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XXVII

DIET AND INFLAMMATION: FOCUS ON THE SMALL INSTESTINE AND ADIPOSE TISSUE

Settore Disciplinare BIO16

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INDEX

1. INTRODUCTION

1.1 Dietary life-style: Western Diet vs High Fat Diet

Extensive scientific evidence indicating that non-optimal diet and sedentary lifestyle constitute some of the behaviors /risk factors associated with the onset of many diseases with significant societal impact, such as inflammatory bowel disease (IBD) and obesity(1;2). On the contrary, the maintaining a balance between physical activity and dietary energy intake is an effective and safe way to prevent these clinical problems, as well as their economic impact. For this reason, numerous official recommendations and education campaigns to sensitize people towards healthy dietary behaviors have been established. Despite these recommendations, IBD and obesity have risen in incidence at an alarming rate over the past decade and are now two of the most serious worldwide health problems. Particularly alarming is the marked increase in obesity among children. The World Health Organization reports that at least 2.8 million people die each year as a result of being overweight or obese, and about 150-200 cases per 100000 from intestinal cancer (3;4;5).

A Western-style diet (WD), defined by elevated fat (20%), low calcium and low vitamin D content, is associated with increased risk of human intestinal cancer (6). A defined rodent WD, formulated by Newmark and M.Lipkinic, reflecting dietary patterns linked to elevated intestinal cancer incidence has been demonstrated to cause neoplastic lesions when fed to wild-type mice for at least 1 year (7;8). Most of the studies mainly investigate the link between WD and colon tumors. Yet little is known about regarding the induction of small intestine tumors. The small intestine is a relatively rare site for primary gastro-intestinal (GI) tract cancer, yet nonetheless recent studies show an increasing incidence of small intestine adenocarcinoma (SIAC) (9;10).

High fat diet, consisting of 60% Kcal from fat, predisposes mice to the development of obesity and other pathological consequences. This allows the study of a wider spectrum of human metabolic disease as well as its associated inflammation in the visceral compartment, that leads to adipose tissue dysfunction (2;11).

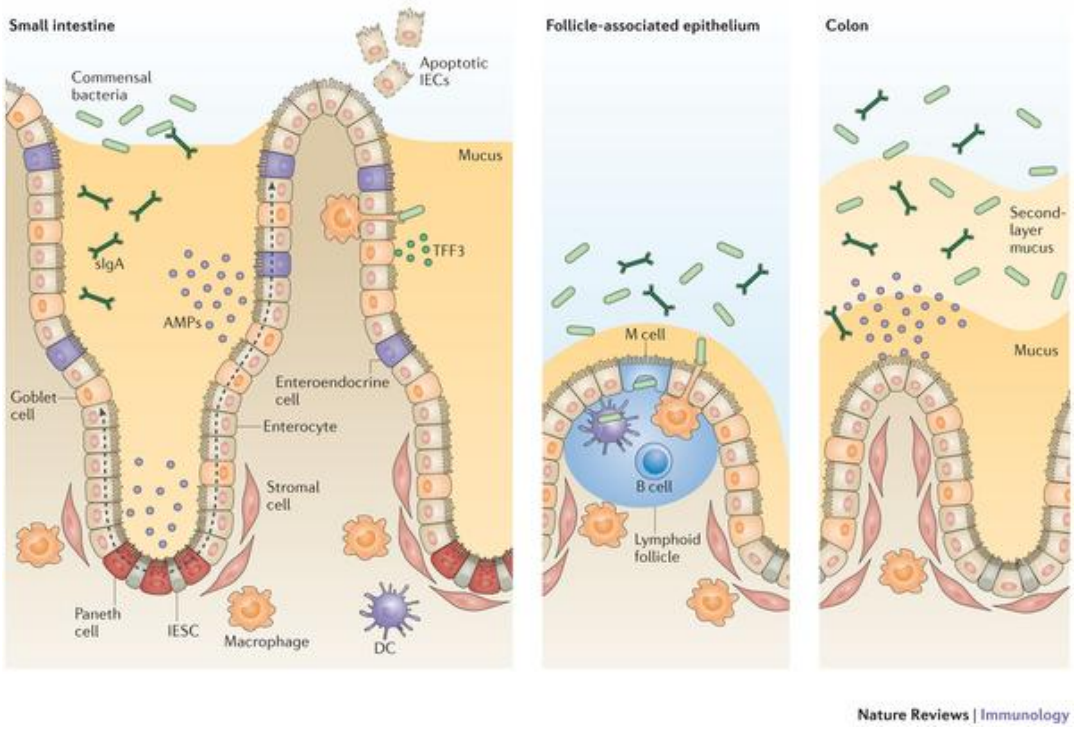
1.2 Defense mechanisms in the gastrointestinal tract.

The intestinal epithelium has a vast surface area that is continuously exposed to dietary and microbial antigens. These antigens need to be tolerated to maintain homeostasis, but pathogens must also be prevented from harming the host. The first line of defense is represented by the

mucosal barrier while the second line by the immune component constituted by the Gut associated lymphoid tissue (GALT), which includes lymphocytes and phagocytes.

1.3 Intestinal mucosa barrier

The intestinal epithelium is a delicate structure organized into invaginations called the crypts of Lieberkuhn and fingerlike protrusions called villi. It is composed of a columnar monolayer of polarized cells, the enterocytes, and other specialized differentiated cell types: goblet cells, neuroendocrine cells, Paneth cells and M cells that all originate from common intestinal epithelial stem cells. The organization of the epithelium in the small intestine and the colon is similar. However, histologically there are three important differences: the colon carries no villi but has a flat surface epithelium and Paneth cells are absent (12). The intestine, in addition to processing and absorbing dietary nutrients, performs other functions that are critical for intestinal homeostasis. Goblet cells produce and secrete glycosylate mucins that block the direct attachment of microbiota to the epithelium, thereby spatially regulating the colonization of commensal bacteria (13). Defects in mucus production alter the morphology of epithelial cells, which results in spontaneous colitis (14). The spatial separation of bacteria from the epithelial surface is further supported by secretion of antimicrobial peptides, such as lysozymes and defensins, secreted by Paneth cells. These cells reside near the stem cell crypt in the villi of the small intestine and can directly sense commensal microbiota through a TLR-MyD88 pathway. More intriguingly, Paneth cells provide essential niche signals to intestinal stem cells and cooperate with the latter to sustain the physiological self-renewal of the small intestine (15;16). The precise control of cell renewal along the cript-villus axis is regulated by different pathways that include WNT, Hedgehog and Notch (17). The Wnt signaling pathway is the dominant mechanism underlying the proliferative activity of the intestinal epithelium. The central player in the canonical Wnt pathway is β-catenin. In the absence of a Wnt signal, β-catenin is targeted for proteasomal degradation through a dedicated destruction complex that consists of adenomatous polyposis coli (APC), casein kinase I (CKI), glycogen synthase kinase 3 (GSK3), and axin. Signalling by WNT factors (Wnt ligands bind to Frizzled receptors and low-density lipoprotein receptor–related protein (LRP) coreceptors) inhibits the APC complex. As a result, β-catenin is stabilized in the cytoplasm and translocates into the nucleus, where it interacts with nuclear TCF transcription factors to drive the transcription of specific target genes (12; 18;19). Aberrant activation of WNT signaling has been directly linked to the development of colon-rectal cancer (20;21). Mice deficient for the TCF-4 completely fail to maintain the stem cells compartment in the fetal small intestinal epithelium (22). Moreover, disruption of EphB2 and EphB3 genes results in redistribution of proliferative cells within the crypt (23;24).



Lance W. Peterson & David Artis, Nature Reviews Immunology 2014

Fig.1: Organization of the intestinal epithelium

1.4 Microbiota and Pattern Recognition Receptors (PRRs)

The total microbial load of the intestine, 10^{13}–10^{14} microorganisms, collectively contain at least 100 times as many genes as the human genome. These bacteria increase in both concentration and complexity from the proximal gastric to the colon. Greater than 99% of the gut microbiota is composed of species within 4 bacterial divisions: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (25). The microbiota is generally viewed as being stable during adult life, during which establish a complex relationship with the host, resulting in the modulation of host physiology and metabolism, and it provides a natural defense against invading pathogens (26).

The physiological importance of the gut microbiota in intestinal development and homeostasis raises the question of how the host senses the myriad of microbial organisms. Two major classes of receptor have been identified that are integral to the host response to bacteria: the Toll-like receptors (TLRs) and the Nod-like receptors (NLRs). Pattern Recognition Receptors (PRRs) are mainly expressed by various immune cells, such as macrophages and dendritic cells, but their expression in intestinal epithelial cells (IEC) has also been reported (27;28;29). Although TLRs and

NLRs differ in their mode of pathogen recognition and function, they share similar domains for microbial sensing and cooperate to elicit immune responses against the pathogen. The TLR family consists of more than 13 members. Structurally, all TLRs are type I integral membrane proteins consisting of an ectodomain comprised of leucine-rich repeats (LRRs) and a cytoplasmic domain containing a Toll/IL-1R homology (TIR) domain, which is required for signaling. In general, TLR-2,-4,-5 and -9 are the ones that recognize bacterial components. Specifically, TLR-2 recognizes peptidoglycan, lipoproteins contained in the cell wall of bacteria, TLR-4 recognizes lipopolysaccharides (LPS), contained in the cell wall of gram-negative bacteria, TLR-5 binds flagellin from bacteria, and TLR-9 recognize non–methylated CpG DNA, abundantly contained in bacterial DNA (30).

The NLR family is composed of 23 cytosolic proteins characterized by 3 domains: a leucine-rich repeats (LRR) domain utilized for ligand sensing, a NATCH domain required for self-oligomerization and activation, and an effector domain at the N-terminus that mediates interactions with other signaling proteins. In particular an NRL family member, NOD2/CARD15, recognizes the smallest fragment common to both Gram-negative and Gram-positive bacteria: the muramyldipeptide N-acetylmuramoyl-L-alanyl-D-glutamate (31).

The activation of TLRs/NLRs is dependent upon the recruitment and association of adaptor molecules, such as Myeloid Differentiation factor 88 (MyD88), TIRAP, TRIF, or TRAM that provide the necessary framework to recruit and activate downstream kinases and transcription factors that regulate the host inflammatory response (32).

