissues were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated in 70% ethanol, and embedded in paraffin. Five- μ m-thick sections were prepared, permeabilized with 0.1% Triton X-100 diluted in PBS for 10 min at RT, rinsed with PBS. Sections were then incubated in peroxidase blocking solution (3% H₂O₂ in PBS) for 30 min to block endogenous peroxidase activity, rinsed with PBS, incubated with 4% goat serum blocking solution (Impress reagent kit, Vectorlabs, Abcys, Les Ulis, France). Sections were subsequently incubated overnight at 4°C with primary antibodies (Anti-macrophages, Santa Cruz Biotechnology Inc., CA, USA). After washes and incubation of an anti-rat secondary antibody (Impress reagent kit) for 1 h at RT, macrophages were detected using AEC Substrate Chromogen (Dako, Trappes, France). Tissue sections were mounted on slides with Glycergel mounting medium (Dako). Immunolabeled sections were examined by two blinded observers on 3 high-power fields (\times 20) of one AT section (n=22 for WT, n=16 for Tg animals). Relative score labeling was determined as follows: (-) = negative labeling, (\pm) = minimal grade labeling (less than 2 positive cells/field), (+) = low-grade labeling (more than 5 positive cells/field), (++) = medium-grade labeling (more than 10 positive cells/field), and (+++) = high-grade labeling (more than 20 positive cells/field).

Paraffin-embedded adipose sections were analyzed after hematoxylin and eosin staining. Three fields were randomly observed at a x10 magnification of optical microscope (PROVIS, OLYMPUS, Rungis, France). Adipocyte size quantification was performed using the ImageJ software (<http://rsbweb.nih.gov/ij/>) on at least > 200 cells per mouse (6-12 animals per group and per genotype have been analyzed), leading to a mean adipocyte surface determination on 5000 to 8000 adipocytes per genotype.

Western Blot analysis

Total protein extracts were prepared from inguinal WAT isolated from mice. Briefly, WAT was lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 30 mM Na pyrophosphate, 50 mM Na fluoride, 1% Triton X-100, and 1X protease inhibitor from Sigma, St. Louis, MO) using a TissueLyser apparatus (Qiagen, Courtaboeuf, France). After protein blotting on an Odyssey nitrocellulose membrane (LI-COR, Lincoln, NE, USA), blots were incubated for 1 h at RT in a blocking buffer (5% fat-free milk in Tris-buffer saline TBS with 0.1 % Tween) before overnight incubation at 4°C with a rabbit anti-MR 39N antibody (1:1000 dilution) and a mouse anti- α -Tubulin antibody (1:5000 dilution, Sigma-Aldrich, Saint Quentin Fallavier, France). Rabbit polyclonal anti-MR antibody (39N) was generated by use of the human MR 1-18 peptide and purified by affinity chromatography (Double X/XP boosting antibody production program; Eurogentec, Seraing, Belgium). After extensive washes, blots were incubated with an IRDye 800-conjugated affinity purified anti-rabbit IgG second antibody (1:15,000 dilution, Perbio Science, Brebières, France) and an IRDye 680-conjugated affinity purified anti-mouse IgG second antibody (1:15,000 dilution, Perbio Science) for 1 h at RT. After washes, proteins were visualized with an Odyssey-Fc apparatus (LI-COR). Specific signals were normalized by the infrared fluorescence of α -Tubulin signals as determined by densitometry using the Image Studio software (LI-COR).

Gene expression analysis

Total RNA was extracted from AT, stroma-vascular fractions (SVF) or isolated macrophages with QIAzol reagent (Qiagen, Courtaboeuf, France) or

TriReagent(Life Technologies, Villebon-sur-Yvette, France), respectively, according to the manufacturer's recommendations. Total RNA (1 µg) was subjected to deoxyribonuclease I amplification Grade treatment (New England Biolabs, Evry, France) and then reverse-transcribed by use of the High-Capacity cDNA reverse transcription kit from Applied Biosystems (Life technologies, Saint Aubin, France). Samples were diluted 10-fold after which 1/20 of reverse transcription was used for reverse transcription-quantitative real time PCR (RT-qPCR) using the Fast SYBR Green Master Mix (Applied Biosystems) containing 300 nM of specific primers (see Supplemental Table 1 for primer sequences). RT-qPCR were carried out on a StepOnePlus Real-Time PCR system (Applied Biosystems). Reaction parameters were as follows: 95°C for 20 sec, then 40 cycles at 95°C for 1 sec and 60°C for 20 sec. For preparation of standards, amplicons were purified from agarose gel and subcloned into pGEMT-easy plasmid (Promega, Charbonnières-les-Bains, France) and sequenced to confirm the identity of each sequence. Standard curves were generated using serial dilutions of linearized standard plasmids, spanning 6 orders of magnitude. Ribosomal 18S were used as an internal control for data normalization. Relative expression of a given gene is expressed as the ratio attomoles of specific gene/picomoles of 18S. Results are mean ± SEM and generally normalized to the relative expression arbitrarily set at 1 for WT animals.

Isolation of stroma-vascular fraction and primary preadipocyte culture

AT from WT and Tg mice were removed, minced into fine pieces with scalpel and then digested with collagenase (0.7 mg/ml in PBS, Sigma-Aldrich, Saint-Quentin Fallavier, France) at 37°C using a shaker for 45 min. Homogenates were passed through a 70 µm mesh to remove undigested clumps and debris. The effluent was centrifuged at 100 g for 10 min. Cellular pellets were defined as the stroma-vascular fractions (SVF) and analyzed by Fluorescence-Activated Cell Sorting (FACS) or plated on 6-well culture dishes. Cells were cultured for 2 days (d) in clonal expansion medium composed of Dulbecco's Modified Eagle Media (DMEM) High Glucose (PAA, Velizy-Villocoublay, France) with 2% PSG (penicillin, streptomycin, glutamin) (PAA), 10% fetal bovine serum (FBS) Biowest, Nuaille, France), 0.2% fungizone (Life Technologies, Villebon-sur-Yvette, France), 0.2 nM 3-isobutyl-1-methylxanthine

(IBMX) (Sigma-Aldrich), 200 nM dexamethasone (Sigma-Aldrich). After 2 d, cells were either maintained in DMEM with 10% FBS (control medium) or cultured in adipocyte differentiation medium composed of DMEM, 10% FBS, with 100 nM insulin (Sigma-Aldrich), 600nM rosiglitazone (Sigma-Aldrich), 2 nM triiodothyronine (T3, Sigma-Aldrich) for 7 d. Afterwards, cells were washed with PBS and total RNA were extracted as described above.

