THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

INVESTIGATION OF THE ASSOCIATION BETWEEN CHRONIC INFLAMMATION AND IMPAIRED EFFEROCYTOSIS IN TWO MICE MODELS

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1. INTRODUCTION

1.1. Apoptosis

Initiating cell death at the right time and to the right extent, and effectively clearing dying cells is an essential for maintaining tissue homeostasis. In mammals, most cell death occurs through activation of the process of apoptosis. The central molecular mechanism of apoptosis was first described in C. elegans. The number of cells in the worms and the pattern of cells dying by apoptosis during their individual development were determined. It is traditionally associated with four different functions: 1) control of morphogenesis, 2) removal of unnecessary tissues and organs, 3) regulation of tissue cell number, 4) elimination of certain cells. This type of cell death is characterized by a series of nearly defined morphological changes that are nearly identical in almost all cells.

The mechanism of apoptosis within the body can be basically divided into three main phases: A) initiation and execution, B) DNA fragmentation, C) apoptotic body ingestion. After activation of the program, the volume of the cell decreases and then its surface becomes blistered, thus detached from adjacent cells and the extracellular matrix when rounded. The chromatin stock in the nucleus is highly condensed, marginalized - and later the DNA is fragmented. Eventually, attachment of the cell membrane results in apoptotic bodies containing cell organelles and nuclei, which are removed by surrounding phagocyte cells. In the process, the integrity of the cell membrane is maintained, but the double phospholipid asymmetry is eliminated, so that the constituents of the inner membrane are released to the surface, which serves as a signal for the phagocyte cells. Activation of the process of apoptosis is primarily of physiological significance in cells that are too large for macrophages, as the program allows the dying cell to disintegrate into small membrane-bounded small pieces and thus be ingested. In the case of small thymocytes, necrotic cell death also leads to ingestion through receptors used for apoptotic cells uptake.

1.2. Efferocytosis: The Clearance Process of Apoptotic Cells

As a result of ongoing cell death, millions of cells are cleared daily without the development of inflammation and immune responses by professional and non-professional phagocytes.

Efferocytosis is not a passive process, but a series of carefully composed steps designed to eliminate cell bodies and limit the release of potentially harmful substances while maintaining immunological tolerance. Efferocytosis can be generally categorized into 4 steps: 1) the release of ‘find-me' signals by dying cells to recruit phagocytes, 2) phagocyte recognition and engagement of ‘eat-me' signals on dying cells, 3) the engulfment of the cellular corpse, and 4) the processing, degradation, and immune response to the engulfed corpse. Upon detection of target cells, the apoptotic cell is able to attract macrophages to the site of death by releasing chemotactic “find signals”. These “find” signs can be soluble or packaged in a small membrane vesicle. Nucleotides such as ATP and UTP are key molecules for phagocytes in finding apoptotic cells. They are involved in activating chemotaxis not as chemotactic signals, but as enhancers of their signaling process, binding to receptors on their own and through their degradation products. Nucleotide release requires activation of pannexin 1 channel by caspase-3 on apoptotic cells. Apoptotic cells are able to attract monocytes to apoptotic cells by secreting lysophosphatidylcholine and sphingosine-1-phosphate molecules. Certain molecules, including the intercellular adhesion molecule 3 (ICAM3) and the proteolytically cleaved form of CX3C-chemokine ligand 1 (CX3CL1), are transported to the microparticles emitted by poptotic cells and transport chemotaxis to macrophages. Macrophage recruitment mediated by such vesicles is characteristic only of certain cell types, such as Burkitt's lymphoma or apoptotic B cells of the germinal center. In addition to attractants released to phagocytes, apoptotic cells also emit “stay away” signals to keep inflammatory immune cells away. Lactoferrin, as a “stay away” signal, prevents neutrophil chemotaxis and activation, and increased expression has been observed in several cell types following induction of apoptosis.

1.3. Effect of Defective Clearance of Dead Cells on Diseases

For some autoimmune processes, experiments and studies in animal and human lines also support a strong association between the development of a given disease state and a disorder of efferocytosis. Most knock out mouse strains in which a disorder of efferocytosis is observed develop autoimmune symptoms similar to those of systemic lupus erythematosus (SLE). Human SLE has also been shown to have impaired removal of dying cells, and there may be evidence of a genetically determined defect in phagocytosis underlying the manifestation of the disease. However, in addition to SLE, a number of other pathological conditions may be associated with impaired efferocytosis. Without wishing to be exhaustive, some of them are:

lung and respiratory diseases (COPD, asthma and cystic fibrosis, atherosclerosis, neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's disease, type 2 diabetes and obesity, and Chronic periodontal disease (periodontitis). Disordered efferocytosis leads to chronic inflammation for two reasons: untreated cells become secondary necrotic and their cell contents, when released into tissues, initiate inflammation, on the other hand, impaired efferocytosis is often associated with disruption of a mechanism that inhibits the inflammatory process activated by efferocytosis.

1.4. Tissue Transglutaminase (TG2)

Tissue transglutaminase is a unique enzyme with various biological activities. Transglutaminases (TG2s) are a family of thiol- and Ca2+-dependent acyl transferases that catalyze the formation of a covalent bond between the c-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the e-amino group of lysine in certain proteins. The reaction results in post-translational modification of proteins by establishing e-(c-glutamyl)lysine cross-linkages and/or covalent incorporation of polyamines and histamine into proteins. Eight distinct enzymatically active transglutaminases have so far been described. TG2 is very unique among them because besides being a transglutaminase it also possesses GTPase, protein disulfide isomerase, and protein kinase enzymatic activities.

TG2 is localized predominantly in the cytoplasm, however, substantial amounts of the protein are also found in the nucleus, plasma membrane, and in the extracellular matrix. Physiologically, the transamidation activity of TG2 is latent and is often manifested in various pathological states accompanied by rise in (Ca2+). The binding of GTP or Ca2+ inhibits, respectively, the transamidation or GTPase function of TG2, which are mutually exclusive enzymatic activities in vivo. Numerous recent observations point to a role of TG2 in cell–matrix interactions. Although TG2 has no leader sequence, hydrophobic domains, or posttranslational modifications for targeting to the endoplasmic reticulum or Golgi apparatus, it is present on the surface of various cell types and in the extracellular matrix. Outside the cell TG2 interacts with the major extracellular protein fibronectin and with integrins through a direct noncovalent interaction with the b1 and b3 integrin subunits and formation of stable ternary complexes with integrins and fibronectin.

This interaction induces integrin clustering regardless of integrin–ligand interaction and might modifiy integrin signaling. To Tissue transglutaminase is a cell surface protein participating in the ‘‘third synapse’’ created between apoptotic cells and macrophages.

1.5. Link Between TG2 and the Apopto-Phagocytosis Program

Fésüs, Falus, and Thomázy suggested in the late 1980s that TG2 may be involved in the apoptosis program of cells, as TG2 expression was found to be particularly high in tissues known to have rapid turnover and higher cell death rates. Since at that time almost nothing was known about the apoptosis program, it was hypothesized that it may have been the trigger. Indeed, in mouse thymocytes, the appearance of the protein precedes nuclear changes characterized by DNA degradation, and induction of TG2 has been detected in several models of in vivo apoptosis. Thus, protein expression is also increased during apoptosis of thymocytes initiated by various signals, during involution of the breast after lactation, during regression of the corpus luteum after birth, or in dying hepatocytes.

Induction of TG2 can also be detected in cells that die during embryonic development or in the dead T lymphocytes of HIV-infected individuals. All of these observations triggered a series of studies in which the dual role of TG2 in the regulation of apoptosis was observed.

1.6. Control of TG2 Expression in the Dying Thymocytes

In vivo expression of TG2 in dying thymocytes is regulated at the transcriptional level, as evidenced by the strong correlation of beta-galactosidase expression in mice carrying the beta-galactosidase reporter gene under the control of a 3.8 kb fragment of the TG2 promoter. endogenous TG2 expression. While TG2 is strongly induced in dying thymocytes in vivo, induction of TG2 is no longer observed in vitro when cell death is induced by the same stimulus. This suggests that signals from the tissue environment contribute to the in vivo induction of the enzyme in thymocytes under apoptosis. The first signal regulating TG2 expression was accidentally discovered when TG2 was found to be TG2-dependent. Because TG2 was known to activate latent TGF-β and TGF-β has a response element in the TG2 promoter, a possible mediating role for TGF-β arose, which was eventually demonstrated by injection of a TGF-β neutralizing antibody. However, TGF-β was only one of the signaling molecules produced by macrophages taking up apoptotic cells, which was found to contribute to the induction of TG2 in thymocytes. Our group showed that both retinoids and adenosine (efferocytosis) are

produced in a dependent manner in macrophages and contribute to the induction of TG2 in vivo.

1.7. Metabolism of Vitamin A

VA homeostasis is regulated by a network of enzymes and proteins involved in the transport, production, and catabolism of retinoids. VA's physiological functions are mainly mediated by its metabolites, retinal, and RA. This is achieved via a series of enzymes catalyzing the conversions. Two oxidation steps occur during the conversion from retinol to retinal (retinaldehyde) and then from retinal to RA. Retinol is reversibly converted into retinal, and retinal is irreversibly converted into RA. Retinol is reversibly oxidized into retinal by two families of enzymes: alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs) or short-chain dehydrogenases/reductases (SDRs). Two RDHs (RDH2 and RDH10) play major roles in this step in different tissues. The produced retinal plays important roles in physiology. For example, vision is mediated by 11-cis retinal conjugated to rhodopsin in response to photon activation. Recently, retinal has been considered an antagonist for the activation of peroxisome proliferator-activated receptor γ (PPARγ). In hepatocytes, RA can be further modified by enzymes such as cytochrome P450 26A1 (CYP26A1) to more hydrophilic products.