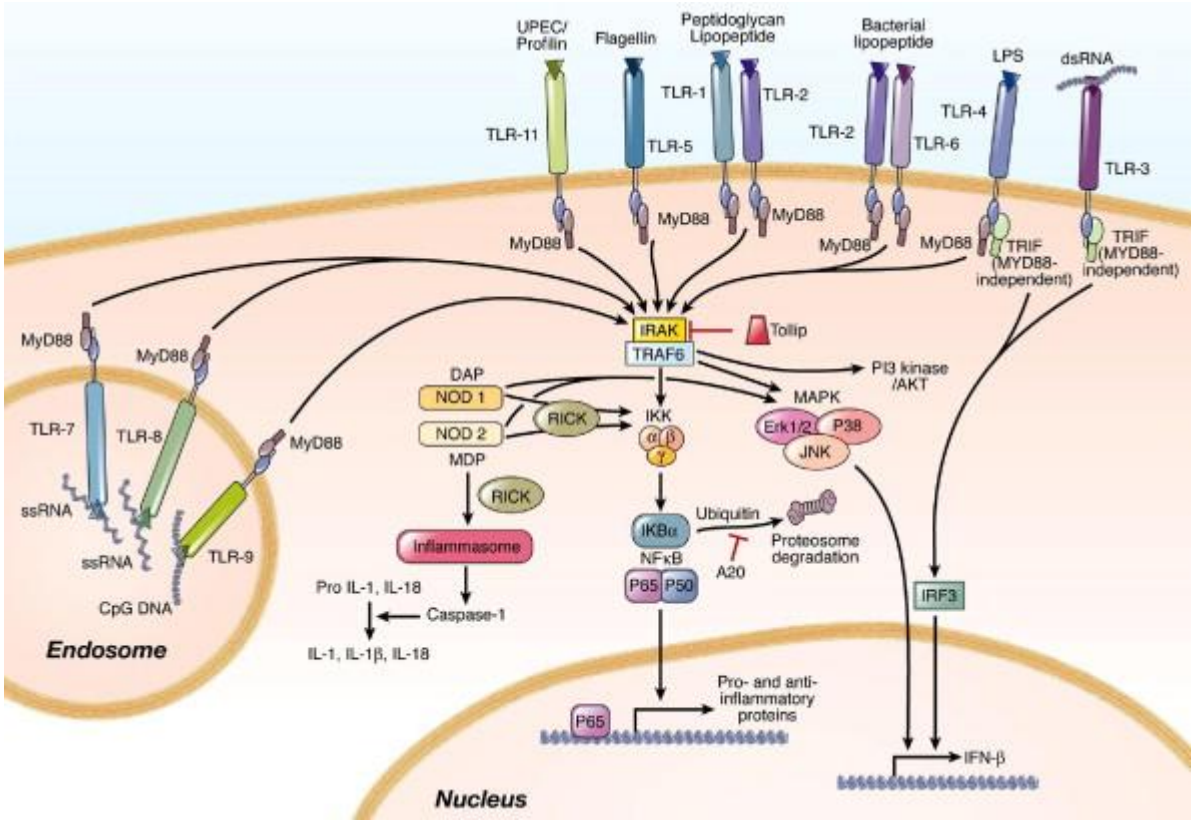
The context of PRR activation is crucial. In the healthy intestine, basal PRRs activation promote epithelial cells proliferation, secretion of IgA into the Gut lumen and expression of antimicrobial peptides, but aberrant PRR activation may lead to IBD (33).

The precise etiology of IBD remains unclear, but several factors that make a major contribution to disease pathogenesis have been identified: genetic factors, the host immune system, and environmental factors such as diet.

On the other hand, epidemiological studies have identified diet as a major variable that contributes to the diversity of human gut microbiota. Altered composition of luminal microbiota is often accompanied by an alteration in immune response and may increase susceptibility to inflammation-induced cancer, in particular intestinal cancer (34,35). Moreover different experimental models demonstrated that PRRs can contribute to development or protection of malignancy. The studies using TLR2 deficient mice showed that TLR2 signaling play a protective role against the development of colorectal cancer (36). In addition, TLR5/MyD88 signaling has been shown to reduce tumor size in a mouse xenograft model for CRC. A role of TLRs in colon carcinogenesis

was demonstrated by Fukata who showed that mice deficient in TLR4 were protected against the development of neoplasia by AOM and DSS (37).

The functions of NLRs in the immune defense of the epithelium are only recently beginning to emerge. There is recent evidence that members of the NLR family play important roles in the immune response against invading pathogens and that NLR genetic variation causes or contributes to the development of inflammatory disorders or increased susceptibility to microbial infection. Indeed, Several mutation in the CARD/NOD2 gene have been associated with increased risk of intestinal cancer (38).



R.Balfour Sartor, Gastroenterology 2008

Fig.2: TLRs/NLRs signaling pathways

1.5 The Gut associated lymphoid tissue (GALT)

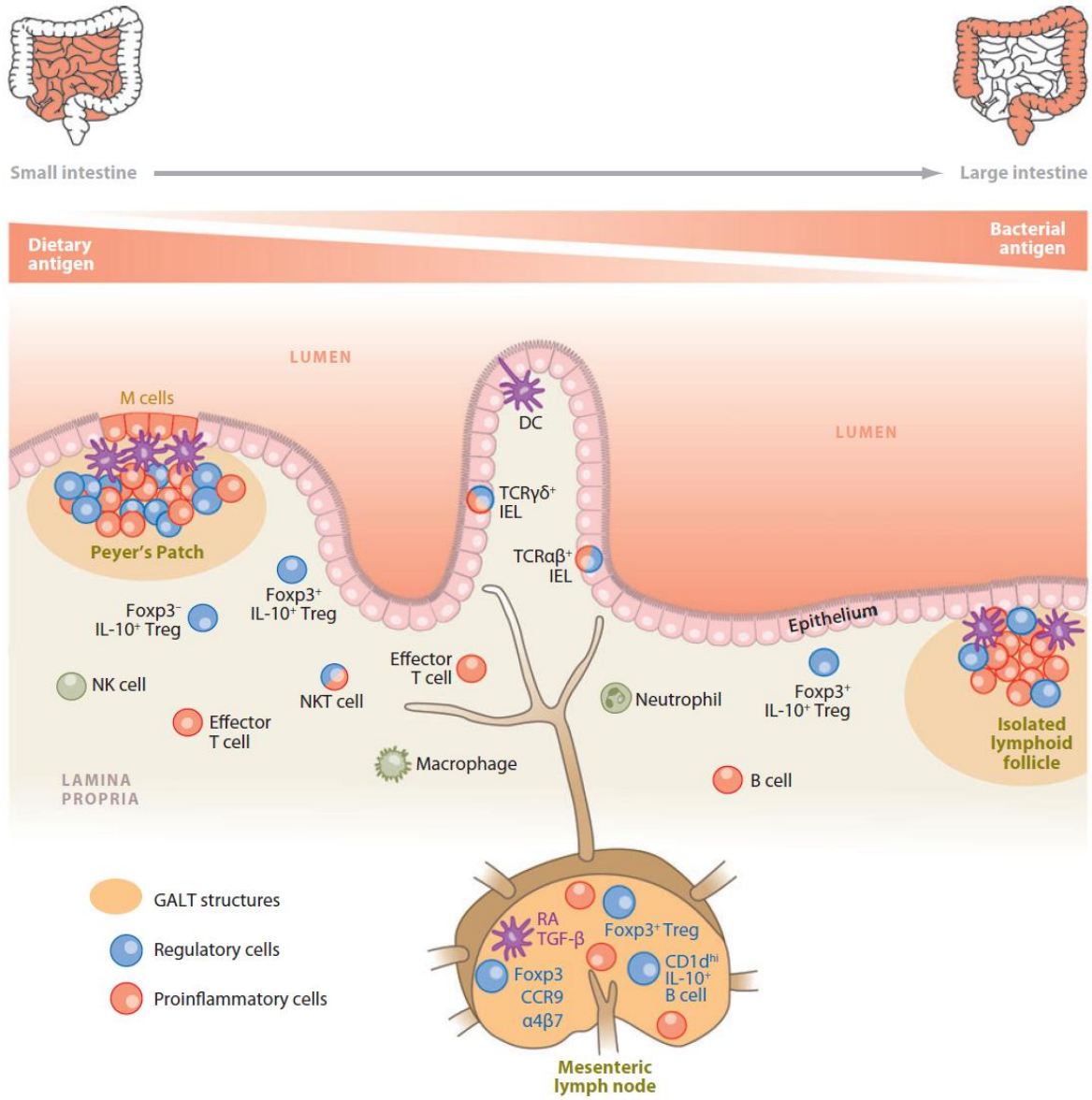
The intestinal epithelium is populated by a large and diverse community of immune cells that are intimately associated with intestinal epithelial cells (IECs). This immunological network, termed the gut associated lymphoid tissue (GALT), consists of unique arrangements of B cells, T cells and Phagocytes. The GALT comprises four distinct lymphoid compartments: the Peyer's patches, the lymphoid follicles associated with the lamina propria, intraepithelial lymphocytes (IELs) and mesenteric lymph nodes (MLN). These lymphoid aggregates come into contact solely with antigens from the gut lumen and serve as sites of induction for intestinal immune responses.

Recent advances have begun to shed light on the role of adaptive immune pathways in the aberrant intestinal inflammatory response in patients with IBD (39). While Crohn's disease (CD) has long been considered to be driven by a classical (Th1) response, ulcerative colitis (UC) has been associated with an alternative (Th2) response (40). Whilst there is no doubt that Th1 and Th2-polarized responses are key drivers of intestinal inflammation, the additional contribution of other subsets of T cells, namely regulatory T (Treg) and Th17 still needs to be fully elucidated.

Regulatory T (Treg) cells, characterized by the expression of the transcription factor Foxp3, are a functionally distinct, immunosuppressive T cell subpopulation that is thought to be engaged in sustaining immunological tolerance and homeostasis (41).

Several groups have undertaken association studies in patients with IBD to provide whether changes in Treg cells can be correlated with disease. Some studies found that patients with CD or UC have decreased numbers of peripheral suppressive CD4+ CD45RO+ CD25+ FOXP3+ Treg cells during active disease, suggesting that the severity of disease is inversely correlated with the frequency of peripheral Treg cells (42-44). In contrast, other studies have found that the lamina propria, mesenteric lymph nodes and intestinal inflamed mucosa of patients with either CD or UC contain increased numbers of Treg cells compared with healthy controls (45;46). The elucidation of the factors regulating Treg localization during immune responses and how they control intestinal inflammation will be key for future therapeutic treatments.