Flow Cytometry Analysis and Fluorescence-Activated Cell Sorting

Spleen, peritoneal fluid, adipose tissues were collected and rinsed in PBS. Spleen and AT were minced in FACS Buffer (PBS with 5% FBS) and passed through a 70 µm mesh. SVF were prepared from collagenase-digested adipose tissues as mentioned previously. Extracellular staining was preceded by incubation with purified anti-CD16/32 antibodies (FcγRII/III block, 2.4G2) to block nonspecific staining. Cells were stained with PerCP-Cy5.5-, APC-, Alexa Fluor700-, APC-Alexa fluor750-, V450 - and V500-labeled antibodies targeting CD11c (N418), CD 206 (ACRR01), B220 (RA3-6B2), CD3 (17A2), F4/80 (BM8) and CD45 (30-F11). All antibodies were purchased from eBioscience (Paris, France), except for CD206 (R&D systems, Lille, France). Cells were finally incubated with a Red Blood Cell (RBC) Lysis Buffer (eBioscience). Flow cytometry acquisition was performed with a FACS ARIA III cytometer (BD Biosciences, Le Pont de Claix, France) and data files were analyzed using FlowJo software (Tree Star Inc, Otten, Switzerland). M1 or M2 macrophages were identified as F4/80-positive/CD11c positive or F4/80-positive/CD206 positive cells, respectively.

Macrophages-Conditioned Media and primary preadipocyte culture

Adult WT or Tg mice were sacrificed by neck dislocation and 5 ml of cold PBS was injected intraperitoneally. After 5 min, peritoneal fluid was collected and macrophages were covered by centrifugation. Macrophage-Conditioned Media (CM) were prepared by incubating macrophages, which were isolated from WT and Tg mice peritoneal cavity, at $2 \cdot 10^6$ cells in 6-well plates in 2 ml of RPMI (GE Healthcare, Vélizy, Villacoublay, France) with 10% FBS and 10 ng/ml LPS (lipopolysaccharides from *Salmonella minnesota* Re 595; L9764, Sigma) for 24h, prior collecting medium. The

conditioned media of macrophages obtained from several individuals per genotype were pooled and stored at -80°C until used. SVF were prepared from collagenase-digested adipose tissues as mentioned previously. Cells were cultured for 2 days in clonal expansion medium as above-described in 12-well plates. Subsequently, cells were cultured in a 1/10 dilution of CM either from WT or Tg macrophages, or in a 1/10 RPMI control medium with adipocyte differentiation medium for 7 days. Afterwards, cells were washed with PBS and total RNA were extracted and gene expression studies performed as described above.

Data Analysis

Data expressed as means \pm SEM, were analyzed using non-parametric Mann-Whitney test with the use of the computer software Prism 5 (Graph-Pad Software, San Diego, CA, USA). Comparisons between time points were analyzed using linear regression, distribution of adipocyte surface and immunolabeling score were analyzed using contingency test (X^2). Values of $p < 0.05$ were considered statistically significant.

RESULTS and DISCUSSION

MR is overexpressed in adipose tissues of Tg mice

Given that the human MR (hMR) was expressed under the control of the proximal promoter of the hMR gene in Tg mice (52), we first examined whether the hMR transgene was expressed in the adipose tissues (AT) of Tg mice and analyzed the consequences on endogenous murine MR (mMR) expression using species-specific primers for qPCR. As expected, hMR transcripts were readily detected in perirenal AT of Tg mice but not of WT animals (Fig. 1A). In addition, hMR was also detected in freshly prepared mature adipocytes isolated from AT of Tg mice as compared to WT animals (Fig. 1B), consistent with an adipocyte-targeted hMR overexpression. This was not associated with any substantial modification of mMR transcript levels that were similar in AT of both genotypes (Fig. 1C).

We next demonstrated by Western Blot analysis using the anti-MR 39N antibody raised against the first highly conserved amino-acids of the primary MR sequence and thus recognizing both murine and human MR (69), that MR protein expression was 3-fold higher in inguinal AT of Tg mice as compared to WT mice (Fig. 1D and 1E). This induction of MR protein expression is similar to that found in cardiomyocytes or neurons derived from MR overexpressing embryonic stem cells (50, 55). These results indicated that MR is indeed overexpressed in Tg mice, notably in AT, irrespective to the site of adipose depots. These findings also confirmed that hMR proximal promoter drives transgene expression in AT, and thus demonstrated that MR is expressed in all MR target cells where hMR proximal promoter is active, as anticipated by our previous studies (49). Finally, our results indicated that Tg mice constitute an appropriate animal model to investigate the functional consequences of global MR overexpression on metabolic balance and energy homeostasis.

Tg mice are protected against HFD-induced obesity

In order to examine the metabolic impact of MR overexpression, 8-wk-old WT and Tg male mice were subjected to SC or HFD during 16 weeks with measurement of body weight (BW) at intervals. As compared to WT mice, hMR overexpression resulted in a lower weight gain when mice were maintained under SC (Fig. 2A, $p < 0.0001$). Indeed,

WT mice gained 5.5 ± 0.7 g, corresponding to a 23.6 % increase in BW, while the BW gain of Tg mice (4.0 ± 0.8 g, i.e. 17.8 % of BW) was lower as compared with WT mice. This difference in BW was not related to a caloric restriction, since we have previously shown that Tg mice had a higher food intake than WT animals (51). In addition, there was no difference in both genotypes either in the tail-nose distance or in the weight of several organs (liver, spleen, heart, kidney, and pancreas) under SC (Table 1). The organ weight to BW ratios did not reveal any significant difference between the two genotypes, notably the weight of fat pads was not significantly altered under SC (Table 1). Thus, global MR overexpression in Tg mice that takes place during early development does not affect organ growth. This finding is in accordance with the normal growth observed in MR knock-out mice ($MR^{-/-}$) during development. Indeed, following the initial dehydration related to urinary sodium loss, the slope of growth curve of $MR^{-/-}$ mice upon sodium supplementation displays similar shape than that of controls (5). Collectively, these observations convey against a major and direct role of MR in the control of body growth and organogenesis.

In an attempt to exacerbate and/or unmask a metabolic phenotype, mice were subjected to a HFD. As expected, 16 weeks of *ad libitum* HFD feeding caused a sharp increase in BW in both genotypes without any difference in the tail-nose distance (Fig. 2B, and Table 1). WT mice gained 22.6 ± 2.0 g under HFD. This corresponded to an 86.7% increase in their BW and a 4-fold higher weight gain as compared to that induced by SC (Fig. 2C). Under similar experimental conditions, Tg mice had a BW increase of 9.1 ± 1.2 g (Fig. 2B), corresponding to an increase of 38.4% (Fig. 2C). Thus, the BW gain observed in Tg mice under HFD was drastically reduced, compared to that of WT mice ($x 2$ vs $x 4$; $p < 0.0001$; Fig. 2B and 2C), indicating that MR overexpressing mice are relatively resistant to HFD-induced obesity.