1.8. Retinol Saturase (RetSat) Enzyme

Retinol saturase (RetSat) is an NADH/NADPH- or FADH-dependent oxidoreductase and strongly expressed in liver, adipose tissue and kidney. Major transcriptional regulators of *RetSat* expression include peroxisome proliferator-activated receptor α (PPARα) and forkhead box O1 (FoxO1) in liver2–4, and PPARγ in adipose tissue, where RetSat's expression is robustly induced during the differentiation of precursor cells into adipocytes5 Retinol saturase performs a stereospecific saturation of the C13–C14 double bond of all-trans-retinol to generate (13R)-all-trans-13,14-dihydroretinol. This compound is found in cells expressing retinol saturase and in the livers of mice fed with retinyl palmitate. All-trans-13,14-dihydroretinol (dihydroretinol) is oxidized in vivo to all-trans-13,14-dihydroretinoic acid, a highly selective agonist of the retinoic acid receptor (RAR), and to 9-cis-13,14-dihydroretinoic acid, a highly selective agonist of the RXR receptor. In fact, it is suggested that 9-cis-13,14-dihydroretinoic acid might be the long-sought physiological RXR ligand. However, it was also

demonstrated that RetSat might perform other biological functions as well, as not all the consequences of its loss could be restored by dihydroretinol administration.

1.9. Involvment of Retinoids in Clearance of Apoptotic Cells

Although retinoids produced by macrophages that during apoptotic cell uptake are involved in regulating TG2 expression in apoptotic cells, this is not the only function of the resulting retinoids. Previous experiments in our laboratory have shown that retinoids and retinoid receptor agonists significantly increase the ability of macrophages to remove apoptotic cells in vitro. This effect is achieved by retinoids enhancing the expression of 5 (including TG2) phagocytosis-associated proteins through RARα activation. Retinoids that also activate RXR receptors have been shown to be more effective, probably because they have also been able to activate the function of PPAR and LXR receptors that enhance the transcription of certain phagocytosis genes through ligation of RXR. During phagocytosis of apoptotic cells, retinoids are produced in macrophages themselves by lipid-sensing nuclear receptors activated by lipids of apoptotic cells to induce both retinol saturase and RALDH expression, which are involved in retinoic acid production, and RARα expression. The resulting retinoids increase the expression of several phagocytosis-associated proteins, in part through the activation of RARR heterodimeric transcription factors that enhance phagocytosis.

1.10. Obesity: Immuno-Metabolic Diseases

Obesity is a metabolic disorder in which the energy balance is shifted towards increasing food intake due to various effects (genetic, nervous, endocrine or environmental). As a result of increased food intake, the number of fat cells increases, and then these cells become larger in size - hypertrophic - due to the storage of increasing amount of fat. In addition to storing energy, adipose tissue also functions as an endocrine organ, secreting various bioactive substances. It is a specific hormone of adipokines, the effects which can be sensed by the liver, muscle tissue and brain, thereby affecting the body's energy balance and metabolic function. However, in obesity, adipokine production of hypertrophic adipocytes is altered, the ratio of pro-inflammatory and anti-inflammatory adipokines is upset: less adiponectin is produced and more resistin and leptin are secreted. The large increase in abdominal fat mass cannot be followed by vascularization processes, so hypoxia develops in the tissue. Under hypoxia,

adipocytes die by Bid-mediated apoptosis, and released chemoattractants, MCP-1, a soluble IL-6 receptor, migrate into the tissue with macrophages.

Ingestion and clearance of dying adipocytes by migrating macrophages is key in activating pathological processes associated with obesity. Adipocyte death is so crucial in initiating obesity-related pathological processes that in Bid-deficient mice, where hypertrophic adipocyte death occurs later, despite the same rate of obesity, pathological processes can only be detected in proportion to adipocyte death. The proportion of macrophages in the adipose tissue of obese individuals can be as high as 50%. The processes result in the development of a chronic low-level inflammation, which is facilitated not only by inflammatory factors in adipocytes but also by the phenotype of macrophages. This is because, in contrast to non-obese macrophages in normal adipose tissue, which inhibit inflammatory processes, there are metabolically activated macrophages in obese adipose tissue. This type of macrophage develops in metabolic diseases with high levels of glucose, insulin, and palmitate, and produces inflammatory cytokines (IL1β, TNFα, MCP-1), contributing to the inflammatory environment in adipose tissue.

1.11. Clearance of Adipocytes by Phagocytosis During Obesity

Because adipocytes are significantly larger in size than macrophages and contain large amounts of lipids, they are not ingested and cleared by conventional phagocytic routes. Macrophages that have migrated into adipose tissue are organized around adipocytes and have a crown-like structure, which are actually two-dimensional histological representations of the simultaneous uptake of a dead adipocyte by a number of macrophages directly surrounding the adipocyte. Hypertrophic adipocytes release triglycerides and non-esterified fatty acids that can be internalized by macrophages. After uptake of large amounts of lipids from macrophages, foaming cells develop, which saturate their metabolic system and are unable to perform their function properly, thereby contributing to the development of metabolic syndrome. Macrophages are able to digest larger particles that bind tightly to the extracellular matrix in an extracellular acidic lytic compartment. This process is exophagia observed in atherosclerosis when examined for degradation of aggregated LDL by macrophages. In obesity, similar mechanisms play a role in the clearance of fat cells. The process was termed lysosomal exocytosis because histological images showed that lysosomes expressing the LAMP1 molecule accumulated at the macrophage adipocyte border, the membrane of which seemed to fuse with the macrophage cell membrane and simultaneously with the adipocyte membrane.

2. AIMS OF THE STUDIES

I. Previous studies have demonstrated that adipocyte apoptosis and impaired clearance of apoptotic adipocytes presumably play a key role in the development of various chronic inflammatory diseases. My aim was to investigate that the high lipid content of the apoptotic adipocyte has any effect on efferocytosis. To answer this question we tested that genetically impaired efferocytosis (loss of TG2) can contribute to the development of metabolic syndrome in mice on HFD.

II. Previous studies from our laboratory have shown that during engulfment of apoptotic cells, retinoids are formed in a retinaldehyde dehydrogenase-dependent manner that upregulate various efferocytosis-related genes, leading to enhanced phagocytic capacity during long-term efferocytosis. Our data also indicated that the retinoids formed might be products of the RetSat pathway. In the second part of my work my goal was to investigate that whether loss of RetSat in mice affects efferocytosis.

3. MATERIALS AND METHODS

3.1. Reagents

All reagents were obtained from Sigma-Aldrich (Budapest, Hungary) except when indicated otherwise.

3.2. Experimental Animals

Animals and diets Eight-week-old male TG2 deficient54 and wild type mice on a C57Bl/6 background generated from heterozygous parents were housed in separated cages (with water and food ad libitum) during the 16 weeks of feeding experiment. In the first 2 weeks, animals were kept on highsucrose/low-fat (HSD; 10% kcal% fat; 17% kcal% sucrose; Research Diets Inc., D12450H) After this run-in period, mice were divided into two groups: the HSD group remained on HSD, whereas the HFD group received highsucrose/high-fat (HFD; 45% kcal% fat, 17% kcal% sucrose; Research Diets Inc., D12451) diet during the following 14 weeks. The control group of mice were kept all the time on normal diet (ND; 13% kcal% fat; 4.6% kcal% sucrose; Special Diets Services, VRF1 (P)). Bone marrow transplanted (BMT) mice were maintained in a specificpathogen-free status (autoclaved top filter cages) for the entire course of experimentation, and antibiotics (amoxicillin antibiotic, clavulanic acid, 500 mg/125 mg l−1 of drinking water) were administered in the drinking water following 4 weeks post-transplantation. Following 2 weeks of ND after BMT, they were kept on HFD diet for 16 weeks. For LXR ligand treatment in vivo, mice were fed after the bone marrow transplantation on HFD supplemented with 20 mg/kg/day GW3965 (AbMole, M1929) as described above. Mice were maintained under a 12 h light: 12 h darkness cycle and had access to food and water ad libitum. The body weight and food intake of the animals were registered weekly. For tissue collection, mice were killed by isoflurane overdose at week 17 in accordance to the University of Debrecen.

Either, experiments were carried out with 4 week, 2–4 month or 1 year old C57BL/6J RetSat+/+ mice and their RetSat−/− littermates. For determining gene expression in the thymus or thymic cell composition, 4 week old mice were injected intraperitoneally with either 0.3 mg dexamethasone acetate (DEX) dissolved in dimethyl sulfoxide (DMSO) or vehicle alone, or exposed to 5 Gy irradiation. For determining the long-term effect of DEX treatment in the

thymus, 4 week old mice were injected with 1 mg/kg DEX daily for 6 days, then exposed to 0.5 Gy irradiation as described by Lauber et al. Mice were maintained in specific pathogen-free conditions in the Central Animal Facility, and all animal experiments were approved by the Animal Care and Use Committee of the University of Debrecen (DEMÁB).