The Th17 subset is a recently-described proinflammatory T helper cell lineage that produces IL-17 and IL-22 to eliminate specific types of pathogens that require a strong inflammatory response. However, an exaggerated Th17 response can give rise to chronic inflammation and autoimmune disorders (47;48).These T cells are thus potent effectors of inflammation, but little is known about the requirements for their differentiation. Consistent with this notion, Ivanov et al. demonstrated that specific commensal microbiota are required for Th17 cell differentiation in the lamina propria of the small intestine. In addition their results suggest that the microbiota regulate the balance between Th17 and Treg cells in the intestinal mucosa and may thus influence intestinal immunity and susceptibility to IBD (49). Moreover, it has been reported that CD4 T cells express TLRs and respond directly to TLR ligands. This suggests that the activation of antigen-specific immune responses is controlled by innate immune recognition (50). It is thus possible that alterations of the microbiota induced by diet may affect components of adaptive mucosal immunity and contribute to the pathophysiology of IBD. Elucidation of the specific role of distinct intestinal microbial species in the regulation of the balance and function of different helper T cell populations, will offer a greater understanding of how the intestinal host-microbial relationship regulate host defense and inflammation.

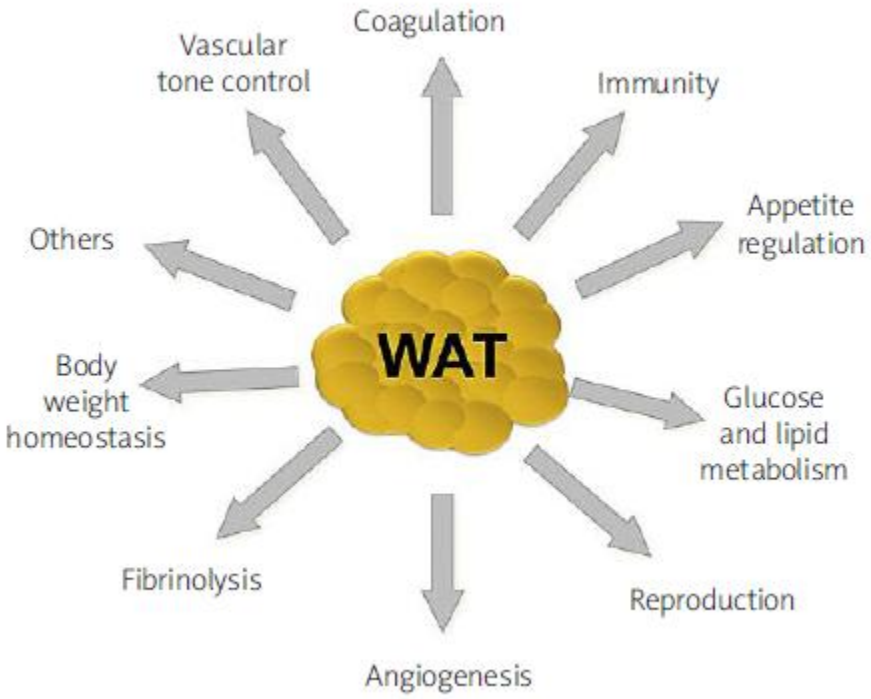


Izcue A. et al,Annual Review of Immunology 2009

Fig.3: Intestinal immune environment.

1.6 White Adipose tissue (WAT)

Research over the past two decades has revealed that adipose tissue is a passive site of energy storage. Yet recently it has emerged that adipose tissue is also an important endocrine and metabolic organ as well as a key player in immunity and inflammation. This dynamic tissue is composed not only of adipocytes, but also of other cell types including stromal-vascular cells and immune cells and it is surrounded by supporting connective tissue that is highly vascularized and innervated. As an endocrine organ, adipose tissue is responsible for the synthesis and secretion of several hormones, known as adipokines, that are implicated in the regulation of many tissue response including hypothalamus, pancreas, liver, skeletal muscle, kidneys (51,52).



Coelho et al, Arch Med Sci 2013

Fig.4: Physiological functions of WAT.

1.7 Obesity and inflammation

The qualitative and quantitative changes in the intake of specific food components have consequences not only on the composition of gut microbiota, but may also modulate the expression of genes in the adipose tissue, liver and skeletal muscle (53). These tissues readily buffer excess nutrients by storing them as triglycerides and glycogen. Chronic over-nutrition dramatically remodels adipose tissue architecture, driving adipocyte hypertrophy, oxidative stress and immune cell infiltration, followed by increased production of proinflammatory adipokines and cytokines that contribute to the progression of a chronic, low-grade inflammatory state (2). Obesity is associated with this inflammatory phenotype and increases the risk of chronic disease, including cardiovascular disease, as well as insulin resistance that predisposes to developing type 2 diabetes (4). These obesity-associated diseases are subsequently linked to premature death, and reinforce the need to further define the complex relationship between inflammation and adipose tissue dysfunction.

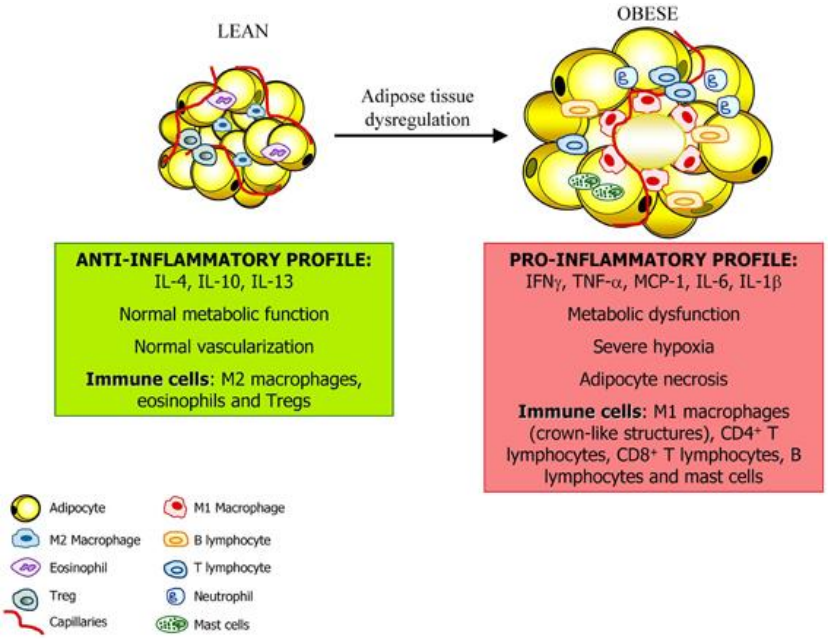
Different studies have identified several candidates for inducers of metabolic inflammation such as endoplasmic reticulum stress due to excess lipid burden (54), hypoxia due to decreased oxygen diffusion into enlarged adipocytes (55), and toll-like receptor activation through free fatty acid sensing (56). In the mid-90s, an important role for inflammatory mediators, in particular TNF-α and IL-6, was revealed, and in ensuing years an active participation by macrophages was described (57;58). In healthy adipose tissue dispersed macrophages display an alternatively activated (M2) anti-inflammatory phenotype and promote insulin sensitivity in adipocytes by secreting IL-10(59). Positive energy balance, such as during diet-induced obesity (DIO), lead to increased macrophage infiltration and shifts toward the classical pro-inflammatory (M1) state. M1 macrophages aggregate around necrotic adipocytes, forming “crown-like structures” and produce pro-inflammatory cytokines perpetuating WAT inflammation and dysfunction contributing to the induction of insulin resistance (60). However, Fujisaka et al have demonstrated that the number of M2 macrophages was also increased in mice fed with HFD. The primary trigger for the recruitment of M1 macrophages is thought to be the secretion of TNF-α from adipocytes (61). In contrast, the precise mechanism of how the increase of M2 macrophages is induced in diet-induced obese mice is not fully understood. These changes in the inflammatory phenotype of macrophages imply that other immune cells, such as T cells, may also participate in WAT inflammation (62,63). However, although T lymphocytes are known to interact with macrophages and regulate the inflammatory cascade, the specific contribution of the different lymphocyte subsets and their cytokines in obesity-induced adipocytes inflammation remains unclear. Nishimura et al showed that CD8+ T cell infiltration is required for adipose tissue inflammation and precedes the accumulation of

macrophages (64). Recent reports demonstrate that during obesity the infiltrating CD4 T cells display a Th1 pattern of activation with enhanced interferon-γ production, and that an imbalance may exist in obese adipose between dominant Th1 responses and reduced Treg or Th2 responses (65-67). Additionally, understanding how T cells are recruited to adipose tissue and activated in obesity as yet to be investigated. The interaction between T cells and adipocytes is multi-faceted: several chemokines and cytokines have been implicated in the recruitment and activation of lymphocytes to the WAT, but a recent report suggests also that adipocytes themselves may be important antigen presenting cells that influence T cell activation in obesity (68).

A better understanding of the crosstalk between metabolism and inflammation may optimize clinical interventions and lifestyle changes to improve human health.

The Council on Science and Public Health of the American Medical Association (AMA) "recognize(s) obesity as a disease state with multiple pathophysiological aspects requiring a range of interventions to advance obesity treatment and prevention".

Reducing inflammation may thus represent a feasible disease-prevention strategy for obesity.



Catalan et al, Front. Physiol.2013

Fig. 5: Adipose tissue dysfunction

2. AIM OF THE STUDY:

The aim of the first part of my work was to investigate a putative role for TLRs/NLRs in triggering events that lead to alteration of the small intestinal mucosa associated with consumption of Western Diet (WD).
In the second part of my work, the aim was to evaluate the effects of X73, a commercial small-molecule anti-inflammatory (the structure of which we cannot disclose in this dissertation for Intellectual Property reasons) in a mouse model of diet-induced obesity (DIO). In particular we analyzed the metabolic and inflammatory status and adipose tissue changes in mice fed with high fat diet (HFD) compared with mice fed with standard diet (SD), and how these events were modified as a result of treatment with X73.