This marked reduction in BW gain of Tg mice correlated with a decrease in the mass of several fat pads measured at sacrifice (Fig. 3 and Table 1). As compared to the WT animals, Tg mice exhibited a decrease of 2.5-, 1.9-, and 3.2-fold in the weight of the perirenal, gonadal and inguinal adipose depots, respectively (Fig. 3A and 3C). This fat mass reduction in Tg mice was maintained and significant even when the

results were normalized to the BW and expressed as AT/BW ratio (Table 1, 44%, 29% and 56% reduction for perirenal AT, gonadal AT and inguinal AT, respectively; Table 1). In contrast, owing to the lower BW of Tg mice, some relative organ weight/BW ratios were slightly enhanced (heart, kidney and pancreas). However, except white fat depots, none of the absolute or normalized weight of other tissues (Brown adipose tissue (BAT), liver, spleen) was decreased in Tg mice under HFD compared to WT mice (Fig. 3D and Table 1). Taken together, these results indicate that MR overexpression in Tg mice causes a relative resistance to HFD-induced obesity through specific alteration of white adipose tissue development. This phenotype was somehow surprising given the well-described pro-adipogenic effect of MR in white and brown adipocytes (9, 61, 76). Indeed, there was clearly a discrepancy between *in vitro* effects of MR facilitating adipocyte differentiation and *in vivo* findings where global MR overexpression partially prevents HFD-induced obesity. Whether the impact on adipose tissue development is related to MR overexpression specifically in adipocytes and/or in other cell types, within or outside adipose tissue remains an open issue that is further addressed in the following sections.

Metabolic and hormonal phenotype

To get further insights into metabolic changes, lipids, glycemia and insulin plasma levels were measured under basal and dynamic conditions. There was no major difference in circulating total cholesterol and triglycerides between the 2 genotypes whatever the diet (Table 1). As expected, non-esterified fatty acid (NEFA) levels drastically increased upon HFD, but without difference between WT and Tg mice.

Under SC, leptin level was similar between the 2 genotypes (Table 1). In accordance with the HFD-induced adipose weight gain, plasma leptin levels increased markedly in WT mice. However, the induction was strongly reduced in Tg mice, consistent with their lower fat pad mass development.

It is well established that glucocorticoids are the physiological ligands for MR in tissues lacking 11HSD2 activity (24). In addition, cells harboring 11HSD1 activities, such as hepatocytes and adipocytes, are able to regenerate active glucocorticoids from 11 dehydro-metabolites, thus amplifying paracrine actions of these hormones

(19). In this context, the circulating corticosterone levels were determined under SC and HFD challenges. Of note, HFD significantly increases corticosterone levels as compared to SC but without any difference between WT and Tg mice (Table 1). The impact of HFD on the hypothalamus-pituitary-adrenal-axis (HPA) activity remains rather controversial and no definite conclusion has been drawn from a recent review of the literature **(2)**. However, whatever the diet and the genotype, basal corticosterone concentrations are sufficient to ensure saturating MR binding and full MR activation in adipose tissues **(40)**. Thus, we propose that phenotypic alterations observed in Tg mice are directly related to MR overexpression rather than adaptive modulations of its physiological ligand levels.

To examine whether the marked differences in BW gain and adiposity between the 2 genotypes were associated with changes in carbohydrate metabolism, we evaluated glycemia and insulinemia under basal (Table 1) and dynamic conditions (Fig. 4). As anticipated, HFD significantly increased fasting glucose levels in both genotypes. However, fasting glycemia was always lower in Tg as compared to WT mice (Table 1). Insulinemia as well as the HOMA-R, an index of insulin sensitivity, were not different between the 2 genotypes yet significantly increased, as expected under HFD. Fasting glycemia is essentially dependent of hepatic glucose production through activation of the neoglucogenic pathway **(58)**. However, since MR is not expressed in hepatocytes, it is unlikely that liver MR transgene expression might contribute to the lower fasting plasma glucose measured in Tg mice. In addition, it is well known that insulin strongly inhibits hepatic glucose production while glucagon and corticosterone activate this metabolic pathway. However, a major contribution of these hormones could be excluded since their plasma levels were not significantly different between both genotypes.

We next performed OGTT after 16 h of fasting in animals fed under HFD. As mentioned above (Table 1), Tg mice exhibited a lower glycemia at baseline as compared to WT mice (111.9 ± 12.2 mg/dl vs 169.0 ± 11.4 mg/dl, $p < 0.01$, Figure 4A). After glucose overload (1 g/kg), plasma glucose levels were always lower in Tg mice as compared to WT mice at all tested time points (Fig. 4A). This correlated with a significant decrease of the area under the curve (AUC) for Tg mice compared to controls (Fig. 4B), consistent with an improvement in glucose tolerance.

To further explore the impact of MR overexpression on carbohydrate metabolism, ITT was performed after a 4 h fasting. Both Tg and WT animals displayed an apparent similar insulin sensitivity with an equal initial slope, indicative of a superimposable glucose uptake or clearance (Fig. 4C).

How these variations in glucose homeostasis could be interpreted in an integrative physiological network? The better glucose tolerance observed in Tg mice well correlated with their lower fat mass development under HFD. An excessive adiposity is generally associated with lipid, cytokine and adipokine profile alterations, which in turn promote low-grade inflammation and insulin resistance (13, 20, 42). Unexpectedly, despite their relative resistance to HFD-induced obesity, MR overexpressing mice did not improve their insulin sensitivity compared to WT mice. These findings suggest that in our model, MR-dependent activation does not markedly alter insulin sensitivity. This does not argue against previous studies showing that MR blockade by eplerenone reverses insulin resistance in genetically determined obese or diabetic mice (33) and prevents pro-inflammatory events prevailing in adipose depots (32). In addition, MR-mediated insulin resistance may be also supported by the higher prevalence of MetS and insulin resistance in patients with primary hyperaldosteronism(22, 23) which remains however controversial (67). The relative resistance to HFD-induced obesity in MR overexpressing mice, associated with the lack of insulin sensitivity improvement, prompted us to further investigate properties of adipose depots that represent an essential insulin target and MR-expressing tissue.

Impact of MR overexpression on adipocyte morphology

We first examined AT histology and quantified adipocyte surface in perirenal adipose depots by histomorphological analysis. Three fields of hematoxylin-eosin stained sections (Fig. 5A) per animal were analyzed at random, adipocyte surface was determined using the Image J software (<http://rsbweb.nih.gov/ij/>) (Fig. 5B). Thereafter, adipocytes were classified in different categories according to their surface (from 100 to 15,000 μm^2). As expected, adipocytes of animals fed a HFD were hypertrophic as compared to those of mice fed a SC (Fig. 5C lower panel vs upper panel). Under SC, 65-70% of adipocytes had a surface lower than 500 μm^2

while they represented only 20% under HFD. Conversely, there were less than 3% adipocytes larger than $2000 \mu\text{m}^2$ under SC, while cell surface exceeded $2000 \mu\text{m}^2$ in more than 60% of adipocytes from HFD mice.