3.3. Bone Marrow Transplantation

Recipient BoyJ, TG2 null and TG2+/+ wild mice (7 weeks old, males) were irradiated with 11 Gy using a Theratron 780C cobalt unit for the ablation of the recipient bone marrow. The animals to be irradiated were immobilized using a circular cage (mouse pie cage) that could hold up to 11 mice (alert mice). Following irradiation, isolated bone marrow cells (in sterile RPMI-1640 medium) flushed out the femur, tibia, and humerus from donor BoyJ, TG2 null or TG2+/+ mice were transplanted into the recipient mice by retro-orbital injection (20 × 106 bone marrow (BM) cells per mouse). This experimental BMT CD45 congenic model allowed us to detect donor, competitor and host contributions in hematopoiesis and repopulation efficiency of donor cells (congenic mice with CD45.1 versus CD45.2). The CD45.1 and CD45.2 contribution were then detected by flow cytometry usually 8–12 weeks following BMT. In short a cut at the tail tip of the mice provided a drop of blood that was placed into 0.5 ml phosphate-buffered saline (PBS) + 1% fetal bovine serum + 10 U ml−1 heparin buffer (Sigma Aldrich H3393) (samples kept on ice). The cells were directly stained by 2 μl mouse anti-mouse CD45.2-FITC (clone 104) and 2 μl rat anti-mouse GR1-PE (clone RB6-8C5) antibodies (BD Pharmingen) and incubated on ice for 30 min. After two washes with ice-cold PBS–fetal bovine serum–heparin buffer, cells were resuspended in 0.5–1 ml BD FACS lysing solution (BD cat no. 349202), incubated for 5 min at RT then centrifuged (400 g, 5 min, 4 °C). The double stained samples were run on FACS (BD FACS Calibur) and the ratio of donor cells was determined. The repopulation was over 95% gated on the granulocyte fraction.

3.4. Isolation and Metabolic Activation of BMDMs

Bone marrow cells were isolated from femurs of 3–6 months old male TG2 null mice and their wild type counterparts. Bone marrow macrophages (BMDMs) were differentiated in DMEM supplemented with 10% FBS (12106C), 2 mM L-glutamine (G7513), 1 mM Napyruvate (S8636), 50 μM 2-mercaptoethanol (M3148) and 100 U/ml penicillin/100 μg/ml

streptomycin (P4333) all from Sigma-Aldrich and 10% L929 fibroblast conditioned media for 7 days. For metabolic activation, differentiated macrophages were treated with a combination of 30 mM D-glucose (G8270), 10 nM insulin (12643) and 0.4 mM sodium-palmitate (P9767) all from Sigma-Aldrich for 24 h (16). Sodium palmitate was prepared by diluting a 200 mM stock solution in 70% ethanol into 10% fatty acidfree, low-endotoxin BSA (Sigma Aldrich, A8806 adjusted to pH 7.4) to obtain a 5 mM palmitate-BSA stock solution that was filtered using a 0.22-μm low-protein binding filter (Millipore). BSA/ethanol was used in control treatments during the protocol. In some experiments during the 24 h metabolic activation BMDMs were treated with PP2 (Sigma Aldrich, 529573), a reversible ATPcompetitive inhibitor of the Src family of protein tyrosine kinases, in 2 μM final concentration or 0.5 mg/ml RGD peptide (Cayman Chemical, 529573).

3.5. Cell Culture and Differentiation of 3T3 Cells

3T3-L1 murine preadipocytes (ATCC:CL-173) were maintained as subconfluent cultures in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 4.5 g/l D-glucose, 2 mM L-glutamine and 10% (v/v) bovine calf serum. For differentiation, after 2 days postconfluency, differentiation was induced by addition of 2 μg/ ml insulin, 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, I5879), and 0.25 μM dexamethasone (Sigma-Aldrich, D4902). The cells were maintained in this medium for 3 days and then for 2 more days in medium supplemented with 1 μg/ml insulin. After 10 days of differentiation, FBS was omitted from the medium and the cells were maintained for another day in order to induce apoptosis55. The basal cell death index was very low (, but following serum withdrawal the percentage of annexin V positive cells increased significantly (≥90 after 24 h).

3.6. Determination of Adipocytes-Like Morphology of Differenciated 3T3-L1

Differenciated 3T3-L1 cells were dyed with 1 μg/ml Nile Red for 30 min. EVOS® FL Cell Imaging microscopy were used to analyse the lipid content of the cells.

3.7. Adypocite Associated (UCP-2 PPAR γ, Leptin, Resistin) Determination In Differenciated 3T3-L1

qRT-PCR was carried outin triplicate using pre-designed FAM-labeled MGB assays (Life Technologies, Budapest, Hungary) on a Roche LightCycler LC 480 real-time PCR instrum qRT-PCR was carried out in triplicate using pre-designed FAM-labeled MGB assays (Life Technologies, Budapest, Hungary) on a Roche LightCycler LC 480 real-time PCR instrument.

QPCR mix:

Nuclease free water: 2,04μl

$MgCl_2$ (25 mM): 1,2μl

Buffer (10x): 1μl

dNTP (2,5 mM): 0,5μl

Random primers (40X oligomix): 0,2μl

Taq Polimease (5 U/μM): 0,062μl

Final volume: 5μl / well

Relative mRNA levels were calculated using the comparative CT method and were normalized to β-actin or GAPDH mRNA.

3.8. Insulin Resistance Test, Intraperitoneal Glucose Tolerance Test (IPGTT), Insulin Determination

Insulin resistance test and IPGTT was performed on week 15 and 16, respectively. After a 6 h fasting period, 0.75 IU/bwkg insulin (ACTRAPID Penfill 100 IU/ml, Novo Nordisk) or 2 g/bwkg glucose was injected intraperitoneally. Blood glucose levels were determined with DCont Trend glucose monitor (DCont, Hungary) at the indicated time points after insulin or glucose injection. Plasma insulin levels were determined by Mouse Insulin ELISA kit (ALPCO, 80-INSMS-E01) according to the manufacturer's instructions.

3.9. Collection of Tissue Samples

At the end of the feeding phase, the animals were sacrificed with isoflurane, body weight, body length, liver, and gonadal white adipose tissue (gWAT) weight were measured. Blood, liver, and gWATsamples were collected for subsequent analysis. Blood samples were allowed to clot and spun at 12.000 rpm for 15 min to collect plasma. For histological analysis, adipose tissue and liver samples were fixed in 4% paraformaldehyde; for gene expression and liver triglyceride determination were frozen in liquid nitrogen and stored at −80 °C prior to extraction.

3.10. Adipocyte And Adipose Tissue Macrophage (ATM) Isolation

gWAT tissue was dissected and washed and kept in transport buffer (DMEM supplemented with 1% Penicillin–Streptomycin solution and 1% bovine serum albumin (BSA). The tissue was minced, digested for 30–60 min at 37 °C in digestion solution (HEPES buffer (pH 7.4, H3375) supplemented with 20 g/L BSA and 0.5 g/L collagenase type 1 (C6885); all from SigmaAldrich, and filtered through a nylon filter (100 μm). After a centrifugation step, adipocytes were collected and stored at −80 °C prior to RNA isolation. After the removal of red blood cells by hemolysis (ACK Lysing Buffer, Thermo Fisher Scientific, A1049201) the stromal vascular cell fraction was resuspended in staining buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA (Sigma-Aldrich, E6758)). ATMs were isolated from SVCs using MACS Technology (F4/80-based positive selection; (Miltenyi Biotech, 130-097-050, 130-117-509) according to the manufacturer’s instructions.

6.11. Liver Triglyceride Levels

Triglyceride concentrations were quantified in saponified, neutralized liver extract (digested in ethanolic KOH (Sigma-Aldrich, P5958) overnight at 55 °C by glycerol enzymatic assay (Free Glycerol Reagent, Glycerol Standard Solution, Sigma-Aldrich, F6428, G7793) according to the manufacturer's instructions.

6.12. Histology And Immunohistochemistry

Hepatic tissues were fixed in 4% neutral buffered formaldehyde (Sigma-Aldrich, F8775) and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin (H&E; Sigma-Aldrich, HT110116, GHS116) stain. Histological sections were analyzed on Leica DMRB/E light microscope (Heerbrugg, Switzerland).

After paraffin embedding, 6 μm thick sections were cut from gWAT tissue samples. The sections were mounted on glass slides, deparaffinized and kept in 10% normal goat serum (Thermo Fisher Scientific, 31872) for 50 min at room temperature. The sections were first incubated with an antibody raised against the macrophage marker F4/80 in rat (diluted 1:1000, Hycult Biotech, HM1066), then transferred into a solution of goat anti-rat IgG conjugated with Alexa Fluor 555 (diluted 1:1000, Molecular Probes, A21434). We have noticed that the fluorescein-labeled anti-digoxigenin antibody offered by the Apoptag fluorescein kit (Millipore, S7110) has strong aspecific binding in our adipocyte tissue sections. This aspecific signal was used to visualize the cellular architecture of gWAT tissue. The immunostained sections were covered with a Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, H-1200). The number of adipocytes and nuclei was determined by ImageJ software, while the number of CLS was counted manually.

6.13. RNA Isolation And Real-Time Quantitative (RT-QPCR)

TRIzol reagent (UD-Genomed, URN0102) was used to isolate total RNA from samples. cDNA was synthesized with High-Capacity cDNA Archive Kit (Thermo Fisher Scientific, 4368813) according to the manufacturer's instruction. Gene expression levels were determined with qRT-PCR using FAM-MGB labeled Taq-Man probes (Thermo Fisher Scientific) by Real-time PCR on Roche Light Cycler 480 platform. Samples were run in triplicates. Expression values were normalized to GAPDH housekeeping gene.

6.14. Time-Lapse Imaging Microscopy

3T3-L1 originated adipocytes were stained with Nile red to identify lipid droplets, while macrophages with Hoechst 33342 (Thermo Fischer Scientific, 62249) to recognize macrophage nuclei and with 10 μM 5-carboxyfluorescein diacetate (CFDA) (Sigma-Aldrich, C4916) to recognize macrophage cytosol. Wild type or TG2 null macrophages were layered on top of the apoptotic adipocytes in a ratio of 5:1. The co-culture was placed in a temperature-, humidity- and CO2-controlled, motorized Olympus IX-81 inverted microscope (Olympus America), which was equipped with a cooled Hamamatsu ORCA-R2 (Hamamatsu Photonics, Hamamatsu City, Japan) highresolution monochrome CCD camera and a DP21-CU 2-megapixel digital color camera (Olympus America). Cells were monitored for 5 h and in every 5 min, an image was taken. Data were converted into a video file with the use of the Xcellence software (Olympus America). Lipid contents of adipocytes induced to undergo apoptosis by serum starvation were labeled by Nile red (Thermo Fisher Scientific, N1142).