3. MATERIALS AND METHODS

3.1 Mice and Western diet

C57BL/6J female and male mice at 8 weeks of age deficient for TLR2, 4, 9, or NOD2 and C57BL/6J wild-type (WT) mice fed with both standard (SD; AIN-76A) and Western Diet (WD;D16365B) have been used. The animals were separated in ten groups as follows: animals fed with WD (TLR2* KO, 4*KO, 9 *KO, NOD 2* KO and WT*) and animals fed with SD (TLR2 KO, 4 KO, 9 KO, NOD2 KO and WT). Mice were monitored daily and weighed weekly. Five mice for group were anesthetized by prolonged CO_2 treatment and sacrificed by cervical dislocation at 3, 6 and 12 months feeding the diets. We will use the WD that has the same high levels of fat (20%) and low calcium and vitamin D of the WD originally formulated by Newmark and Lipkin and reflects dietary patterns linked to human intestinal cancer incidence (7).

3.2 Mice and High Fat Diet

We have used C57BL/6J female mice at 8 weeks of age. Ten animals were fed with SD (SD, AIN-76A) and 30 mice were fed with High Fat Diet (HFD, D12492) for three months. Then, for other two months, only the mice fed with HFD were separated in four groups as follows: 9 animals continued to be fed with HFD, 7 animals returned to SD, 7 animals continued to be fed with HFD and 7 returned to SD but they received 30mg/kg of X73, a commercial small-molecule anti-inflammatory treatment (the structure of which we cannot disclose in this dissertation for Intellectual Property reasons) in their drinking water. The water was changed every third day to maintain dosage. Body weight was monitored weekly. At the end of the experiment the mice were anesthetized by prolonged CO_2 treatment and sacrificed by cervical dislocation.

3.3 Processing of samples

After collection, the adipose tissue and small intestine tissue samples were fixed in 4% formalin at 4°C o/n. Then we proceeded with 2 rinses in PBS half-hour after each and were placed in 70% ethanol for at least 12 hours. Finally the samples were dehydrated by successive steps in an ascending scale of alcohols, placed in xylene for 2 hours and finally embedded in paraffin and sectioned at 3µm.

3.4 Hematoxylin and eosin staining (H&E)

Hematoxylin-eosin staining was performed on sections obtained from paraffin-embedded samples. The samples were deparaffinized in xylene, rehydrated through descending scale of alcohol, left for 5 minutes in hematoxylin, washed under running water and then placed in eosin for 6 minutes. After washing the sections were dehydrated through ascending scale of alcohols and xylene and then mounted with coverslips using Eukitt (Sigma). All samples were observed and photographed with a microscope Olympus BX53 with a digital camera.

3.5 Ki67 immunohistochemistry

The intestinal sections on slides are deparaffinized in xylene and hydrated through descending scale of alcohol. Antigen retrieval was performed by boiling sections for 10' in 0,005M sodium citrate buffer (pH 6) in a microwave oven. Endogenous peroxidase was blocked by incubation with 3% H_2O_2, 50% methanol solution in PBS 1x 20' at room temperature (RT), after which the sections were washed with PBS 1x (three washes of 5 minutes each). Sections were incubated with primary antibody anti-Ki67 (rat anti-mouse, Dako TEC-3) 1:50 in PBS for 30' at RT, followed by 3 washes for 5 minutes with PBS 1x. After washing, the sections were incubated with biotinylated secondary antibody rabbit anti-rat 1:100 (Abcam, 6733) diluted in PBS 1x for 30' minutes at RT. We proceeded with further washes for 5 minutes with PBS 1x, then the sections were incubated with ABC-kit (Vector) and immunoreactivity was visualized with 3,3-diaminobenzidine (DAB ,Biocare Medical). The development has been blocked with distilled H_2O. Sections were counterstained with hematoxylin for 5 minutes at RT and finally dehydrated through ascending scale of alcohols and xylene, then mounted with coverslips using Eukitt (Sigma).

3.6 Friezzle-5 immunohistochemistry

The intestinal sections on slides are deparaffinized in xylene and hydrated through descending scale of alcohol. Antigen retrieval was performed by boiling sections for 10' in 10 mM sodium citrate buffer (pH 6) in a microwave oven. Endogenous peroxidase was blocked by incubation with 3% H_2O_2, 50% methanol solution in PBS 2x15' RT. Nonspecific binding was blocked for 45' with 10% goat serum in PBS 1x, after which the sections were incubated with primary antibody anti-Fzd5 (Abcam, 75234) 1:1000 in PBS for 60' at 37°C, followed by 3 washes for 5 minutes with PBS 1x. After washing, the sections were incubated with biotinylated secondary antibody anti-rabbit 1:500 (BA-1000, Vector) diluted in PBS 1x for 30' minutes at RT. We proceeded with further washes for 5 minutes with PBS 1x, then the sections were incubated with ABC-kit (Vector) and DAB ,Biocare

Medical).The development has been blocked with distilled H_2O. Finally sections were dehydrated through ascending scale of alcohols and xylene, and then mounted with coverslips using Eukitt (Sigma).

3.7 Quantitative real-time PCR

Total RNA was extracted from adipose and small intestine tissues using PureZol RNA Isolation Reagent (Bio-Rad, Hercules, CA, USA) and incubated for 15 minutes. RNA was separated with chloroform and centrifuged for 15 minutes at 4°C, 12000g; then aqueous phase was separated and isopropanol was added to precipitate RNA. Pellet of RNA was cleaned with ethanol 75%, recentrifuged and dissolved in RNAase free water. The cDNAs were synthesized using High capacity cDNA Reverse Transcription Kit and the gene expressions were quantified using the TaqMan Master Mix and the Viia7 Real-time PCR machine (Applied Biosystems, Foster City, CA, USA) in accordance with manufacturer's instructions. Primer sets for individual genes were purchased from Applied Biosystems. 18S was used as a housekeeping gene, levels of which did not change with the various diets or genotypes. Relative amounts of the target gene were calculated using the ΔΔCt method. The primers sets used are listed in table 1.

Primer	code
CD3	Mm00599684_g1
Foxp3	Mm00475162_m1
RoRgt	Mm01261022_m1
CD4	Mm00442754_m1
F4-80	Mm00802529_m1
IL-6	Mm00446190_m1
IL-10	Mm00439614_m1
IL-12	Mm00434174_m1
IFN-γ	Mm01168134_m1
IL-4	Mm00445259_m1
IL-13	Mm00434204_m1
TNF-α	Mm00443258_m1
CCL2	Mm00441242_m1
18S	Mm03928990_g1
HIF1-α	Mm00468869_m1

Tab.1: List of primers.

3.8 Oral Glucose tolerance test (OGTT)

This test provides information on how the body copes when exposed to high blood glucose levels. OGTT was determined after oral glucose load at a dose of 1 g/kg body weight by oral gavage. Blood glucose was measured at intervals of 0, 30, 60 and 120 min using Accu-check glucometer (Roche diagnostics).

3.9 Insulin assay

Serum insulin concentration was measured by ELISA (EZRMI-13K, mouse/rat insulin ELISA kit, Millipore, MA) according to the manufacturer's instructions. Briefly, 10µl of serum samples were added into each well and incubated at RT with Detection Antibody for 2 h followed by repeated washes. Substrate was added for 10 min and absorbance was measured at 450 nm. Standard samples provided with the kit were prepared and used to generate a calibration curve to interpolate sample data values. Insulin content in control and treated samples was expressed as ρg/ml.

3.10 IL-6 ELISA assay

Serum IL-6 concentration was measured by ELISA (DY406 ELISA kit, R&D System) according to the manufacturer's instructions. Briefly, 50 µL per well of the Capture antibody were used to coat the 96-well microplate, and incubated overnight at RT. After wash, 100 µL of sample or standards were added in Reagent Diluent per well. The plate was covered with an adhesive strip and incubated 2 hours at RT, followed by aspiration/wash. 100 µL of the Detection Antibody was added to each well and incubated 2 hours at RT, followed by aspiration/wash. 100 µL of the working dilution Streptavidin-HRP was added to each well and incubated for 20'RT. After the aspiration/wash 100 µL of the Substrate Solution was added and incubated for 20'RT, followed by 50 µL of Stop Solution. The optical density was determined using a microplate reader set to 450 nm. Standard samples were used to generate a calibration curve to interpolate sample data values. IL-6 content in control and treated sample was expressed as ρg/ml.

3.11 Glucose Assay kit

Serum glucose concentration was measured by the Glucose Assay Kit according to the manufacturer's instructions. Serum can be directly diluted in Glucose Assay Buffer. Typically 0.5-2 µl/assay is sufficient (normal serum contains ~5 nmol glucose/µl). 50 µl/well of reaction mix is required, and should contain the following components: 46 µl of Glucose Assay Buffer, 2 µl of Glucose Probe and 2 µl of Glucose Enzyme Mix. The reaction mix was added to each well containing a diluted Glucose Standard or test samples and incubated 30 min at 37°C, protect from light. The optical density was determined using a microplate reader set to 570 nm. Glucose concentrations of the test samples were calculated based on the standard curve generated.

3.12 Quantification of adipocytes size

Tissue sections from mesenteric adipose tissue were stained with H&E. Five fields for each mouse sections were included in the analysis. ImageJ software was used to measure adipocytes area, which is represented as μm^2.

4. RESULTS

4.1 Western Diet modifies small intestinal mucosa morphology in TLR KO mice

Diet is considered one of the main factors contributing to the diversity of gut microbiota. As TLRs/NLRs recognize bacterial products, there is a possibility that changes in bacterial composition induced by diet can promote a different response by TLRs/NLRs and thus may increase susceptibility to intestinal inflammation. Although several studies reported that Western Diet induces intestinal neoplastic lesions when fed to wild-type mice for 1 year, there are no studies regarding TLR/NLR KO mice. In order to elucidate a putative role for TLRs/NLRs in triggering events that lead to alteration of the small intestinal mucosa associated to consumption of western diet (WD), we utilized C57BL6/J wild-type (WT) mice and TLR/NLR KO mice (TLR2, 4, 9 and NOD2 KO mice) fed with standard diet (SD) or WD for 3, 6 and 12 months. Body weight was monitored weekly (Fig.1) and histological analyses were performed in small intestinal sections in order to investigate the effect of WD on the morphology of the small intestine (Fig.2).

After 3 months the body weight in WT group fed with SD remained constant around 20 g/mouse (left graph, fig.1A).All mice on WD, except TLR2 KO-WD, gained more body weight than mice fed with SD (right graph, fig.1A). In particular, there was a significant difference between WT and NOD2 KO fed with WD compared to WT and NOD2 KO mice fed with SD (Fig.1B).