Of interest, when comparing adipocyte size within the same diet, we observed that MR overexpression influenced adipocyte size distribution. Under SC (Fig. 5C, upper panel), Chi-square analysis on more than 5000 independent determinations shows that perirenal adipose depots from Tg mice displayed a significantly higher proportion of small adipocytes as compared to those of WT mice ($p < 0.0001$).

Under HFD (Fig. 5C, lower panel), we observed a very different distribution of adipocyte size of perirenal AT between the 2 genotypes. Indeed, small adipocytes ($< 2000 \mu\text{m}^2$) were more abundant in Tg mice as compared to controls, while few large adipocytes ($> 7000 \mu\text{m}^2$) were detected in MR overexpressing mice. In parallel, there was a significant decrease of the mean adipocyte surface in Tg mice as compared to WT mice ($3452 \pm 46 \mu\text{m}^2$ vs $3703 \pm 41 \mu\text{m}^2$, respectively; $p < 0.001$).

Thus, the higher proportion of small adipocytes in Tg mice could reflect differences in adipocyte development and/or function.

Dysregulation of gene expression in perirenal AT of Tg mice

To determine if the lower body fat mass and the higher proportion of small adipocytes in Tg mice could be explained by an alteration in adipogenesis, we studied the expression of the transcription factor PPAR γ 2, (Peroxisome Proliferator-Activated Receptor Gamma 2), the master adipogenic regulator gene. Under SC, Fig. 6A shows that PPAR γ 2 expression was reduced by 40% in the perirenal adipose depot of Tg mice, as compared to controls. Likewise, under HFD, PPAR γ 2 expression remained significantly lower in Tg mice, compared to WT animals (Fig. 6B) (147 ± 14 vs 245 ± 22 amol/pmol 18S under SC, 68 ± 6 vs 82 ± 6 amol/pmol 18S under HFD). Similar results were also observed in inguinal fat depot of Tg mice (Supplemental Fig. S1A). These findings were in agreement with the disturbed adipocyte development of Tg fat depot, exacerbated by HFD and well correlated with the lower circulating leptin levels (see Table 1). However, the downregulation of adipocyte PPAR γ 2 expression was in the same order of magnitude in Tg mice while adipose tissue development was drastically reduced under HFD. This suggests that under these conditions,

additional events, either cell autonomous or environmental factors, may be involved in the resistance to obesity in Tg as compared to WT mice.

To test this hypothesis, we further studied whether MR overexpression might influence local glucocorticoid signaling, notably GR (Fig. 6C and 6D) and 11HSD1 (Fig. 6E and 6F). Surprisingly, Tg mice fed a SC displayed a 32% reduction in GR mRNA steady state levels (Fig. 6C). This reduction in GR expression in Tg mice was attenuated under HFD yet remained significant as compared to WT mice (Fig. 6D) even though absolute AT GR expression in WT animals was similar whatever the diet (122 ± 11 vs 124 ± 8 amol/pmol 18S, respectively under SC and HFD). Likewise GR mRNA levels down-regulation was also detected in inguinal AT of Tg mice (Supplemental Fig. S1B). Of interest, 11HSD1 transcript levels were also significantly decreased in the perirenal AT of Tg mice under SC (Fig. 6E) but this difference was abolished under HFD (Fig. 6F) as a consequence of a drastic reduction of 11HSD1 expression in both genotypes (5.44 ± 0.39 vs 8.64 ± 1.03 amol/pmol 18S under SC, 0.21 ± 0.02 vs 0.21 ± 0.02 amol/pmol 18S under HFD). Such a reduction in 11HSD1 expression and activity has already been reported in adipose depots of mice fed a HFD (43). Thus, in Tg mice fed a HFD, a complete down-regulation of adipocyte 11HSD1 cannot account for decreased local corticosterone regeneration owing to high circulating corticosterone levels. However, we could not exclude that impaired GR activation in Tg animals might affect fat mass development. Collectively, these results suggest that in MR overexpressing mice, adaptive regulations in the glucocorticoid signaling pathway may account, at least in part, for resistance to HFD-induced obesity.

***In vitro* preadipocyte differentiation capacity is not altered by hMR overexpression**

Given the apparent discrepancy between the pro-adipogenic role of MR in preadipocyte cell lines (9, 61, 76) and the partial defect of HFD-induced fat mass development in Tg mice, we examined whether the alteration in adipocyte differentiation was an intrinsic property of MR-overexpressing preadipocytes. To this aim, stroma vascular fractions (SVF) were isolated from inguinal, perirenal and gonadal adipose depots and tested *in vitro* for their adipogenic capacity (Fig. 7). As

shown in Fig.7A, freshly isolated SVF of adipose depots were composed of immature undifferentiated preadipocytes (upper panels), which could undergo adipocyte differentiation under appropriate differentiation cocktail exposure for 7 d (Fig. 7A, lower panels). Interestingly, we showed that preadipocytes originated for Tg adipose depots had an identical or even higher adipocyte differentiation ability as evidenced by appearance of multilocular, cytoplasmic lipid droplets, compared to WT preadipocytes (Fig. 7A, lower panels). As expected, this was accompanied by an increased or equivalent PPAR γ 2 expression in differentiated adipocytes derived from inguinal, perirenal or gonadal SVF of Tg compared to WT mice (Fig. 7B). In addition, adipogenesis is known to be associated with an increased expression of important nuclear receptors including MR (9, 29). Accordingly, it was not surprising that mMR expression was induced in hMR overexpressing adipocytes (Fig. 7C). Of note, inguinal preadipocytes seem to exhibit the highest differentiation capacity as compared to other perirenal and epididymal adipose depots. Altogether, these results demonstrate that isolated preadipocytes from Tg mice have identical or even enhanced differentiation abilities compared to those from WT animals. In any case, we could exclude that hMR overexpression impairs preadipocyte differentiation commitment. Thus, we propose that relative resistance to HFD-induced obesity in Tg mice is not explained by defective intrinsic adipocyte maturation but rather due to adipocyte-independent mediators affecting adipose tissue development.