6.15. Western Blot

Mme BMDMs were washed with PBS, dissociated by lysis buffer (50 mM Tris (T5941) pH 6.8, 2% SDS (L3771), 5% glycerol (G5516), 2 mM DTT (D0632), 2.5 mM EDTA, 2.5 mM EGTA (E3889), all reagents from Sigma-Aldrich), supplemented protease and phosphatase inhibitor cocktail (Sigma-Aldrich, P8340, 524629). Samples were boiled for 10 min with SDS-sample buffer at 100 °C. The samples were separated by 12% SDS-PAGE and transferred to PVDF membrane (Bio-Rad, 1620177). The membrane was blocked with TBST buffer (10 mM Tris, pH 8.0, 0.15 M NaCl (S7653), and 0.05% Tween 20 (P1379); all reagents from Sigma-Aldrich) containing 5% BSA for 1 h at room temperature and then probed with anti-phospho(Tyr416)- Src, c-Src (Cells Signaling Technology, 2101, 2108) and beta-actin (Sigma-Aldrich, 5441) primary antibodies overnight at 4 °C. Beta-actin was used for the loading control. After washing with TBST buffer, blots were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Proteins were detected with chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Merck Millipore, WBKLS0500). The pixel density of bands was determined by Image J software.

6.16. Flow Cytometry Analysis of Freshly Isolated Thymocytes

At the indicated time points after the various treatments of mice, thymocytes were isolated, washed twice, and resuspended in ice-cold PBS before staining either with phycoerythrin (PE)-labeled anti-CD4 and Cy5-conjugated anti-CD8 (Pharmingen, San Diego, CA, USA) antibodies or with fluorescein isothiocyanate (FITC)-labeled annexin V in binding buffer. Cell-bound fluorescence was analyzed in a blinded fashion using a FACSCalibur (Beckton Dickinson).

6.17. Thymocyte Apoptosis in Vitro

Isolated thymocytes (105 cells/mL) were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, 1 mM Na-pyruvate, and 5 × 10−5 M 2-mercaptoethanol at 37 °C/5% CO2. Apoptosis was induced by addition of 0.1 μM DEX. After 6 h, the extent of cell death was determined by Annexin V-FITC labeling of apoptotic cells. Cell-bound fluorescence was analyzed by FACSCalibur (Beckton Dickinson).

6.18. Retinoid Measurement by High-performance Liquid Chromatography Mass Spectrometry (HPLC/MS/MS)

Four-week-old RetSat-null mice were injected i.p. with either 0.3 mg dexamethasone acetate (Dex) dissolved in DMSO alone or with N,N-diethylaminobenzaldehyde (DEAB) (0.24 mg/g body weight) or vehicle. After 24 h, thymi were removed in the dark, snap-frozen in liquid nitrogen, and stored at −70 °C. Concentrations of retinoic acids were determined in mouse thymi by our HPLC-MS-MS method. In summary, 100 mg of the thymic samples (if samples were under 100 mg, water was added to attain a 100 mg sample) were diluted with a threefold volume of isopropanol, the tissues were minced using scissors, vortexed for 10 s, put in an ultrasonic bath for 5 min, shaken for 6 min, and centrifuged at 13,000 rpm in a Heraeus BIOFUGE Fresco (Kendro Laboratory Products, East Coast, Southern, US) at +4 °C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany) at 30 °C. The dried extracts were resuspended with 60 μL of methanol, vortexed, shaken, diluted with 40 μL of 60 mM aqueous ammonium acetate solution, transferred into the autosampler, and subsequently analyzed using HPLC-MS-MS equipment. In addition, we focused on detecting novel dihydro-retinoic acid derivatives, and we switched our MS-MS to single ion recording (SIR) mode, focusing on 303 m/z signals in relative intensity.

6.19. Bone-Marrow-Derived (BMDM), Peritoneal, or Thioglycolate-elicited Macrophage Generation, Cell Culture and Treatment

Bone marrow progenitors were obtained from the femur of 2 to 4 month old mice by lavage with sterile physiological saline. Cells were differentiated for 5 days in DMEM medium supplemented with 10% conditioned medium derived from L929 cells, as a source for macrophage colony stimulating factor (M-CSF), 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in 5% CO2. Non-adherent cells were washed away every second day. BMDMs from both wild type and RetSat-null mice were collected at the end of differentiation to determine their total mRNA expression levels and every day during their differentiation alone or in the presence of all-trans-13,14-dihydroretinol (Santa Cruz Biotechnology, Heidelberg, Germany) for qRT-PCR analysis of the expression of their various genes. Tissue-resident peritoneal macrophages were isolated by peritoneal lavage with RPMI 1640 medium. For generating thioglycolate-elicited peritoneal macrophages, mice were treated with 4 mL of 4% thioglycolate (in PBS), and four days later, macrophages were collected by peritoneal lavage. Peritoneal macrophages were then seeded on 24-well plates in RPMI 1640 medium containing 10% fetal calf serum (FCS) (1 × 106 cells). BMDMs or tissue-resident peritoneal macrophages were treated with various compounds as indicated in the figure legends, and their phagocytic capacity or mRNA expressions were also determined.

6.20. In Vitro Apoptotic Cell Phagocytosis

BMDMs or peritoneal macrophages were stained for 24 h with 10 μM 5(6)-carboxyfluorescein diacetate (CFDA-SE) (Invitrogen, Carlsbad, CA, USA). To generate apoptotic thymocytes, the thymus was collected from 4 week old C57BL/6 mice, thymocytes were isolated and cultured for 24 h (107 cells/mL) in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2.5 μM Deep Red dye (Invitrogen, Carlsbad, CA, USA) in the absence of serum. The percentage of apoptotic thymocytes determined by annexin V labeling was ≥ 80%. Stained apoptotic thymocytes were added to the macrophages in a 5:1 (apoptotic cells:macrophage) ratio for 1 h. After coculture, apoptotic cells were washed away and macrophages were detached by trypsinization. For long-term phagocytosis assays, macrophages were first exposed to unstained apoptotic cells for 5 h and then to the stained apoptotic cells for an additional hour. Cells were analyzed using either FACSCalibur or confocal microscopy.

6.21. Confocal Microscopy

Peritoneal and bone-marrow-derived macrophages from wild type (WT) and RetSat−/− mice were plated in 8-well chamber slides (3 × 105/well) (Gräfelfing, Germany). Phagocytosis assays were carried out as described above. Macrophages were then washed and fixed in 1% paraformaldehyde. Calculations and statistical analyses were based on microscopic images. Fluorescence confocal images were taken with a Zeiss LSM 880 invert microscope using appropriate excitations and emission filters corresponding to the different fluorescence markers (objective: C-Apochromat 40x/1.2 W Korr; 488 nm excitation with 493–600 nm emission range; 633 nm excitation with 638–755 nm emission range). Pixel size and sampling was fit to an appropriate wavelength and followed the Nyquist rate. A high resolution tile scan automatic focus readjusting system was used. Further image analysis was undertaken in Zeiss Zen and ImageJ to determine the number of ingested apoptotic cells.

6.22. mRNA Sequencing

To obtain global transcriptome data of wild type and RetSat-null BMDMs, high-throughput mRNA sequencing analysis was performed on an Illumina sequencing platform. The quality of total RNA samples was checked on an Agilent BioAnalyzer using the Eukaryotic Total RNA Nano Kit according to the manufacturer's protocol (Agilent, Santa Clara, CA, USA). Samples with RNA integrity number (RIN) values > 7 were accepted for the library preparation process. RNA-Seq libraries were prepared from total RNA using the TruSeq RNA Sample preparation kit (Illumina) according to the manufacturer's protocol. Briefly, poly-A RNAs were captured by oligo-dT conjugated magnetic beads, and then the eluted mRNAs were fragmented at 94 °C. First-strand cDNA was generated by random priming reverse transcription, and after a second-strand synthesis step, double-stranded cDNA was generated. After repairing ends, A-tailing and adapter ligation steps, adapter ligated fragments were amplified in enrichment PCR, and finally, sequencing libraries were generated. Sequencing runs were executed on an Illumina HiSeq2500 instrument using single-end 50 bp sequencing. Gene ontology (GO) term enrichment analysis of the 117 differentially expressed genes was carried out using Search Tool for the Retrieval of Interacting Genes v10 (STRING), covering both physical interactions and functional associations between proteins (JensenLab, https://string-db.org/).

6.23. Anti-Nuclear Antibody Detection by Indirect Immunofluorescence Assay

Anti-nuclear antibodies were detected on HEp-2 cells using an indirect immunofluorescence assay kit (Euroimmun GmbH, Lübeck, Germany) according to the manufacturer's instructions, with the only modification of replacing the original conjugate solution with a goat anti-mouse IgG (H+L) antibody labelled with Alexa Fluor 488 (A-11001, Thermo Fisher Scientific, Rockford, USA, 1:400 dilution). The fluorescence pattern and intensity of each well were read visually on a fluorescence microscope with an LED light source (Eurostar II Plus). The fluorescence intensity was recorded on a semi-quantitative scale (negative, 1+, 2+, 3+, 4+ positive).