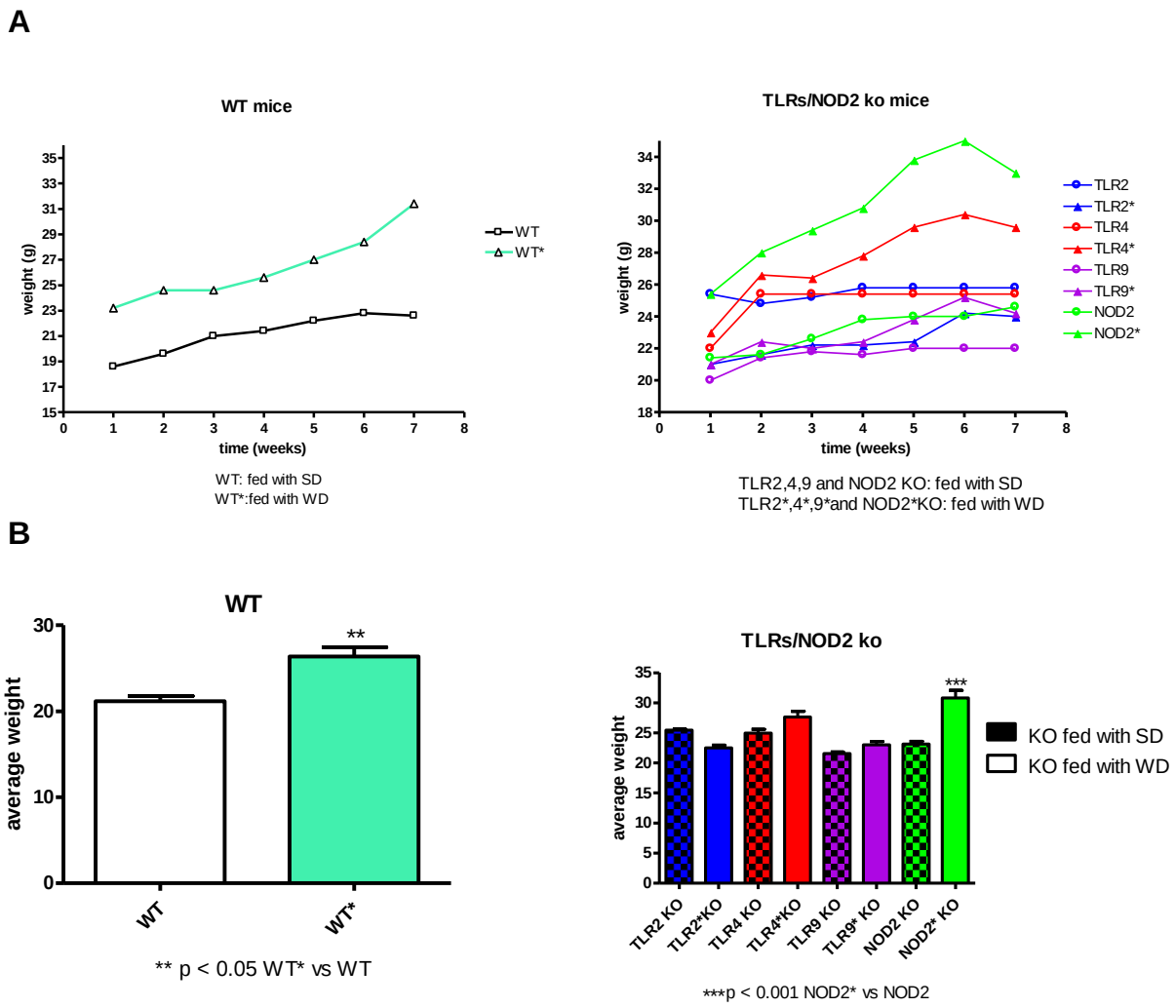


Fig.1: Trend (A) and statistical analysis (B) of body weight of WT and KO mice fed with SD and WD. (*) indicates WD-fed animals.

Anatomo-pathological observations revealed that WT mice fed with WD did not show any morphological alteration compared with WT mice fed with SD. Among TLR 2, 4, 9 and NOD 2 KO mice fed with WD, the group showing more significant alterations including villus atrophy and crypt disorganization were the TLR9 KO mice. In contrast, NOD2 KO mice showed a normal mucosa as compared to NOD2 KO mice fed with SD. TLR2 and 4 KO mice on WD showed only mild alterations (Fig.2). Evaluation of H/E stained sections from all KO mice fed with SD did not reveal changes of the mucosa architecture (Fig.2).

We next evaluated the small intestinal morphology of WT and KO mice fed with SD and WD at six and twelve months of WD feeding. At six months we did not observe alterations in intestinal epithelial cell architecture (data not shown). The results at 12 months showed that WT mice fed

with SD and WT and NOD2 KO mice fed with WD spontaneusly developed small intestinal tumors. Unexpecdedly, we did not observe tumor formation in WD-fed TLR 9 KO mice, the groups displaying the most marked alterations at three months after consuption of WD (data not shown). Overall, the histological analysis suggests that after only three months, WD is able to induce alterations in the small intestinal epithelium in KO mice, in particular in TLR9 KO.

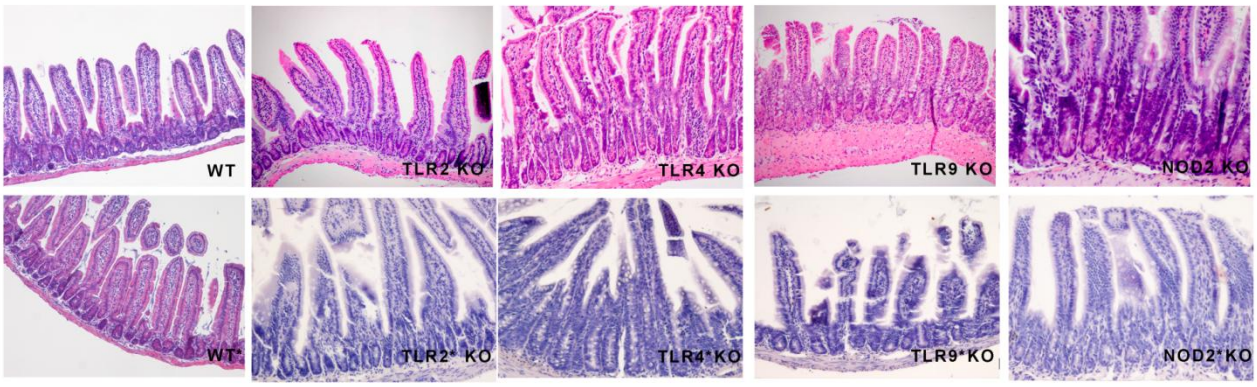


Fig.2: WD induces morphological alteration of the mucosa in TLR9 KO mice.
Representative sections of the small intestine from wild-type and transgenic adult mice stained with hematoxylin/eosin (WT, TLR2 KO, TLR4 KO, TLR9 KO, NOD2 KO fed with SD; WT*, TLR2 *KO, TLR4 *KO, TLR9* KO, NOD2* KO fed with WD)

4.2 Western Diet affects cellular proliferation and Wnt signaling in KO mice

Homeostasis of the intestinal epithelium is strongly dependent on the balance between cell proliferation, cell cycle arrest and cell differentiation. These processes are intimately coupled to cell migration in a precise, spatially organized manner. At the bottom of the crypts, stem cells give rise to a transient population of undifferentiated cells that vigorously proliferate as they migrate toward the lumen of the intestine. Absorptive, enteroendocrin and goblet cells migrate toward the villus while Paneth cells occupy the bottom of the crypts (12). We evaluated the effects of WD on small intestinal homeostasis. Determination of the proliferation rate of crypt cells, performed by immunohistochemistry for the nuclear proliferation marker Ki-67, revealed a trend for the reduction of proliferation in all mice fed with WD compared with mice fed with SD; this reduction was significant for WD-fed NOD2 KO mice (Fig.3). We next assessed proliferative cell localization (Fig.4). In WT mice fed with both SD (WT) and WD (WT*) proliferative cells were distributed as expected in the transit–amplifying zone (TA). It is noteworthy that, in KO mice fed with WD (TLR2*, 4*, 9* and NOD2* KO) proliferative cells are irregularly located also at the base of crypts

among Paneth cells, where intestinal epithelial stem cells are normally found. Moreover in TLR9 KO mice fed with WD (TLR9*), proliferative cells are often detected in the villus epithelium normally occupied by differentiated cells (Fig.4). Yet all KO mice fed with SD showed a normal localization of proliferative cells in the TA zone as observed in WT mice (Fig.4).

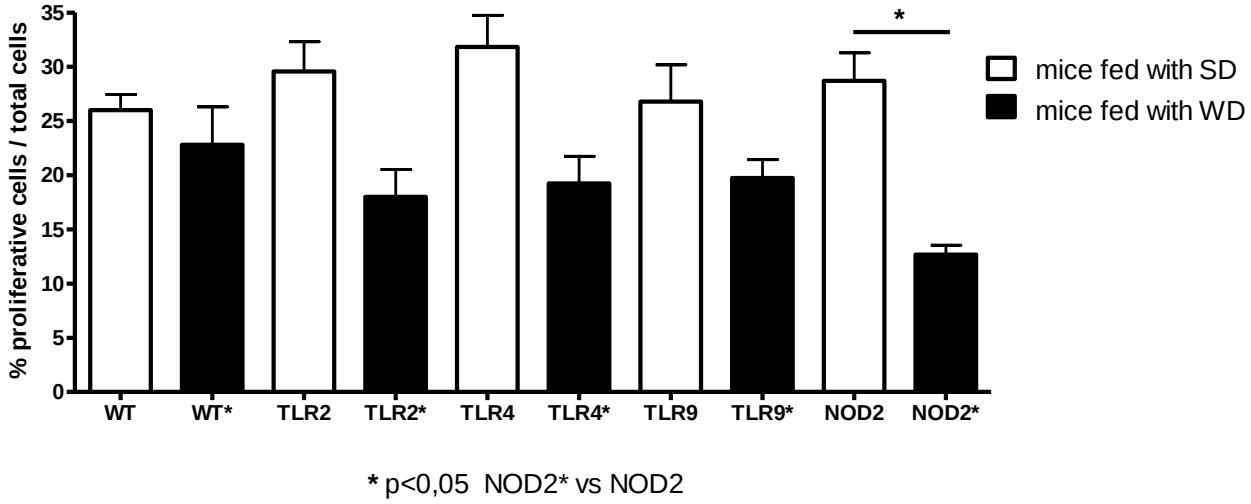


Fig. 3: Trend for the reduction of proliferative cells in all groups of mice fed with WD.
Mitotic cell count**:** WT, TLR2, 4, 9, NOD2 KO mice fed with SD; WT*, TLR2*, 4*, 9*, NOD 2* KO mice fed with WD.