Involvement of macrophage polarization in the resistance to HFD-induced obesity in Tg Mice

Two main arguments led us to focus our attention to macrophages. On one hand, obesity is considered as a chronic low-grade inflammatory state (7, 72, 75), in which AT itself seems to be a site of inflammation (72) leading to a systemic pro-inflammatory state, oxidative stress and insulin resistance (20). Incidentally, MR and GR exert opposite effects on inflammation and adipocyte differentiation (9, 34) given that MR promotes AT inflammation whereas GR is an anti-inflammatory factor. Interestingly, obesity is associated with an increased AT accumulation of macrophages that participate to inflammation and contribute to obesity-linked comorbidities(72, 75). Macrophages can polarize to exert either pro- (M1) or anti-

inflammatory (M2) effects, depending on the surrounding tissue microenvironment and the subset of circulating monocyte precursors from which they derive (17). A phenotypic switch from a M2 to M1 polarized macrophage state has been also described in obesity (41). On the other hand, MR is expressed in macrophages and seems to be a pro-inflammatory factor. Administration of MR antagonists reduces macrophage accumulation in a number of disease models (26, 56), while selective macrophage MR deletion protects against mineralocorticoid-mediated cardiac fibrosis, despite normal macrophage recruitment (4, 60). Accordingly, administration of eplerenone, a selective MR antagonist, to genetically obese (*ob/ob*) and diabetic (*db/db*) mice decreases their AT inflammation (32, 33). Furthermore, myeloid-specific MR knock-out led to a transcription profile of alternative macrophage activation (68) and suppressed classically activated M1 macrophage markers while partially preserving alternatively activated M2 markers (28). These findings suggest that MR activation plays an important role in controlling immune cell function during the inflammatory responses, thereby participating to the metabolic phenotype of our MR overexpressing mice. This prompted us to examine whether macrophage polarization could account for the resistance to HFD-induced obesity.

Immunohistochemistry analysis using an anti-macrophage antibody and semi-quantitative scoring of macrophage infiltration in AT (Fig. 8A), did not reveal any significant difference between the 2 genotypes, despite a lower fat pad mass in Tg mice under HFD. However, immunohistochemistry is a semi-quantitative and low sensitive method that does not allow precise characterization of the inflammatory profile of the resident and/or infiltrating macrophages. We first confirmed that macrophages purified from spleen by Fluorescence-Activated Cell Sorting (FACS) and isolated from peritoneal cavity expressed F4/80 macrophage marker (Fig. 8B, left panel) while, as expected, the transgene hMR expression was only detected in macrophages originating from Tg mice (Fig. 8B, right panel). We further investigated macrophage percentage (Fig. 8C, left panel) by using FACS analysis (see material and methods session). Surprisingly, under SC, Tg mice exhibited a significant lower proportion of F4/80 positive cells in spleen, a major immune organ ($5.1 \pm 0.6\%$ vs $6.7 \pm 0.9\%$ $p < 0.01$ compared to WT mice; Fig. 8C, middle panel). Similar reduction was detected when considering either high or intermediate F4/80 subsets

independently (data not shown). In HFD animals, a trend towards reduction in splenic macrophages proportion was also observed but not statistically significant (Fig. 8C, middle panel). We next evaluated the proportion of macrophages in AT and found no difference presumably due to the low proportion of these immune cells in adipose depots and the low fat mass recovered from these animals under SC. However, differences emerged in HFD animals showing increased proportion of macrophages in SVF isolated from gonadal AT of Tg mice ($17.3 \pm 1.5\%$ vs $8.8 \pm 0.2\%$ in WT mice $p < 0.05$; Fig. 8C, right panel). Collectively, spleen macrophage depletion together with higher AT macrophage infiltration in Tg mice strongly suggest a distinct macrophage distribution between WT and Tg mice consistent with the fact that MR-overexpression was associated with a stimulation of macrophage homing in AT, thereby contributing to resistance to HFD-induced obesity.

Macrophage polarization was further studied by FACS using CD11c antibody as M1 marker and CD206 antibody as M2 marker, enabling us to distinguish three cell subpopulations composed of F4/80/CD11c-positive cells (pro-inflammatory macrophages), F4/80/CD206-positive cells (alternatively activated macrophages), and F4/80/CD11c/CD206-positive cells (intermediate macrophage phenotype) (Fig. 8D, left panel). We investigated splenic macrophages, which may represent precursors of tissue macrophages. As showed in Fig. 8D, right panel, Tg mice displayed a distinct macrophage pattern with a trend towards higher M2 differentiation as compared to WT cells. In this context, a higher proportion of M2 macrophages in AT may protect from developing obesity.

We next performed RT-qPCR analysis in perirenal fat tissues recovered from WT and Tg animals subjected to SC or HFD. Under SC, despite a sharp reduction in F4/80 gene expression in perirenal AT from Tg animals as compared to WT (Fig. 8E, left panel), CD206 (M2) marker was predominantly expressed while CD11c (M1) was mildly detected (Fig. 8E, right and middle panels) whatever the genotype. Interestingly, HFD differently affected M1/M2 ratio in WT and Tg animals. As expected, under HFD, F4/80 mRNA levels drastically increased in both genotypes (fold increase was essentially similar), suggesting that macrophage recruitment was efficiently induced in both animals, and consistent with chronic low-grade inflammation associated with obesity. However, whereas CD11c mRNA was

increased in both WT and Tg animals (reflecting that inflammatory process are equally induced), CD206 mRNA expression was reduced in WT but preserved in Tg animals (Fig. 8E). The higher proportion of macrophages among SVF of Tg animals and reduced macrophage expression in whole AT could be explained by a lower cellularity of the SVF in Tg animals compared to WT mice. Thus, both FACS and gene expression studies converge to suggest that MR transgene expression promotes a distinct macrophage polarization.

Taken together, these results indicate that macrophage number, infiltration and polarization are readily affected in MR-overexpressing animals, very likely dependent upon tissue location (spleen vs adipose depots) and existing prior diet-induced obesity. This subtle and significant difference in macrophage phenotype is probably underestimated given the complexity of the transition states of macrophage activation, M1 vs M2 further subdivided in M2a, M2b and M2c (44), which is finely tuned in response to different microenvironments. This phenomenon is further entangled with the dynamic aspects of the macrophage transition associated with fat mass development. Whether such a distinct macrophage polarization facilitates development of other immune cells such as helper T lymphocytes, or modulates natural killer or T regulatory lymphocytes remains to be established (48). We thus propose that MR overexpression both in preadipocytes and mature adipocytes as well as in macrophages leads to distinct modulation of both adaptive and innate immune responses to obesity. Further investigations are needed to better define plasticity and heterogeneity of macrophage phenotype in the context of immunometabolism. Moreover, it would be of interest to examine whether MR-overexpression actually modulates the recruitment and infiltration of macrophages within AT, or preferentially acts on the population of resident macrophages.

Tg macrophage-conditioned medium partially impaired preadipocyte differentiation *in vitro*.