6.24. Anti-dsDNA Antibody ELISA

Samples were tested for anti-dsDNA antibodies using the immunometric, direct ELISA test kit (ORG 604) from Orgentec Diagnostika GmbH, Mainz, Germany. In order to detect mouse autoantibodies, the original conjugate was substituted with a sheep anti-mouse IgG antibody conjugated with peroxidase (1:10000 dilution). After stopping the chemical reaction in the wells, the optical densities (OD) were read at 450 nm on an ETI-MAX 3000 ELISA processor.

6.25. Caspase-3 Immunohistochemistry

Spleens were removed from 1 year old wild type or RetSat−/− mice and fixed in formaldehyde (4% in phosphate buffer) for 1 day. Dehydration was carried out before embedding into paraffin according to standard protocol. After paraffin became solid, blocks were cut with a microtome to obtain 3–4 micrometer thick sections. After the deparaffination and hematoxylin and eosin (HE) staining, immunohistochemical staining was performed using anti-Cleaved Caspase-3 (Cell Signaling Technology, Inc, Global Headquarters USA) primary monoclonal antibody at a dilution of 1:300 and an horse radish peroxidase (HRP)-labelled polymer anti-rabbit secondary antibody (Dako, Glostrup, Denmark). The intensity and distribution of immunostaining was assessed by light microscopy (Leica DM2500 microscope, DFC 420 camera and Leica Application Suite V3 software; Leica).

6.26. Detection of IgM-Containing Immune Complexes

The kidney was removed from 1 year old RetSat+/+or RetSat−/− mice and horizontally sliced, and then tissue blocks were snap-frozen with isopentane in liquid nitrogen. Four-micrometer cryosections were cut, fixed in cold acetone, and incubated with FITC-labeled anti-mouse IgM diluted in PBS (1:40). After rinsing in PBS, slides were mounted in buffered glycerol. Immunostaining was assessed by light microscopy (Leica DM2500 microscope, DFC 420 camera and Leica Application Suite V3 software; Leica).

6.27. Determination of Serum Urea Concentration

The method described by Rahmatullah and Boyde was used.

6.28. Statistical Analysis

Data are presented as mean ± SD for all data. All statistical analyses were performed using GraphPad Prism 6.01 and a P-value < 0.05 was considered as significant and is indicated by asterisk (*). For differences between 2 groups 2-tailed unpaired Student's t-test, for comparisons n > 2 groups one- ANOVA (with Turkey's multiple comparisons test) was used. n = 8 mice/group were used for the experiments except for BM transplanted population, where n = 5 mice were investigated. For datasets split on two independent factors, 2-way ANOVA was used.

7. RESULTS

7.1. Effect of TG2 deficiency on pathological changes induced by high fat diet in mice

7.1.1. Effect of TG2 deficiency on adipocyte clearence and high fat diet-induced pathological changes

Wild type mice exposed to either high sucrose/high fat diet (HFD) or to high sucrose diet (HSD) developed marked obesity compared with animals kept on a standard control diet (ND) (40.0±5.5 versus 33.9±2.6 versus 27.2±0.6g body weight of mice kept on HFD, HSD or ND, respectively, $p<0.01$). Loss of TG2 did not affect significantly the weight gain of the animals kept on the three different types of diet As seen in Fig. 1b, the gonadal fat tissue (gWAT) weight also increased significantly in both wild type and TG2 null mice exposed to HSD or HFD as compared to mice kept on control diet. However, in case of TG2 null mice kept on HFD the gonadal fat was significantly less than in wild type mice kept on HFD very likely due to the enhanced adipocyte apoptosis in TG2 null gonadal fat at this time point (see later). Loss of TG2 did not affect the obesity-related alterations in the expression levels of the adiponectin and resistin, but obesity-induced expressions of TNFα, MCP-1, and leptin were significantly enhanced in the gWAT adipocytes of TG2 null animals as compared to wild type mice (Fig. 1c). HSD and HFD significantly induced the mRNA expression of the pro-inflammatory cytokines in adipose tissue macrophages as well, which was more pronounced in the absence of TG2. All these data indicate that though loss of TG2 does not affect weight gain, inflammation is much more pronounced in the gWAT of TG2 null animals during diet-induced obesity.

7.1.2. Enhanced Adipocyte Apoptosis In The Gonadal Fat During Diet-Induced Obesity In TG2−/− Mice

Enhanced adipocyte apoptosis in the gonadal fat during diet-induced obesity in TG2−/− mice As obesity promotes adipocyte death, we decided to determine the expression of various pro- and antiapoptotic genes in the adipocytes of gWAT.In the gWAT adipocytes not only the expression of the pro-apoptotic Bid, but the expression of the pro-apoptotic Bim was also induced during diet-induced obesity. Interestingly, not only the pro-apoptotic, but mRNAs of two anti-apoptotic genes, Bcl-2 and Bcl-xl, were also induced during diet-induced obesity. But the levels of Bcl-2 mRNA were significantly dropped in the TG2 null mice kept on HFD as compared to their wild type counterparts. On the other hand, the anti-apoptotic Mcl-1 levels were decreased, while the expression of TG2 was not altered in the gWAT adipocytes during diet-induced obesity (Supplementary Fig. S1f). Altogether these data are indicative for more dietinduced adipocyte death in the gWAT of mice kept on HFD in the absence of TG2. Indeed, in the gWAT of TG2 null mice kept on either HSD or HFD significantly more dying cells were detected than in their wild type counterparts by identifying dead adipocytes as labeled by CLS.

7.1.3. Loss of TG2 Enhances Hepatic Steatosis And Insulin Resistance In Mice During Diet-Induced Obesity

Since increased gonadal fat apoptosis and high proinflammatory cytokine production are associated with ectopic fat storage including hepatic steatosis10 and insulin resistance16, we measured the development of hepatic steatosis, alterations in glucose tolerance and in insulin resistance in these mice. Together with the enhanced gWAT adipocyte apoptosis) in TG2 null mice kept on HFD both the liver weight and the triacylglycerol content of the livers were significantly higher than that of the wild type mice kept on HFD indicating translocation of more lipids from the gonadal fat into the liver. Tissue sections of livers stained with haematoxylin eosin confirmed these findings. Glucose tolerance tests demonstrated that as compared to mice fed on ND, HSD and HFD decreases the glucose tolerance. However, loss of TG2 did not worsen the results of the glucose tolerance tests. Based on insulin tolerance tests, however, animals lacking TG2 fed on HFD are more insulin resistant These results were corroborated by enhanced fasting plasma insulin levels as compared to their wild type counterparts.

7.1.4. Loss of TG2 In Non-Bone Marrow-Related Cells Leads To Enhanced Circulating Plasma Insulin Levels And Insulin Resistance

TG2 is expressed not only by macrophages, but also by adipocytes and hepatocytes. To determine, whether loss of TG2 in bone marrow-derived cells or in other cell types is responsible for the above changes, wild type or TG2 null mice were terminally irradiated, and their bone marrow was replaced by a bone marrow originated from either wild type or TG2 null mice in each combination. To confirm successful ablation and reconstitution, we involved wild type BoyJ (a CD57BL/6 variant strain) mice in these experiments, which express the CD45.1 allelic variant of CD45, while C57BL/6 mice are CD45.2 positive. Hematologic and flow cytometric analysis of these mice following bone marrow transplantation demonstrated a leukocyte repopulation over 95%. Irradiated CD57BL/6 mice gained significantly less weight, than their non-irradiated counterparts by the end of the 16 weeks feeding, in line with previously published studies33,34. As compared to the wild type counterparts, loss of TG2 in the non-bone marrow-derived cells did not affect significantly the percentage of dying adipocytes, the gonadal weight, gWAT adipocyte Bid expression, liver weight, liver triacylglycerol content or adipocyte pro-inflammatory cytokine or adipokine mRNA expression either, with the exception of TNF-α. On the other hand, the amount of resistin and IL-6 produced by adipose tissue macrophages were significantly higher in mice lacking TG2 in in the non-bone marrow-derived cells with no alterations in their TNF-α or MCP-1 production. In addition, we could detect significantly increased fasting circulating plasma insulin levels and increased insulin resistance by using insulin tolerance test. Altogether our data indicate that loss of TG2 in non-bone marrowderived cells induces early insulin resistance without significantly affecting the development of adipocyte cell death, inflammation and hepatic steatosis induced by HFD.

7.1.5. Loss of TG2 From Bone Marrow-Derived Cells Is Responsible For The Obesity-Related Adipocyte Cell Death, Inflammation And Hepatic Steatosis In TG2 Null Mice

Absence of TG2 in bone marrow-derived cells, however, promoted adipocyte death in the gWAT of mice exposed to HFD, compared to mice transplanted with wild type bone marrow, possibly due to the enhanced TNFα and leptin production in these mice. In addition, significantly larger gonadal fat weight, increased liver weight and TAG content could be detected in animals lacking TG2 in the bone marrow compartment. Increased hepatic steatosis was confirmed by haematoxylin eosin stained liver sections as well. In line with these data, in the absence of bone marrow-derived cells' TG2 a significantly higher expression of adipose tissue TNF-α, IL-6, MCP-1, leptin and Bid mRNA-s were detected in mice exposed to HFD. Similarly, gWAT macrophage IL-6, MCP-1, TNFα mRNA levels were also significantly higher in the absence of TG2. As shown later in, circulating plasma levels were also higher in the absence of bone marrow derived cells in TG2 null mice. Taken together, our data indicate that as compared to wild type mice, loss of TG2 from bone marrow-derived cells drives in TG2 null mice the enhanced inflammation and the more severe pathological consequences of HFD-feeding.