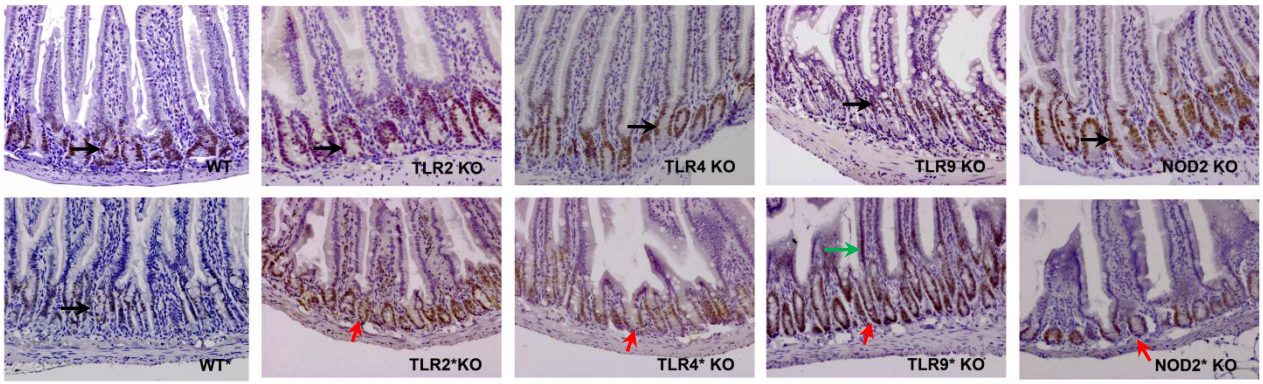


Fig.4: KO mice fed with WD show an ectopic localization of proliferative cells.
Immunohistochemical analysis of small intestine sections stained with an antibody against proliferating cell antigen Ki-67. Proliferative cells were distributed as expected in intestinal crypts (TA) of the WT and KO mice fed with standard diet (black arrow), but were present in an ectopic position at the base of the crypts among Paneth cells in KO mice fed with WD (red arrow). Moreover in TLR9 fed with WD proliferative cells were often detected in villus epithelium normally occupied by differentiated cells (green arrow).

To further understand this aberrant cell localization we decided to investigate the WNT/β-catenin pathway. Wnt signalling has a key role in the formation of normal crypt-villus and aberrant activation of Wnt signaling is strongly associated with the development of intestinal cancer (19). In the intestine, Frizzled 5 (Fzd5), a component of WNT signaling, is necessary for regulation of Paneth cell differentiation and is normally confined to expression in crypt cells. Recent reports demonstrated that WD induces changes in villus cells that include ectopic expression of Paneth cell markers (a lineage normally confined to the bottom of small intestinal crypts) and elevated expression of the Wnt receptor (21).We performed immunohistochemistry for Fzd5 on WT and TLR/NLR KO mice. Its expression, in WT and KO mice fed with both SD and WD for 3 months was very low at the base of the crypt (Fig.5). Interestingly, only in TLR9 KO mice fed with WD was Fzd5 expression detected along the villus cells (Fig.5). Relevant to these findings, Wang et al demonstrated that WD induces ectopic expression of Fzd5 in WT mice fed with WD for one year (21). It is noteworthy that we observed this alteration at three months in TLR9 KO mice. Moreover, aberrant expression of FZD5 in TLR9 KO mice correlates with the morphological alterations and the ectopic localization of proliferative cells that we observed before. These findings demonstrate that only three months WD are sufficient to induce alterations in the distribution of proliferating cells that can lead to defect in Wnt-βcatenin signaling. Importantly, these results suggest a critical role for TLRs, especially TLR9, in the control of small intestinal homeostasis.

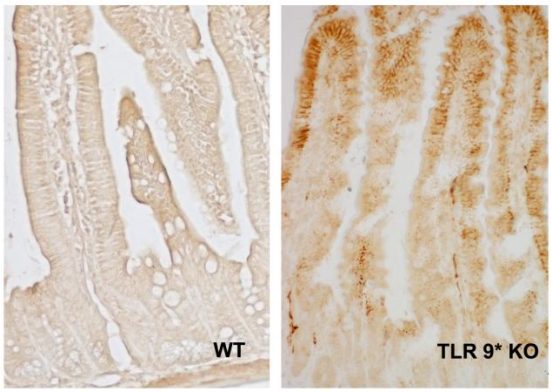
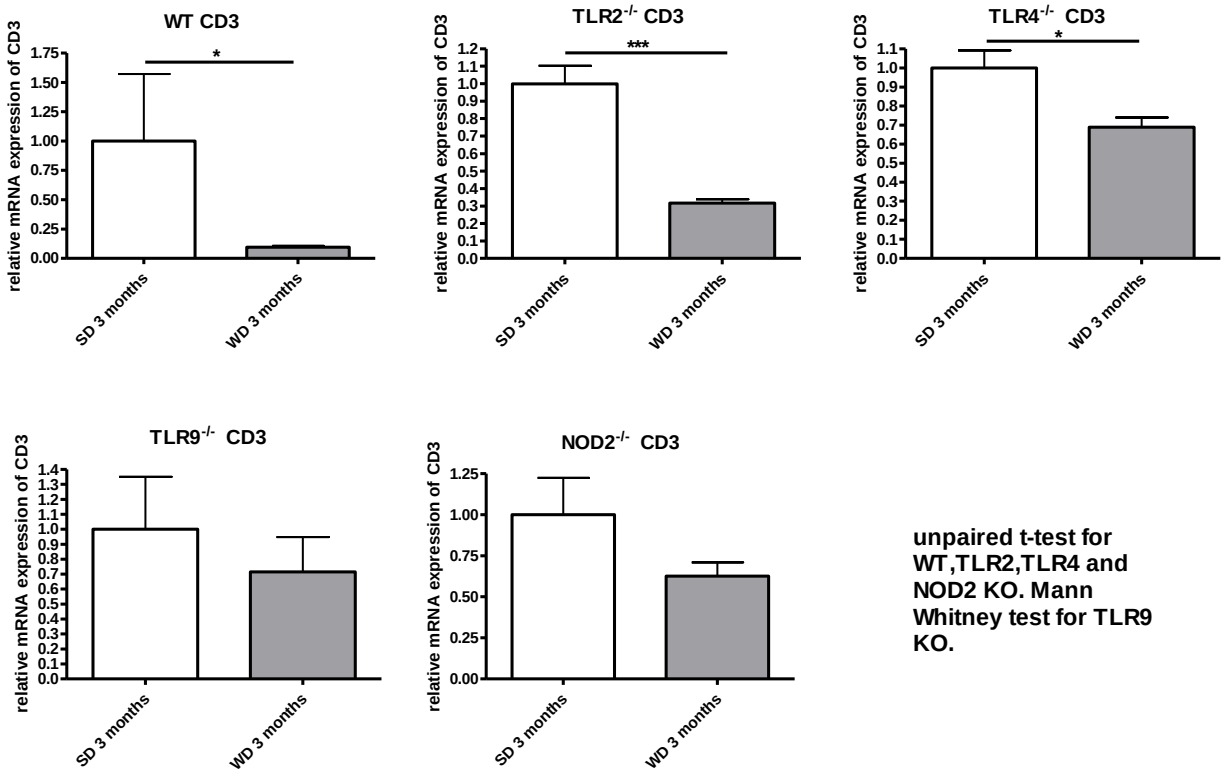


Fig.5: **Aberrant expression of Frizzled 5 (marker of Paneth cell differentiation) in TLR9* KO mice.** Immunohistochemical detection of Fzd 5 in mice fed with SD and WD. Fzd5 expression was detected along the villus cells only in TLR9* KO mice fed with WD. Fzd5 expression in WT and in TLR/NLR KO mice fed with both SD and WD for 3 months was very low at the base of the crypt (representative image of WT fed with SD).

4.3 Effects of Western diet on T cell gene expression

In order to evaluate the contribution of T cells to the alterations in the small intestinal mucosa associated to consumption of WD, we performed quantitative real time (RT)-PCR on small intestine samples of WT and TLR/NLR-KO mice fed with SD compared to mice fed with WD. In particular, we evaluated CD3e expression, a marker of all T cells, Foxp3 expression, the transcription factor expressed by immunosuppressive regulatory T (Treg) cells and RoRgt expression, transcription factor for the pro-inflammatory Th17 cells. We observed a reduction of CD3 in all mice fed with WD compared to mice fed with SD, significantly so in WT, TLR2* and TLR4*KO (Fig.6A). We calculated the Foxp3/CD3 and RoRgt/CD3 ratio, as an indicator of the relative abundance of the respective T cell subpopulations. The results showed that there is a significant increase in Treg cells compared to the total lymphocytes population only in WT and TLR2*KO fed with WD (Fig.6B). In TLR2*KO and TLR4*KO fed with WD we observed a significant increase of Th17 compared to the total T cell population (Fig.6C). The Foxp3/RoRgt ratio showed a significant reduction of Foxp3 in TLR2*KO mice fed with WD (Fig.6D).