To better support the role of MR-overexpression in macrophages on the adipogenesis alteration, we used an *ex-vivo* approach in which conditioned medium (CM) from either WT or Tg macrophages were tested *in vitro* for their ability to alter adipogenic capacity of SVF-isolated preadipocytes. These CM were prepared by

incubating macrophages, which were isolated from the peritoneal cavity of WT (WT CM) and Tg (Tg CM) mice, and activated for 24h with 10 ng/ml LPS. As anticipated, both WT CM and TG CM were able to partially inhibit adipocyte differentiation (Fig 9A) as evidenced by a significant reduction in PPAR γ 2 (Fig.9B) and FAS (Fig.9C) expression in differentiated adipocytes as compared to control medium ($p < 0.0001$ in both conditions). Interestingly, PPAR γ 2 expression was decreased by 40% in WT CM condition and by 50% in Tg CM condition yet did not reach statistical significance between genotype ($p = 0.08$). However, a significantly higher reduction in FAS mRNA levels was observed after Tg CM exposure (56%) compared to WT CM (47%) ($p = 0.015$), suggesting that macrophages from MR overexpressing mice secreted higher amounts of some unidentified cytokines which alter adipocyte differentiation capacity. These important data clearly support the involvement of macrophages in the resistance to high fat diet-induced obesity. Tg CM had a higher inhibiting effect on preadipocytes differentiation as compared to WT CM and indicating that MR overexpression in macrophages could induce a distinct macrophage function which could explain, in part, AT development alteration. Identification of the Tg macrophage-derived secretory molecules that promote these anti-adipogenic properties remains a future challenge.

Figure 10 summarizes the main findings of this study. We provide further support for the proadipogenic properties of MR as evidenced by enhanced adipocyte differentiation capacities of MR overexpressing preadipocytes *ex vivo*. However, the relative resistance to HFD-induced obesity observed in Tg mice led us to hypothesize that MR overexpression in innate immune cells, and may be other cell types, could alter macrophage polarization which in turn could impair adipogenesis (**10, 31, 37, 39, 65, 66**).

The phenotype observed in our transgenic model is the consequence of global MR-overexpression. We cannot precisely delineate the relative contribution of different cell types in which the MR P1 proximal promoter is functional. However, this transgenic model is relevant to mimic a global, pharmacological, often non tissue-selective, activation of MR. Clearly, the metabolic phenotype relied not only on specific MR function on adipocytes and macrophages but very likely on other MR

target tissues such as endothelial cells or neurons. Despite the complexity of the underlying mechanisms, this model provides a unique opportunity to unravel unexpected MR physiological properties *in vivo*.

CONCLUSION

There is now a large body of evidence that corticosteroid effects in AT are mediated by both GR and MR. It was reported that while GR mediates an anti-inflammatory response, MR activation promotes a pro-inflammatory and diabetogenic adipokine expression profile (32–34). The balance between gluco- and mineralocorticoid action could play a key role in AT homeostasis and in obesity- and type 2 diabetes-associated metabolic and cardiovascular complications. Thus, selective modulation of GR or MR may offer new avenues for the treatment of insulin resistance and type 2 diabetes. One major challenge would be to dissociate the putative beneficial effects of such drugs from their immuno-modulatory properties. Interestingly, a recent work has identified the so-called compound A (cpdA) as a GR-modulating substrate that preserves anti-inflammatory properties, but has for instance no side effects on bone formation (59). We anticipate that such selective MR modulators could be a relevant strategy in metabolic diseases through an appropriate pharmacological targeting of the pro-inflammatory properties of MR.

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FIGURE LEGENDS

Figure 1: MR expression in adipose tissue and isolated adipocytes of WT and Tg mice

Expression of human MR (hMR) in perirenal AT (**A**) and in adipocytes isolated (**B**) from WT mice (n=12) and Tg mice (n=5) that were submitted to a 16-wk high fat diet (HFD) and murine MR (mMR) in perirenal AT from the same animals (**C**), analyzed by RT-qPCR. hMR was only expressed in Tg mice without modification of endogenous MR (mMR) expression. Results are means \pm SEM and values measured in Tg mice were arbitrarily set to 1. Relative hMR expression was 7.6 ± 0.9 amol/pmol18S and 22.1 ± 4.3 amol/pmol18S in AT and isolated adipocytes from Tg mice, respectively. MR expression in inguinal AT from WT (n=10) and Tg (n=11) mice analyzed by Western Blot using 39N antibody that recognizes both mMR and hMR (**D**) with quantification of the Western Blot bands (**E**). Results are means \pm SEM and represent MR to α -tubulin ratio, arbitrarily set to 1 for WT mice. MR protein expression was 3-fold higher in inguinal AT of Tg mice as compared to WT mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2: Mice overexpressing hMR are resistant to HFD-induced obesity

Body weight of WT and Tg mice during 16 wks time course of SC (**A**) or HFD (**B**) (n=10-11 for each group). Under SC, hMR overexpression resulted in a lower weight gain in Tg mice as compared to WT mice (BW at the end of SC: 27.5 ± 0.5 g vs 29.2 ± 0.6 g). This difference was exacerbated under HFD, consistent with a resistance to HFD-induced obesity in Tg mice ($33.6 \text{g} \pm 0.9$ g vs $48.5 \text{g} \pm 2.4$ g). Linear regression was performed for each curve, and regression coefficients were compared. *** $p < 0.001$. **C**, Percentage of body weight gain in WT and Tg mice under SC and HFD. As expected, a HFD feeding for 16 wks caused a sharp increase in BW gain in both genotypes. Yet, the doubling of BW gain observed in Tg mice under HFD was drastically reduced as compared to that of WT mice. Results are expressed as mean \pm SEM. †† $p < 0.01$, ††† $p < 0.001$ HFD vs SC (within the same genotype), *** $p < 0.001$ Tg vs WT mice (within the same diet).

Figure 3: Decrease of fat pad mass under HFD in Tg mice

Perirenal (A), gonadal (B), inguinal (C) and brown (D) fat mass of the left side of the animal was removed at sacrifice, and weighed. As compared to the WT animals, Tg mice exhibited a 2.5-, 1.9-, and 3.2-fold decrease in the weight of the perirenal, gonadal and inguinal adipose depots, respectively. The marked reduction in BW gain of Tg mice correlated with the decrease in the fat pad mass. No significant difference in brown fat mass was observed. Results are expressed as mean \pm SEM, ** $p < 0.01$, *** $p < 0.001$.

Figure 4: Improved glucose tolerance in Tg mice

A, OGTT performed after 16 wks of HFD in Tg (n=11) and WT (n=9) mice subjected to 16 h fasting. After glucose overload (1 g/kg), plasma glucose levels were always lower in Tg mice as compared to WT mice at all tested time points. This correlated with a significant decrease of the area under the curve (AUC) for Tg mice compared to controls (**B**), consistent with an improvement in glucose tolerance. * $p < 0.05$, ** $p < 0.01$. **C**, ITT after 16 wks of HFD in Tg (n=11) and WT (n=10) mice subjected to 4 h fasting. Glycemia were measured at all indicated time points after insulin injection (0.75 U/kg), and expressed as a percentage of basal glycemia. Baseline plasma glucose levels were slightly but significantly lower in Tg mice (135.1 ± 7.5 mg/dl) than in WT mice (177.0 ± 12.8 mg/dl) ($p = 0.005$). The 2 groups of animals displayed apparent similar insulin sensitivity with an equal initial slope, indicative of a superimposable glucose uptake or clearance. Results are expressed as mean \pm SEM, * $p < 0.05$.