7.1.6. Loss of TG2 In Macrophages Results In A More Efficient Lysosomal Exocytosis

Since our initial hypothesis was that the enhanced obesity-relatedinflammation in TG2 null mice is the result of impaired apoptotic cell phagocytosis, we decided to determine whether macrophage TG2 is required for the clearance of apoptotic adipocytes. For this purpose, adipocytes differentiated from 3T3 fibroblasts were made apoptotic using serum deprivation, exposed to both wild type and TG2 null bone marrow-derived macrophages and their clearance as a function of time was followed by laser scanning cytometry. Surprisingly, loss of macrophage TG2 did not delay, but accelerated lysosomal exocytosis of dead adipocytes. We have revealed that adipocytes entering apoptosis form first lipid-containing vesicles surrounded by phosphatidylserine positive membranes indicative of cell death. Macrophages attach to the dead adipocyte as it was described, and continuously engulf these lipid-containing vesicles. Onewildtype macrophageacting on one dead adipocyte keeps taking up these vesicles during the whole 5h time frame of the video, but does not complete the clearing. TG2 null macrophages, however, take up these lipid-containing vesicles, and trigger the initiation of the classical apoptosis program characterized by membrane blebbing much faster. After the whole

cytosolic content of the adipocyte is cleared, TG2 null macrophages digest finally the degrading nucleus. None of the wild type adipocytes could reach this final engulfment stage during the video time frame.

7.1.7. Metabolically Activated TG2 Null Macrophages Produce More Pro-Inflammatory Cytokines Than Their Wild Type Counterparts Due To Enhanced C-Src Signaling

Previous studies have demonstrated that in the absence of TG2 integrin β3 signaling is altered leading to enhanced c-Src tyrosine kinase activity35. Accordingly, in TG2 null macrophages the amount of phosphorylated c-Src, β3 integrin levels and c-Src-dependent integrin β3 phosphorylation are enhanced leading to decreased basal IκB levels, and consequently to enhanced pro-inflammatory cytokine transcription following exposure to lipopolysaccharide36. Similar to lipopolysaccharide, palmitate was also shown to activate TLR417 and c-Src37. Thus we determined the pSrc levels in MMe macrophages that we generated in vitro as it was previously described16, and found increased levels in the absence of TG2. Next we determined the mRNA levels of integrin β3 in gWAT macrophages and found that its levels are increased during HSD and HFD, and both the basal and the HSD/HFD-induced levels are significantly higher in the absence of TG2. Similarly, in the absence of TG2 we detected a significantly increased expression of integrin β3 in MMe macrophages as well induction of integrin β3 expression in MMe macrophages was completely c-Src-dependent, as PP2, a Src inhibitor, prevented it.

7.1.8. Metabolic activation also induced significantly higher TNF-α, IL-1β, and IL-6 mRNA levels in TG2 null MMe macrophages than in the wild type ones

LXR agonist treatment reverts the HFD-induced phenotype in mice lacking TG2 in bone marrow-derived cells with less hepatic steatosis than in wild type mice Liver X receptors (LXR) are nuclear receptors that play a key role in regulating whole-body cholesterol, fatty acid and glucose metabolism38. Activation of LXR receptors is also known to strongly suppress macrophage inflammation. Though LXR agonist treatment has been shown to affect metabolism significantly during diet-induced obesity resulting in attenuated weight gain, induction of white adipocyte lipolysis and fatty acid oxidation40, hepatic steatosis41 and improved insulin sensitivity, we reasoned, if loss of TG2 in macrophages affects primarily

inflammation, LXR agonist treatment will result in a similar phenotype in mice carrying and in mice lacking TG2 in bone marrow-derived cells exposed to HFD. Thus, we exposed bone marrow transplanted mice expressing or not TG2 in their bone marrow-derived cells to the LXR agonist GW3965 mixed with their high fat containing food during the whole feeding period, as described44. In agreement with a previous publication, LXR agonist treatment prevented body and gonadal fat weight gain in HFD-exposed mice. This was related to an enhanced white adipocyte apoptosis assessed by the enhanced Bid and Bim expression of gWAT adipocytes. In contrast, the percentage of dying adipocytes observed on tissue sections decreased in he gonadal fat of LXR-treated mice, if TG2 was missing from the bone marrow-derived cells, perhaps due to their more efficient clearance. Similarly, significantly increased LXR activation-related hepatic steatosis was detected only in wild type mice. LXR agonist treated TG2 null gWAT macrophages produced significantly less MCP-1 and resistin, than their wild type counterparts, while the gWAT adipocytes from the same mice significantly more adiponectin reflecting an improved insulin sensitivity. Indeed, though there was no difference in the fasting circulating plasma insulin levels in the LXR agonist treated mice, animals lacking TG2 in bone marrow-derived cells, showed increased insulin sensitivity.

7.2. Consequences of Loss of Retinol Saturase for Efferocytosis

7.2.1. Loss of Retinol Saturase Does Not Affect the Induction of Retinoid-Regulated Genes or the Thymic Apopto-Phagocytosis Program

Our previous studies indicated that in macrophages, during engulfment of apoptotic cells the expression of at least 5 efferocytosis-related genes (TG2, CD14, C1qb, Tim-4, and ABCA1) is induced in a retinoid-dependent manner; the expression of these genes is enhanced in the apoptosing thymus, but the classical retinoic acids could not be detected, unlike a potential dihydroretinol derivative. Therefore, we decided to determine whether the induction of the same genes is affected in the absence of RetSat in the apoptosing thymus. Induction of none of the tested genes was altered by the loss of RetSat in the apoptosing thymus. In addition, we could detect the appearance of the same retinaldehyde dehydrogenase-dependent retinoid peak of unknown identity in the apoptosing thymus that we suspected to be a dihydroretinol derivative (data not shown). Thus, we concluded that either RetSat-related retinoids do not play a determining role in the induction of retinoid-related genes, or in the absence of RetSat, they can

be replaced by other retinoids in the mouse thymus. Since the apopto-phagocytosis program plays a crucial role in shaping the T cell repertoire and retinoids were shown by us to influence the thymic T cell selection processes, we investigated whether loss of RetSat affects the composition of the thymus. However, as shown in, loss of RetSat did not affect the cellularity or the size of the various thymocyte populations. Since RetSat was induced in engulfing macrophages, we also tested whether loss of RetSat affected the ability of macrophages to clear apoptotic thymocytes in vivo. RetSat-null thymocytes induced to die in vitro entered apoptosis with the same speed. Similarly, induction of cell death either by dexamethasone or by γ-irradiation did not significantly affect thymocyte loss or the percentage of annexin-V-positive uncleared thymocyte population, indicating that neither the apoptosis of thymocytes nor their phagocytosis was significantly affected in vivo by the loss of RetSat.

7.2.2. RetSat-Null Macrophages Are Characterized by Impaired Long-Term Phagocytosis

Since in vivo various macrophage populations use different phagocytic receptors and bridging molecules for efferocytosis, we decided to test the in vitro efferocytotic capacity of RetSat-null peritoneal and bone-marrow-derived macrophages (BMDMs). Short term phagocytosis determined following a 1 h uptake of apoptotic cells was not affected by the loss of RetSat. However, if phagocytosis was determined after 5 h of continuous efferocytosis, the phagocytic capacity of both types of RetSat-null macrophages was decreased as compared to their wild type control. These data indicate that RetSat contributes to the efficient efferocytosis of apoptotic cells.

7.2.3. RetSat-Null Macrophages Express Less MFG-E8

To determine the reason behind the long-term phagocytic defect, we applied total RNA sequencing and identified 117 differentially expressed genes (DEGs) between RetSat+/+ and RetSat−/− BMDMs (based on at least a 1.5-fold change and corrected p value < 0.05). A total of 59 transcripts showed decreased gene expression and 58 transcripts showed increased gene expression in the RetSat−/− cells. The list of DEGs is shown in Table 1. The mean fold change (FC) of decreased and increased transcripts was –15.1 ± 97.1 and 3.1 ± 2.7, respectively. The median FC value of decreased and increased transcripts was –2 and 2.1, respectively. Functional analysis revealed that genes related to monocyte differentiation are overrepresented among the

DEGs. Among the genes showing decreased expression in RetSat-null macrophages, we found only one related to phagocytosis of apoptotic cells; this was MFG-E8, the expression of which was about 2.5 times less than that of the wild types cells. To verify the finding, we determined the mRNA expression of MFG-E8 in BMDMs, peritoneal, and thioglycolate-elicited RetSat-null macrophages by qRT-PCR and found decreased expression as compared to wild type ones. Testing in BMDMs, we found that exposure to apoptotic cells for 5 h did not increase the expression of MFG-E8, but the initial difference in MFG-E8 expression remained constant.

Since our initial analysis indicated that the expression of some differentiation-related genes was altered in RetSat-null macrophages, we decided to determine whether MFG-E8 expression is altered during the differentiation of monocytes and whether administration of dihydroretinol alters it. There was no difference in the expression of MFG-E8 of wild type and RetSat-null monocytes, and the expression of both RetSat and MFG-E8 was induced during monocyte differentiation. However, the induction of MFG-E8 expression was less pronounced in RetSat-null cells. Surprisingly, administration of dihydroretinol could not overcome the defect in the induction of MFG-E8. These data indicate that functions of RetSat other than dihydroretinol production during monocyte/macrophage differentiation regulates the expression of MFG-E8 in macrophages.

Though we did not find a difference in the expression of the retinoid-related genes in the RetSat-null BMDMs, we also determined whether the administration of dihydroretinol during their differentiation affected their expression. The expression of TG2 was induced during monocyte differentiation, and administration of dihydroretinol significantly induced its expression. However, we did not find any difference in the TG2 expression or in the dihydroretinol response of wild type and RetSat-null cells. The expression of the other four retinoid-regulated genes, on the other hand, was not induced by dihydroretinol during differentiation either in wild type or in RetSat-null cells. Interestingly, despite of the lack of induction of these genes, dihydroretinol added during differentiation enhanced the short-term phagocytosis of macrophages, demonstrated by both FACS analysis and confocal microscopy.However, since we did not find a difference in the short-term phagocytosis of wild type and RetSat-null BMDMs without adding dihydroretinol, these data indicate that despite the fact that RetSat expression is induced during monocyte/macrophage differentiation, dihydroretinoids might not have been produced in significant amounts.