A



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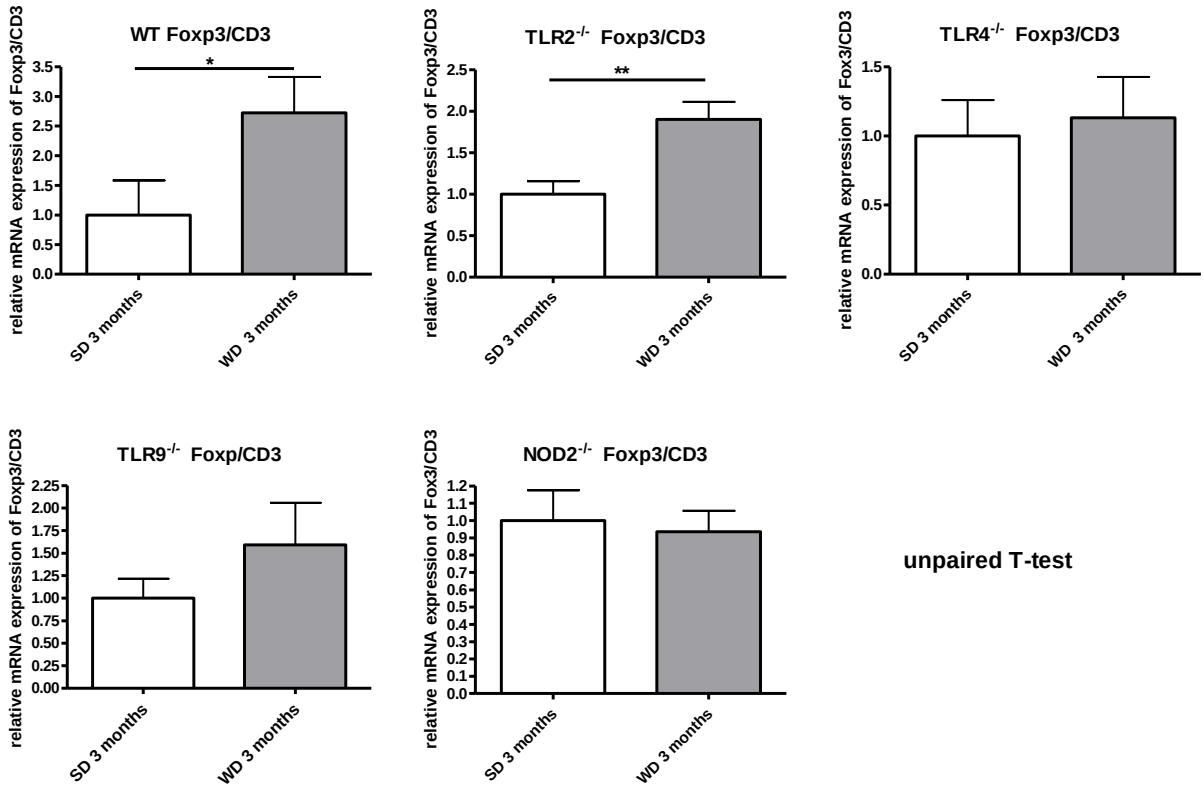
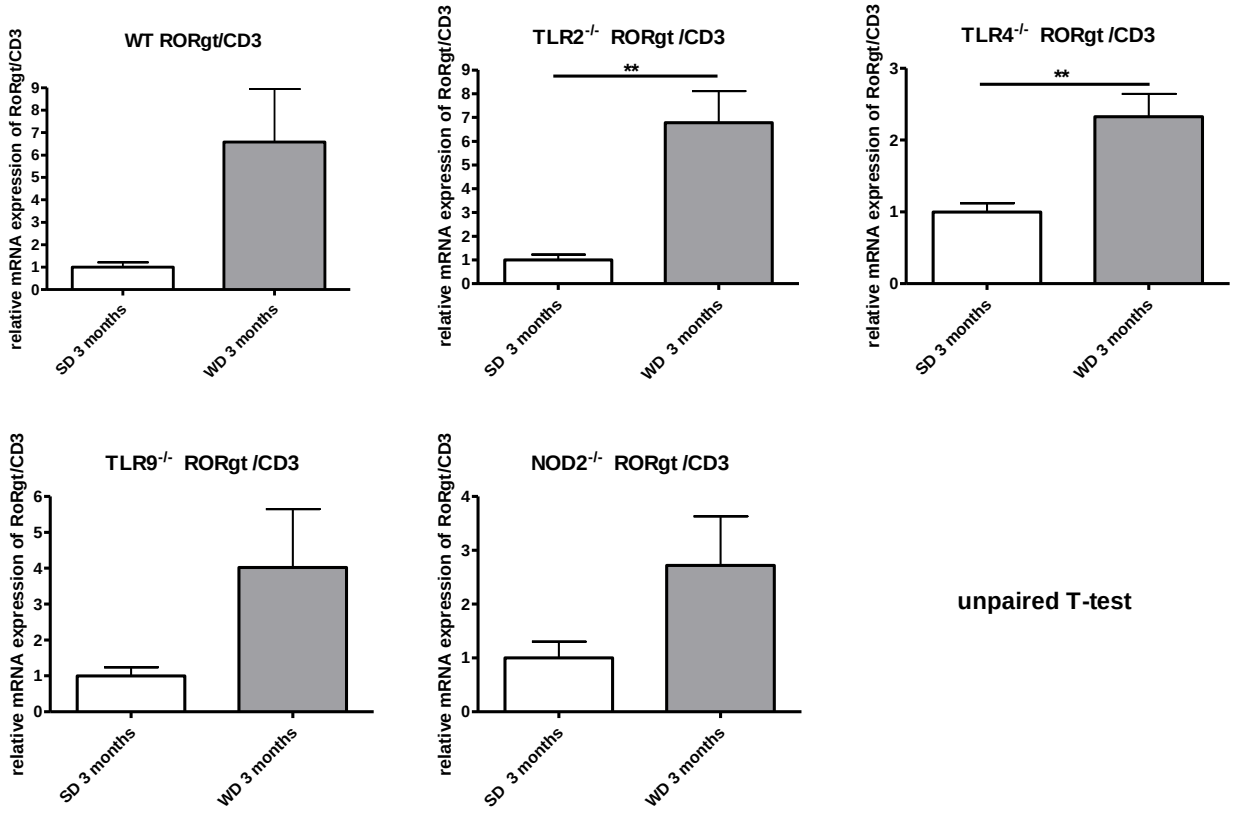

WT Foxp3/CD3
relative mRNA expression of Foxp3/CD3
*
SD 3 months
WD 3 months
TLR2-/- Foxp3/CD3
relative mRNA expression of Foxp3/CD3
**
SD 3 months
WD 3 months
TLR4-/- Foxp3/CD3
relative mRNA expression of Fox3/CD3
SD 3 months
WD 3 months
TLR9-/- Foxp/CD3
relative mRNA expression of Foxp3/CD3
SD 3 months
WD 3 months
NOD2-/- Foxp3/CD3
relative mRNA expression of Fox3/CD3
SD 3 months
WD 3 months
unpaired T-test

C


WT RORgt/CD3
TLR2-/- RORgt /CD3
TLR4-/- RORgt /CD3
TLR9-/- RORgt /CD3
NOD2-/- RORgt /CD3
relative mRNA expression of RoRgt/CD3
**
SD 3 months
WD 3 months
unpaired T-test

D

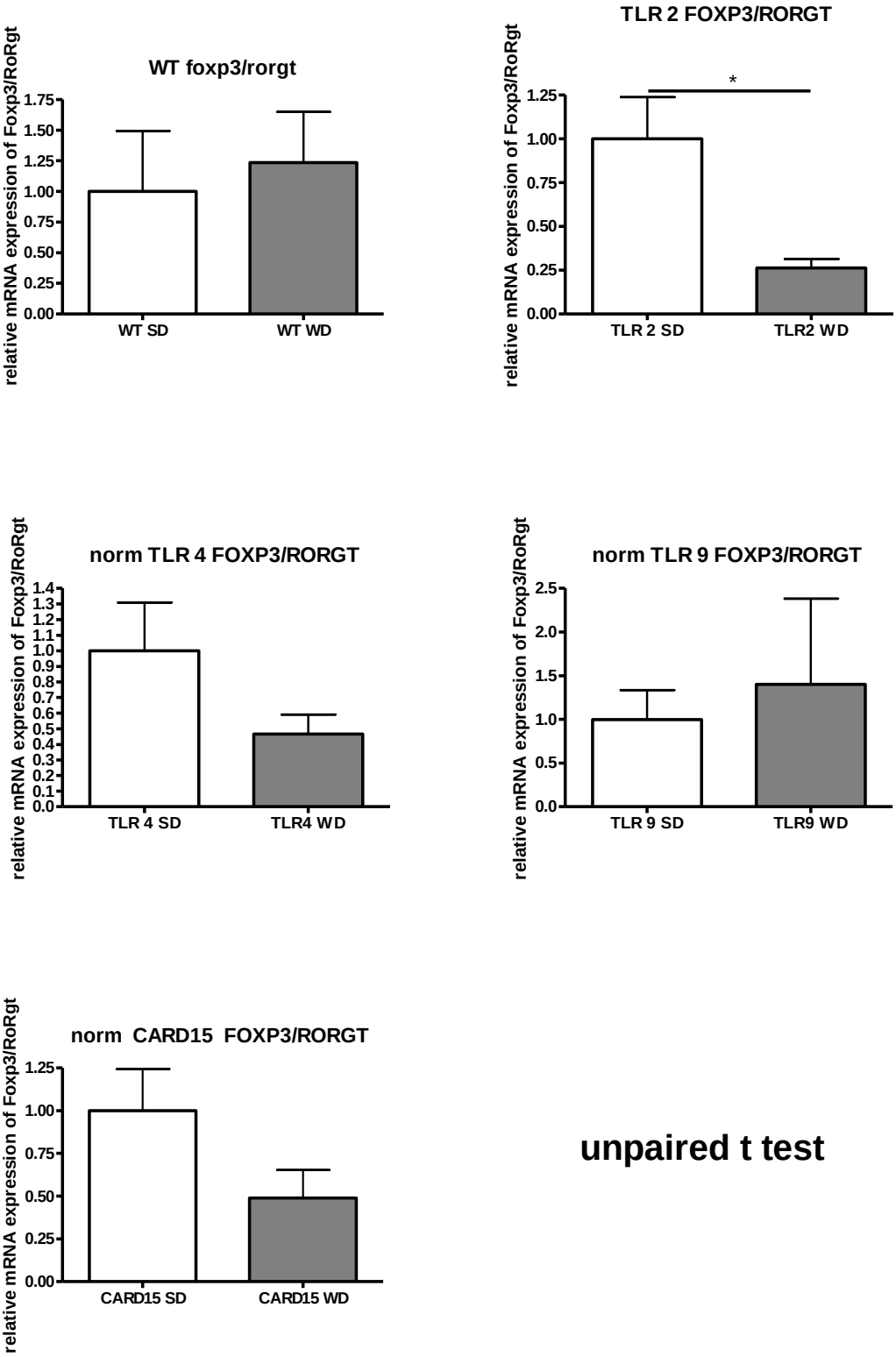


Fig.6: Gene expression analysis by real-time qPCR of T cell markers in small intestine of WT and KO mice fed with SD compared to WD. Data were normalized according to 18S mRNA level and presented as a value relative to that for SD fed mice. A: CD3 decrease in all mice fed with WD, significant in WT, TLR2

and TLR4 KO. B: increase in Treg cells in WT and TLR2*KO fed with WD. C: increase of Th17 in TLR2*KO and TLR4*KO fed with WD. D: reduction of Foxp3 in TLR2*KO mice fed with WD.