Figure 5: Adipocyte Surface Modification in perirenal AT of Tg mice

(A), Representative image obtained after hematoxylin-eosin coloration of perirenal AT (Magnification $\times 10$) from WT and Tg animals fed either SC and HFD. (B) Distribution of adipocyte surfaces in perirenal AT in WT and Tg mice under SC (upper panel) or HFD (lower panel). Both under SC and HFD, Tg mice exhibited a higher proportion of small adipocytes, yet a lower proportion of large adipocytes under HFD, suggesting differences in adipocyte development associated with hMR overexpression. χ^2 test was performed for each histogram, $p < 0.0001$.

Figure 6: Gene expression in perirenal AT of WT and Tg Mice under SC and HFD analyzed by RT-qPCR

Expression of *PPAR γ 2* (A,B), *GR* (C,D), *11HSD1* (E,F) genes normalized to 18S rRNA levels, were quantified by RT-qPCR in perirenal AT of 6-mo-old male WT mice (n=10) and Tg mice (n=10-11), both under SC (A,C,E) and HFD (B,D,F). Results are expressed as mean \pm SEM and values measured in WT mice were arbitrarily set to 1. *PPAR γ 2* expression was significantly reduced in Tg animals as compared to WT animals, in agreement with a disturbed adipocyte development of Tg fat depot. Tg mice fed a SC displayed a reduction in *GR* mRNA steady state levels (B). This reduction of *GR* expression in Tg mice was attenuated under HFD yet remained significant as compared to WT mice (E). *11HSD1* transcript levels were also significantly decreased in the perirenal AT of Tg mice under SC (C) but this difference was abolished under HFD (F). * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, Tgvs WT animals. In WT mice under SC, relative expression of *PPAR γ 2* was 245.1 ± 22.3 amol/pmol of 18S, of *GR* was 122.2 ± 11.1 amol/pmol of 18S, of *11HSD1* was 8.6 ± 1.0 amol/fmol of 18S. Under HFD, in WT animals relative expression of *PPAR γ 2* was WT 81.7 ± 6.3 amol/pmol of 18S, of *GR* was 123.7 ± 8.2 amol/pmol of 18S, and of *11HSD1* was WT 206.8 ± 16.3 amol/pmol of 18S.

Figure 7: *In vitro* preadipocyte differentiation capacity of preadipocytes isolated from WT and Tg adipose depots

A, Isolated SVF of adipose depots were composed of immature undifferentiated preadipocytes (upper panels). Under differentiation cocktail exposure for 7 d preadipocytes undergo differentiation into mature adipocyte (lower panels). The adipocyte differentiation ability of hMR overexpressing preadipocytes was similar or even higher compared to WT preadipocytes, as evidenced by appearance of cytoplasmic lipid droplets (lower panels). *PPAR γ 2* (B) and *mMR* (C) expression in differentiated adipocytes derived from inguinal, perirenal or gonadal SVF of Tg compared to WT mice. Adipogenesis was accompanied by an increased or equivalent *PPAR γ 2* and *mMR* expression in differentiated adipocytes derived from inguinal (WI), perirenal (WP) or gonadal (WG) SVF of Tg compared to WT mice.

hMR overexpression does not impair the ability of isolated preadipocytes to differentiate compared to those from WT animals. Results are expressed as mean \pm SEM and values measured in WT cells were arbitrarily set to 1, ** $p < 0.01$ *** $p < 0.001$, Tg vs WT cells. In WT animals, relative expression of PPAR γ 2 was 67.3 ± 10.2 , 33.5 ± 3.6 and 23.7 ± 3.9 amol/pmol18S, in subcutaneous, perirenal and gonadal AT, respectively. Relative expression of mMR was 1.2 ± 0.1 , 0.8 ± 0.1 and 0.8 ± 0.1 amol/pmol18S subcutaneous, perirenal and gonadal AT of WT, respectively.

Figure 8: Macrophages infiltration and polarization in adipose tissue of Tg and WT animals under HFD

A, Illustrations of macrophage immunostaining labeling in perirenal AT from HFD fed mice. Examples of score labeling considering as negative (left panel) or highly positive (+++) (middle panel), using an anti-macrophage antibody. Comparison of labeling score for macrophage immunostaining in perirenal AT from Tg and WT animals (right panel). The macrophage infiltration score was not different between genotypes (Chi-square analysis, $p = 0.25$). This similar macrophage labeling score in Tg mice was somehow unexpected given their lower fat pad mass as compared to HFD-fed WT animals. **B**, A pool of splenic macrophages purified by FACS and macrophages isolated from peritoneal cavity of WT and Tg animals express identical levels of the F4/80 marker (left panel). hMR mRNA expression was only detected in macrophages originating from Tg mice, as determined by RT-qPCR using species-specific primers (see Material & Methods section, right panel). **C**, Quantification of F4/80-positive cells by FACS analysis (left panel). The percentage of F4/80 expressing cells determined in spleen from animals under SC (middle panel) or in gonadal SVF (right panel) obtained from HFD-fed mice. While Tg mice displayed a significant reduction of splenic F4/80-positive cells percentage, a 2-fold increase in macrophage infiltration was found in gonadal SVF of Tg mice, reflecting a marked difference in macrophage distribution between WT and Tg animals. **D**, Polarization characterization by FACS of macrophage isolated from spleen. F4/80/CD11c-positive cells (M1 polarization), F4/80/CD206-positive cells (M2 polarization) and F4/80/CD11c/CD206-positive cells (DP: double positive cells, intermediate macrophage phenotype) were quantified in both WT and Tg animals (left and middle

panels). As showed in the right panel, Tg mice exhibited a distinct macrophage pattern with a trend towards a higher proportion of M2 macrophages as compared to WT cells. **E**, Gene expression of F4/80, CD11c and CD206 (left, middle and right panels, respectively) was studied by RT-qPCR in perirenal AT of WT and Tg animals under SC and HFD. mRNA levels were always lower in Tg mice as compared to controls whatever the diet. While HFD stimulated F4/80 and CD11c gene expression in both genotypes, CD206 mRNA levels were significantly reduced in WT mice under HFD but remained unchanged in Tg mice. ** $p < 0.01$, *** $p < 0.001$ Tg vs WT mice (within the same diet); † $p < 0.05$, ††† $p < 0.001$ HFD vs SC (within the same genotype). NS : No significant difference, HFD Tg vs SC Tg. F4/80 (WT vs Tg): 195 ± 5 vs 50 ± 6 amol/pmol 18S under SC, 696 ± 116 vs 221 ± 33 amol/pmol 18S under HFD; CD11c (WT vs Tg): 16 ± 2 vs 6 ± 1 amol/pmol 18S under SC, 234 ± 29 vs 81 ± 19 amol/pmol 18S under HFD; CD206 (WT vs Tg): 329 ± 46 vs 118 ± 11 amol/pmol 18S under SC, 191 ± 27 vs 100 ± 11 amol/pmol 18S under HFD.