Since we previously detected the induction of efferocytosis-related genes by retinoic acids in differentiated macrophages, we also tested their response to 24 h dihydroretinol treatment.

Again, we detected a significant increase only in the expression of TG2 (2.8-fold), but not in that of the other retinoid-sensitive genes in both types of macrophages (data not shown).

Since we found that neuropeptide Y (NPY) is hardly expressed by RetSat-null macrophages but NPY was reported to promote the M2 phenotypic change of macrophages, which is known to be associated with enhanced phagocytic capacity, we also tested whether lack of NPY expression and MFG-E8 production could be related. We found that NPY expression is also induced during the differentiation of wild type monocytes, but surprisingly, it was downregulated during the differentiation of RetSat-null monocytes. Administration of dihydroretinol did not alter this pattern. We also tested whether administration of NPY could restore the defect in MFG-E8 expression during monocyte differentiation. However, administration of the neuropeptide at three different concentrations (10−10, 10−9, 10−8 M) to trigger different NPY receptors did not alter the MFG-E8 expression either in wild type or in RetSat-null cells (data not shown). Interestingly, we could not detect a defect in NPY expression in other tissues tested.

7.2.4. Lower MFG-E8 Production Is Responsible for the Defect in Long-Term Phagocytosis of RetSat-Null Macrophages

To prove that decreased MFG-E8 production is responsible for the defect in long-term efferocytosis of RetSat-null macrophages, their long-term efferocytosis was tested in the absence and presence of recombinant MFG-E8. Administration of recombinant MFG-E8 restored the defect in long-term efferocytosis of both RetSat-null BMDMs and peritoneal macrophages, while it had no effect on the efferocytosis of wild type cells. These data indicate that the impaired long-term phagocytosis of apoptotic cells detected in vitro is indeed related to an impaired MFG-E8 production by RetSat-null macrophages.

Previous studies have shown that MFG-E8 is not expressed by thymic macrophages, thus it is very likely not involved in the clearance of apoptotic thymocytes, probably explaining why we did not see an efferocytosis-related phenotype in the thymus of RetSat-null mice. However, it was also reported that MFG-E8-null mice show an efferocytosis-related phenotype in their thymus if they are treated for a week with a daily low dose of DEX in order to induce MFG-E8 expression in macrophages and only then are exposed to X-ray irradiation to induce thymic apoptosis. We repeated these experiments by using RetSat-null mice and found a significant increase both in the remaining total number and in the number of annexin-V-positive cells

following irradiation, indicating accumulation of uncleared apoptotic thymocytes in the absence of RetSat.

7.2.5. Female RetSat-Null Mice Are Prone To Develop Mild Systemic Lupus Erythematosus (SLE)-Like Autoimmunity

It is well known that impaired clearance of apoptotic cells is associated with increased sensitivity to develop SLE-like autoimmunity. This is related to the fact that uncleared apoptotic cells undergo secondary necrosis and induce inflammation. Their released autoantigens also induce autoantibody formation, demonstrated by repeatedly injecting apoptotic cells into mice. In addition, proper phagocytosis should induce various anti-inflammatory mechanisms that become disturbed when efferocytosis is impaired. Efferocytosis receptor- or bridging-molecule-null mice are characterized by splenomegaly, anti-DNA and anti-nuclear autoantibodies, and immune complex deposits into the kidney, leading to glomerulonephritis. Like humans, female mice are more prone to develop autoimmunity than male mice. If loss of RetSat impairs the in vivo clearance of apoptotic cells by macrophages, it is expected that RetSat-null female mice are also more prone to develop SLE-like autoimmunity. Thus, we decided to investigate 1 year old female mice and compared them to age-matched wild type controls. The average spleen weight of RetSat-null female mice was significantly higher than that of the wild type females, and out of the 12 RetSat-null mice, 6 had bigger spleen weights than the average of the controls. Previous studies have shown that enlarged spleens in MFG-E8 null mice are associated with impaired clearance of apoptotic cells by tingible body macrophages that engulf apoptotic B cells generated in the germinal centers. If apoptotic cells are not cleared properly, they remain for a longer time in the tissue and the number of activated caspase-3-positive cells increases. Thus, we determined the occurrence of activated caspase-3-positive cells in the spleens and found that it is significantly increased in the large spleens as compared to their wild type controls. While we could not detect significantly increased anti-dsDNA antibody titers, 10 out of the 12 RetSat-null mice produced anti-nuclear antibodies. Immune complex deposits could easily be detected in the kidneys of mice with enlarged spleens, while no immune complex deposits could be detected in six randomly selected wild type kidneys. Glomerular dysfunction was indicated in the mice with enlarged spleens by high serum concentrations of urea (52 to > 100 mM versus the normal 11 ± 3 mM).

8. DISCUSSION

In my dissertation, I examined the disruption of dead cell clearance and the pathological consequences in two knock out (TG2 deficient and RetSat deficient) mouse models.

Previous studies have demonstrated that impaired clearance of apoptotic cells is associated with the development of various chronic inflammatory diseases. Since TG2 has been shown to participate in the apoptotic cell clearance and obesity is associated with enhanced adipocyte apoptosis, we decided to investigate whether loss of TG2 affects diet-induced obesity-related pathologies. Our data indicates that loss of TG2 indeed enhances obesity-related pathologies such as adipose tissue inflammation, adipocyte death, hepatic steatosis and insulin resistance in mice exposed to either HSD or to HFD, and all this is a result of the loss of TG2 from bone marrow-derived cells. Since our hypothesis was that the enhanced inflammation in TG2 null mice is related to impaired apoptotic cell clearance, we tested whether TG2 was also required for the clearance of the large, lipid-rich apoptotic adipocytes. Since macrophages were reported to interact only with dead adipocytes and the process involved actin polymerization15, we expected that TG2 might be involved in the process by promoting possible integrin β3/apoptotic adipocyte interaction, as it does during the classical efferocytosis process. To our surprise, however, we found that dead adipocyte clearance is more effective by TG2 null macrophages. Our results clearly demonstrate that the apoptosis program of adipocytes is initiated with packing the cytosolic content into lipid-rich extracellular vesicles covered by phosphatidylserine positive membrane, and the lysosomal exocytosis of macrophages is a process of a continuous uptake of these vesicles that, according to Haka et al., might occur directly into the lysosome. It is interesting to note that that living adipocytes also release lipid-containing extracellular vesicles (exosomes) that are taken up by the surrounding macrophages and the exosomes contributes to the macrophage metabolic programming. Since TG2 null macrophages are much more efficient in lysosomal exocytosis, impaired clearance of dead adipocytes cannot explain the observed HFD-induced phenotype of TG2 null mice, though we cannot exclude that enhanced lipid uptake itself might be pro-inflammatory. However, we could demonstrate that metabolically activated TG2 null macrophages produce more proinflammatory cytokines due to their enhanced integrin β3/c-Src signaling. Our observations are in line with other reports, which also demonstrated that αvβ3 integrin signaling can induce NFκB activation in macrophages, and that c-Src activity in macrophages is associated with various inflammatory responses. Interestingly, we found that both pro-inflammatory cytokine formation and

lysosomal exocytosis, both TLR2dependent, were enhanced in TG2 null macrophages. TLR2 ligands are molecules with diacyl and triacylglycerol moieties. Integrin β3 is part of the pre-formed TLR2/ TLR1 signaling complex and is essential for triacyl lipopeptide engagement of TLR2 by recruiting triacyl lipopeptide bound to vitronectin. In addition, αvβ3-integrin signaling can boost the MYD88-dependent TLR2 signaling. What is more, phosphatidylserine positive vesicles were already shown to be taken up by MFG-E8/integrin β3-dependent manner at least by dendritic cells51. Thus enhanced integrin β3 expression and signaling might be the explanation for the enhanced lysosomal exocytosis of TG2 null macrophages. Finally, we also compared the response of mice to LXR agonist treatment during HFD-induced obesity with the expectation that after attenuation of adipose tissue inflammation the phenotype of mice carrying or not TG2 in their bone marrow-derived cells will be similar. LXR agonist treatment prevented HFD-induced obesity in both types of mice. In addition, as it was reported by others.

Lack of gonadal weight gain as a result of LXR treatment was reported to be the consequence of the LXR agonist-induced activation of adipocyte LXRα which is known to promote lipolysis and fatty acid oxidation in adipose tissue41 leading finally to reduced adipocyte cell number. While, however, the rate of adipocyte apoptosis, based on the mRNA expression levels of Bid, was not higher in LXR agonist-treated wild type mice, their hepatic steatosis was significantly more pronounced than that of the LXR agonist-treated mice in which TG2 was absent from the bone marrow-derived cells. Previous studies indicated that the LXR agonist-induced hepatic steatosis is the result of activation of hepatocyte LXRα which triggers SREBP-c1-dependent triacylglycerol synthesis. However, in our mice there was no difference in the genotype of the hepatocytes. Thus our observations indicate that TG2 null macrophages can better buffer metabolic tissues from the damage caused by ectopic accumulation of saturated free fatty acids53 very likely due to their enhanced lipid handling. Thus it is interesting to speculate whether LXR agonist treatment in combination with enhancing lysosomal exocytosis could be a therapeutic strategy in obesity. Previous studies from our laboratory have shown that during engulfment of apoptotic cells, retinoids are formed in a retinaldehyde dehydrogenase-dependent manner that upregulate various efferocytosis-related genes, leading to enhanced phagocytic capacity during long-term efferocytosis. Our data also indicated that the retinoids formed might be products of the RetSat pathway. Indeed, we were able to demonstrate the induction of RetSat enzyme both in vivo in the mouse thymus following thymocyte apoptosis induction and in macrophages following LXR activation, though the expression of the enzyme in macrophages is about 60x and 20x less than in the liver and kidney, the two tissues that express the highest

amount of RetSat, respectively. We investigated whether loss of RetSat in mice affects efferocytosis in vitro and in vivo. Data shows that macrophages differentiating in the absence of RetSat produce significantly less MFG-E8 than their wild type counterparts.

MFG-E8 is a 66 kDa glycoprotein that contains a signal sequence for secretion, two N-terminal epidermal growth factor (EGF) domains, and two C-terminal discoidin domains with homology to the C1 and C2 domains found in blood-clotting factors V and VIII. The second EGF domain contains an arginine–glycine–aspartic (RGD) integrin-binding motif that engages αvβ3/αvβ5 integrins to facilitate integrin-mediated signal transduction, while the C-terminal discoidin domains mediate attachment to PS on apoptotic cells . Thus MFG-E8 acts as a bridging molecule for the αvβ3/αvβ5 integrins during efferocytosis, and its integrin β3 binding is further promoted by TG2, which acts as a coreceptor for integrin β3. Loss of MFG-E8 leads to development of autoimmunity that is specifically due to defects in apoptotic cell engulfment by tangible body macrophages in germinal centers.

The data presented by us demonstrate that macrophages from RetSat-null mice behave similarly to that of MFG-E8 null macrophages. In short-term efferocytosis experiments, they do not show efferocytosis defects, as was reported for the MFG-E8 null mice, but they have impaired efferocytosis in the long term. We believe that this might be related to the fact that MFG-E8 secretion is accelerated upon apoptotic cell exposure. In accordance, administration of recombinant MFG-E8 did not affect long-term efferocytosis by wild type macrophages, but it enhanced efferocytosis by RetSat-null BMDMs and peritoneal macrophages. In addition, as found with MFG-E8 null mice, a delayed efferocytosis was detected in the thymi of RetSat-null mice following X-ray exposure if they were pre-exposed to a daily dose of DEX for a week to induce MFG-E8 expression.

Like MFG-E8-null mice, aged female RetSat-null mice also develop SLE-like autoimmunity characterized by enlarged spleens, accumulation of apoptotic cells in the spleen, and anti-nuclear antibodies and immune complex deposits in the kidneys. Interestingly, aged TG2 (the integrin β3 coreceptor that promotes MFG-E8 binding)-null mice have a similar phenotype . In the absence of proper clearance, autoimmunity develops not only due to the accumulation of apoptotic cells that undergo secondary necrosis, but also because uptake of apoptotic cells induces various anti-inflammatory mechanisms that also become impaired. In this respect, it is worth noting that both MFG-E8 and NPY, which is not produced by RetSat-null macrophages, have anti-inflammatory properties. In addition, engulfing macrophage-derived MFG-E8

strongly contributes to the M2 polarization of the inflammatory macrophages, and proper macrophage M2 polarization is a requirement for the timed inflammatory resolution process. Interestingly, RetSat-null macrophages also express lower endogenous CSF-1 and Gpr68/OGR1, and this might also decrease their M2 polarization potential as CSF-1 promotes macrophage differentiation into the M2 direction, while Gpr68-deficient macrophages express fewer M2 markers.

Increasing evidence indicates that MFG-E8 bound to αvβ3,5 integrins not only promotes efferocytosis but is strongly coupled to fat metabolism as well. Thus, MFG-E8 enhances fatty acid uptake and is also involved in the regulation of enterocyte lipid metabolism. In the lipid turnover of the adipose tissue, adipose tissue macrophages continuously take up and metabolize lipids released from adipocytes in the form of exosomes. Exosomes, by being PS positive, also utilize MFG-E8 to promote their cellular uptake. In this context, it is worth noting that RetSat-null mice show increased adiposity. It is interesting to speculate whether this phenotype could also be related to a decreased MFG-E8 production by adipose tissue macrophages by lowering the amount of exosomal lipids taken up and metabolized by them. It has been shown that the expression of MFG-E8 is induced by granulocyte/monocyte colony-stimulating factor in macrophages. We also detected the induction of MFG-E8 mRNA expression during monocyte differentiation, but the induction in RetSat-null cells was less than in wild type macrophages. We tested the role of dihydroretinol and found that the reduced expression of the gene is not related to dihydroretinol production by RetSat during this late differentiation process. Our data indicate that RetSat itself or its activity might contribute to the general expression level of MFG-E8, without affecting its inducibility. Interestingly, in RetSat-null macrophages we found a significantly decreased expression of GATA2 (4.5-fold), a zinc finger transcription factor that contributes to the proper monocyte/macrophage differentiation process, as well as that of C/EBPβ. GATA2 was shown to regulate C/EBPβ expression, while C/EBPβ enhances MFG-E8 transcription. In addition, we found that not only MFG-E8 but other differentiation-related genes are affected by the loss of RetSat. Since we did not see evidence for dihydroretinol production during monocyte/macrophage differentiation, we believe that RetSat or its products might affect macrophage differentiation at an earlier phase, and thus, they might influence the differentiation of other monocyte-derived cells as well. In this context, it is worth noting that the expression of the dendritic cell-specific transmembrane protein (DC-STAMP) is also lower in RetSat-null macrophages. We did not determine the expression of DC-STAMP in monocyte-derived dendritic cells, but DC-STAMP-null mice are also more sensitive to the development

of autoimmunity, very likely due to altered dendritic cell functions. Thus, we cannot exclude that a possibly altered DC-STAMP expression in dendritic cells might also contribute to the development of autoimmunity in RetSat-null mice.

Cumulatively, our data indicate that RetSat affects the differentiation of macrophages, but it might not be related to its ability to produce dihydroretinol, at least not during the monocyte/macrophage differentiation phase. Though administration of dihydroretinol during differentiation could enhance the short-term phagocytic capacity of macrophages, this was not related to the induction of retinoic acid receptor-regulated phagocytic genes, despite the fact that dihydroretinoids can selectively activate RAR and RXR receptors. Since RetSat was induced during our monocyte differentiation protocol, if dihydroretinol was produced, it should have enhanced the short-term efferocytotic capacity of wild type macrophages. Since, however, loss of RetSat did not affect the short-term phagocytic capacity of macrophages generated and we did not find a difference in the expression of TG2, a dihydroretinol-sensitive gene, our observations indicate the lack of significant dihydroretinol production during monocyte differentiation at least during the in vitro protocol. However, we described an efferocytosis-related phenotype of these mice, which seems to be related to less efficient MFG-E8 and perhaps also to the lack of neuropeptide Y production by RetSat-null macrophages.

9. SUMMARY

Previous studies have shown that a disorder in the removal of apoptotic cells is associated with the development of a number of chronic inflammatory diseases (Fond and Ravichandran, 2016). In my dissertation, I present several hitherto unknown elements of the relationship between the development of chronic inflammation and inadequate efferocytosis through the study of two knock out (TG2, RetSat deficient) mouse strains.

A study of the TG2-deficient mouse strain on a high-fat diet showed that TG2-deficient enhances pathological changes in animals on a high-fat diet such as inflammatory cytokine production, insulin resistance, and fatty liver development. We have discovered that the apoptosis of adipocytes occurs in a unique way: dying adipocytes first package their lipid content into lipid-containing extracellular vesicles bounded by a phosphatidylserine-positive membrane and the macrophages first take up this and then form the classical apoptosis. TG2-deficient macrophages take up these vesicles much more efficiently, but they also produce more inflammatory cytokines upon lipid uptake. The increased susceptibility to inflammation is thought to be due to the compensatory expression of multiple integrin β3 receptors by TG2-deficient macrophages, which partly increases the production of inflammatory cytokines, partly as part of the TLR1 / 2 complex. In the case of chronic inhibition of inflammatory cytokine production by an LXR agonist, more efficient lipid vesicle uptake by macrophages in TG2-deficient mice maintained on a high-fat diet reduces ectopic fat loading in peripheral tissues. While macrophages phagocytose apoptotic cells, they increase the expression of some of their phagocyte receptors to maintain effective phagocytosis. Some of these are induced by retinoids produced by macrophages. Although our preliminary experiments suggested that these retinoids may be products of the RetSat pathway, the absence of RetSat did not affect the induction of retinoid-dependent genes. However, RetSat-deficient macrophages cannot completely clear dead cells because they produce fewer bridging molecules involved in phagocytosis (MFG-E8), resulting in a disorder of macrophage differentiation. Due to the reduced production of MFG-E8 by macrophages, some female mice develop SLE-like autoimmune disease in the long term. It is hypothesized that decreased MFG-E8 production may explain another phenotype in these mice as increased obesity, as MFG-E8 may contribute to the uptake and metabolism of lipid-containing vesicles produced by adipocytes by macrophages, thus reducing adipocyte fat load.

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11. PUBLICATIONS

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Candidate: Tibor Sághy
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List of publications related to the dissertation

1. **Sághy, T.**, Köröskényi, K., Hegedűs, K., Antal, M., Bankó, C., Bacsó, Z., Papp, A., Stienstra, R., Szondy, Z.: Loss of Transglutaminase 2 Sensitizes for DietInduced Obesity-Related Inflammation and Insulin Resistance due to Enhanced Macrophage c-Src Signaling. *Cell Death Dis. 10*, 1-14, 2019.
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