Figure 9: Alteration of preadipocyte differentiation by Tg macrophages-conditioned media

A, SVF isolated from adipose depots were composed of immature undifferentiated preadipocytes (basal medium, left upper panel). Under differentiation cocktail exposure for 7 days, preadipocytes undergo differentiation into mature adipocytes (Differentiation medium, right upper panel). The adipocyte differentiation ability was reduced in presence of WT and Tg macrophages-conditioned media (WT CM and Tg CM, respectively; lower panels). Expression of *PPAR γ 2* (**B**) and *FAS* (**C**) genes normalized to 18S rRNA levels, were quantified by RT-qPCR in preadipocytes, isolated from SVF, cultured in presence of WT CM, Tg CM and RPMI-10% FBS diluted at 1/10 with adipocyte differentiation medium (control medium) or basal medium. Results are expressed as mean \pm SEM from 3-6 independent determinations and values measured in RPMI-10% FBS diluted at 1/10 with adipocyte differentiation media were arbitrarily set to 1. *PPAR γ 2* mRNA levels were significantly reduced in CM conditions (WT or Tg) as compared to control medium, but there is a trend toward to a higher decrease with Tg CM as compared to WT CM ($p = 0.08$). *FAS* expression was reduced more importantly by Tg CM (54%) as

compared to WT CM (47%) ($p=0.015$). * $p<0.05$, *** $p<0.001$. PPAR γ 2 (control medium) : 0.078 ± 0.005 amol/fmol 18S; FAS (control medium) : 1.462 ± 0.068 amol/fmol 18S.

Figure 10: Graphical summary of immuno-metabolic actions in MR overexpressing mice

It has been demonstrated that MR promotes adipocyte differentiation *in vitro* and *ex vivo*. However, in an *in vivo* model of global MR overexpressing mice, especially in innate immune cells, MR could lead to an early inflammation state which in turn could impair adipogenesis.

Supplemental Figure S1: Gene expression in inguinal AT of WT and Tg Mice under HFD analyzed by RT-qPCR

Expression of PPAR γ 2 (A) and GR (B) genes normalized to 18S rRNA levels were quantified by RT-qPCR in inguinal AT of 6-mo-old male WT mice ($n=10$) and Tg mice ($n=10$) under HFD. Results are expressed as mean \pm SEM and values measured in WT mice were arbitrarily set to 1. PPAR γ 2 expression (A) was significantly reduced in Tg animals as compared to WT animals, in agreement with a disturbed adipocyte development of Tg fat depot. GR transcript levels were also significantly decreased in the inguinal AT of Tg mice under HFD (B). *** $p<0.001$, Tgvs WT animals. Under HFD, relative expression of PPAR γ 2 was in WT animals 0.2660 ± 0.0296 amol/fmol of 18S, of GR was 0.2069 ± 0.017 amol/fmol of 18S.

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SC

HFD

	WT	Tg	WT	Tg
Animals (n)	8	10	22	16
Height (mm)	104.9 ± 1.2	98.4 ± 0.7	103.7 ± 1.8	99.0 ± 0.7
Weight (g)	29.4 ± 1.1	27.4 ± 0.5 *	42.5 ± 2.5 ††	30.6 ± 1.4 *** ††
Organs Weight (mg)/BW (g)				
Liver	46.5 ± 1.5	49.5 ± 2.6	36.0 ± 2.4 ††	43.0 ± 2.3
Spleen	4.9 ± 0.4	5.9 ± 0.8	2.9 ± 0.3 ††	4.5 ± 0.7
Heart	6.6 ± 0.4	6.3 ± 0.1	5.0 ± 0.3 †	7.3 ± 0.6 **
Kidney	7.4 ± 0.2	7.2 ± 0.3	5.3 ± 0.3 ††	8.0 ± 0.4 ***
Pancreas	8.4 ± 0.6	9.9 ± 0.5	5.7 ± 0.4 ††	7.5 ± 0.4 * ††
Perirenal AT	2.1 ± 0.2	1.9 ± 0.2	17.0 ± 1.4 ††	9.5 ± 1.7 ** ††
Gonadal AT	4.4 ± 0.4	4.4 ± 0.7	38.3 ± 3.3 ††	27.3 ± 4.6 * ††
Sub-cutaneous AT	3.2 ± 0.2	2.8 ± 0.5	40.3 ± 2.6 ††	17.7 ± 3.1 *** ††
Brown AT	ND	ND	5.4 ± 0.8	5.0 ± 0.8
Cholesterol (g/l)	1.45 ± 0.09	0.98 ± 0.18 **	1.35 ± 0.08	1.44 ± 0.08 ††
Triglycerides (µmol/l)	159.8 ± 14.9	200.3 ± 43.4	203.5 ± 27.3	224.6 ± 39.1
Non Esterified Fatty Acids (µmol/l)	194.6 ± 14.6	196.1 ± 17.2	797.0 ± 71.8 ††	929.4 ± 100.2 ††
Leptin (ng/ml)	0.89 ± 0.17	1.33 ± 0.26	31.13 ± 6.57 ††	6.65 ± 1.62 ** ††
Corticosterone (ng/ml)	185.2 ± 51.3	158.2 ± 18.8	431.7 ± 91.6 ††	354.0 ± 47.5 ††
Glycemia (mg/dl)	83.9 ± 4.4	70.9 ± 5.7 *	131.9 ± 9.5 ††	101.4 ± 8.8 * ††
Insulin (ng/ml)	0.16 ± 0.06	0.05 ± 0.02	1.37 ± 0.39 ††	1.11 ± 0.47 ††
HOMA-R	0.88 ± 0.26	0.24 ± 0.09	11.16 ± 4.06 ††	8.35 ± 4.73 ††

Table 1: Clinical and biological parameters in WT and Tg mice under SC and HFD

These parameters were obtained at sacrifice after 16 wks of diet (SC or HFD), except for insulin plasma level and glycemia measured at basal time of OGTT.

Results are expressed as mean ± SEM.

* p<0.05, ** p<0.01, *** p<0.001 Tgvs WT mice (within the same diet)

†p<0.05, †† p<0.01, ††† p<0.001 HFDvs SC (within the same genotype)

AT: Adipose Tissue, BW: Body Weight, ND: Not Determined, SC: Standard Chow, HFD: High Fat Diet, HOMA-R: HomeostasisModel Assessment of Insulin Resistance (insulin (mUI/l)*glycemia (mmol/l)/22.5).