4.4 Effect of HFD and anti-inflammatory X73 treatment on the body weight and on metabolic parameters

A loss of glucose tolerance is a key component of a pre-diabetic condition in human. Often this state is associated with an increase of body fat and with adipose tissue inflammation, two important and interconnected aspects of obesity (53). Interventions which limit adipose tissue inflammation may thus represent a feasible disease-prevention strategy for obesity. For this reason, we decided to evaluate the effects of X73, a commercial small-molecule anti-inflammatory treatment (the structure of which we cannot disclose in this dissertation for Intellectual Property reasons) in a mouse model of diet-induced obesity (DIO).We used C57BL/6J female mice fed for three months with SD (10 mice) or with High Fat Diet (30 mice – see Methods for diet details). Then, for a further two months, the mice fed with HFD were separated in four groups as follows: 9 animals continued to be fed with HFD, 7 animals returned to SD, 7 animals continued to be fed with HFD with the addition of 30mg/kg of X73 in their drinking water and, finally, 7 returned to SD with the addition of 30mg/kg of X73 in their drinking water. Body weight was monitored weekly (Fig.7). The metabolic and inflammatory status was evaluated by histological, molecular and biochemical analysis in all mice.

Mice on SD and HFD had the same body weight at the onset of the experiment (Fig.7A). The body weight in the SD-fed group remained constant around 20 g/mouse (fig.7A, black line). By week 5 and for the remainder of the experiment, mice fed with HFD showed statistically significant increases in body weight ($P<0.001$) relative to the SD-fed group (fig 7A blue line). X73 treatment induced a statistically significant decrease in body weight in mice fed with HFD (fig.7B red line) compared to untreated HFD-fed controls. However, as there was a non-significant but nonetheless notable reduction of weight in the mice that received HFD and X73 post-diet change, we cannot exclude that this difference is an arbitrary effect of mouse weight variation between batches of mice. Indeed the rate of change of weight gain in HFD-fed, X73-treated mice did not alter, unlike all other groups with the exception of the HFD-fed group. As expected, mice that returned to standard diet displayed a statistically significant decrease in body weight compared to the HFD-fed group (fig.7B pink line). The group with X73 treatment that was returned to SD showed a trend similar to the untreated group returned to SD (fig.7B green line). From these results we conclude that X73 treatment in HFD-fed animals does not appear to have a biologically-relevant effect on weight.

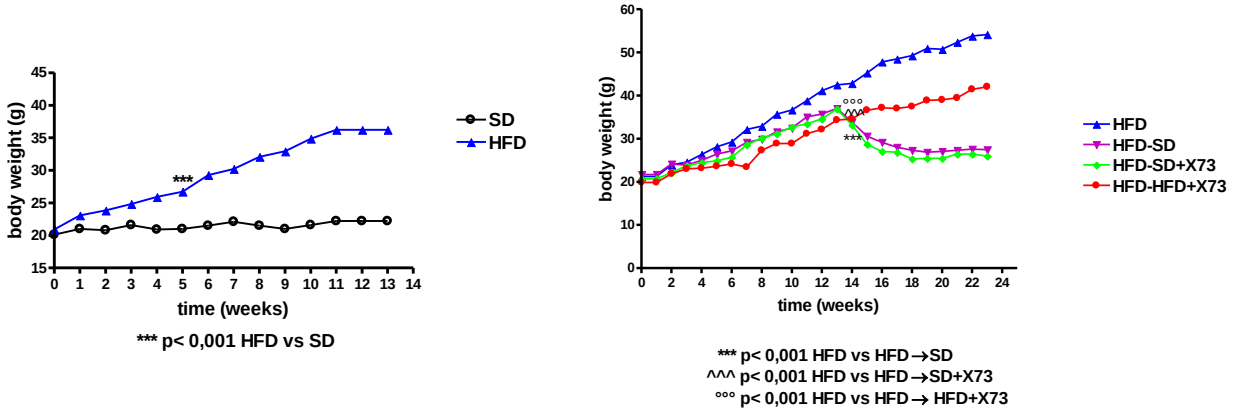


Fig.7: Effect of X73 treatment on body weight. A: SD-fed mice (black line) and HFD-fed mice (blue line); B: HFD→SD (pink line); HFD→SD+X73 (green line); HFD→HFD+X73 (red line).

To determine if X73 treatment improved glucose tolerance, we evaluated blood glucose and insulin levels in all mice using commercial kits. After three months, the HFD-fed group showed a significant increase in insulin level compared with mice fed with SD (fig.8A). Moreover, oral glucose tolerance test showed that glucose level in mice fed with HFD was significantly higher at all times as compared to lean control ($p<0,001$) (fig.8.B). Data was confirmed by measurement of serum glucose level (fig.8C). Next, we evaluated insulin and glucose levels after diet changing. As expected, mice group that returned to SD showed a significant reduction of insulin compared with mice fed with HFD. Similar results were observed for mice treated with X73 and fed with SD. It is interesting to note that X73 treatment significantly decreased hyperinsulinemia level in mice fed with HFD (fig.9 A-B). This reversal reached the insulin levels of SD-fed mice; the magnitude of this effect suggests that it is very unlikely, though not formally impossible, that the effect could have been due to the marginal yet significant difference in weight mentioned above. In summary, these results suggest that X73 treatment reverses insulin resistance in mice fed with HFD.

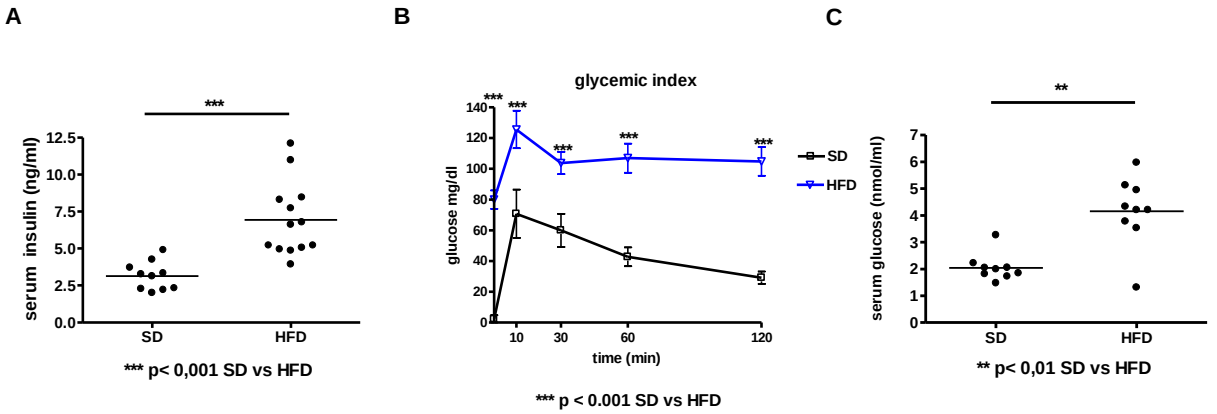


Fig.8: Serum insulin (A), Glycemic index (B) and serum glucose level (C) after three months of feeding with SD and HFD.

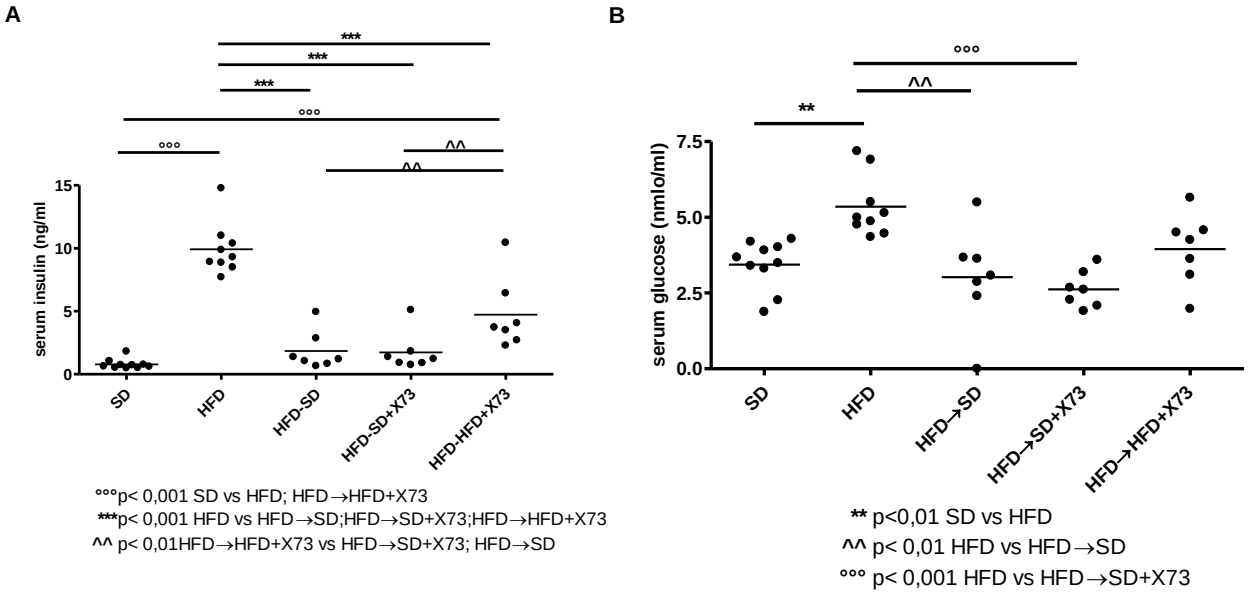


Fig.9: X73 treatment decrease hyperinsulinemia. Insulin level (A) and serum glucose (B) after two months of changing diet.

4.5 Histological characterization of mesenteric adipose tissue after treatment with X73

We assessed whether the metabolic improvements induced by X73 were accompanied by alterations in adipose tissue morphology. Morphological analysis on adipose tissue sections stained with H/E revealed large adipocytes in mice fed with HFD compared with mice fed with SD (Fig.10), as demonstrated by measurement of adipocyte size (Fig.11). The groups that returned to SD showed morphology similar to the control group. It is noteworthy that X73 treatment significantly reduced adipocyte hypertrophy in mice fed with HFD (Fig.10,11).
This data suggests that X73 treatment can exert anti-adiposity effects.

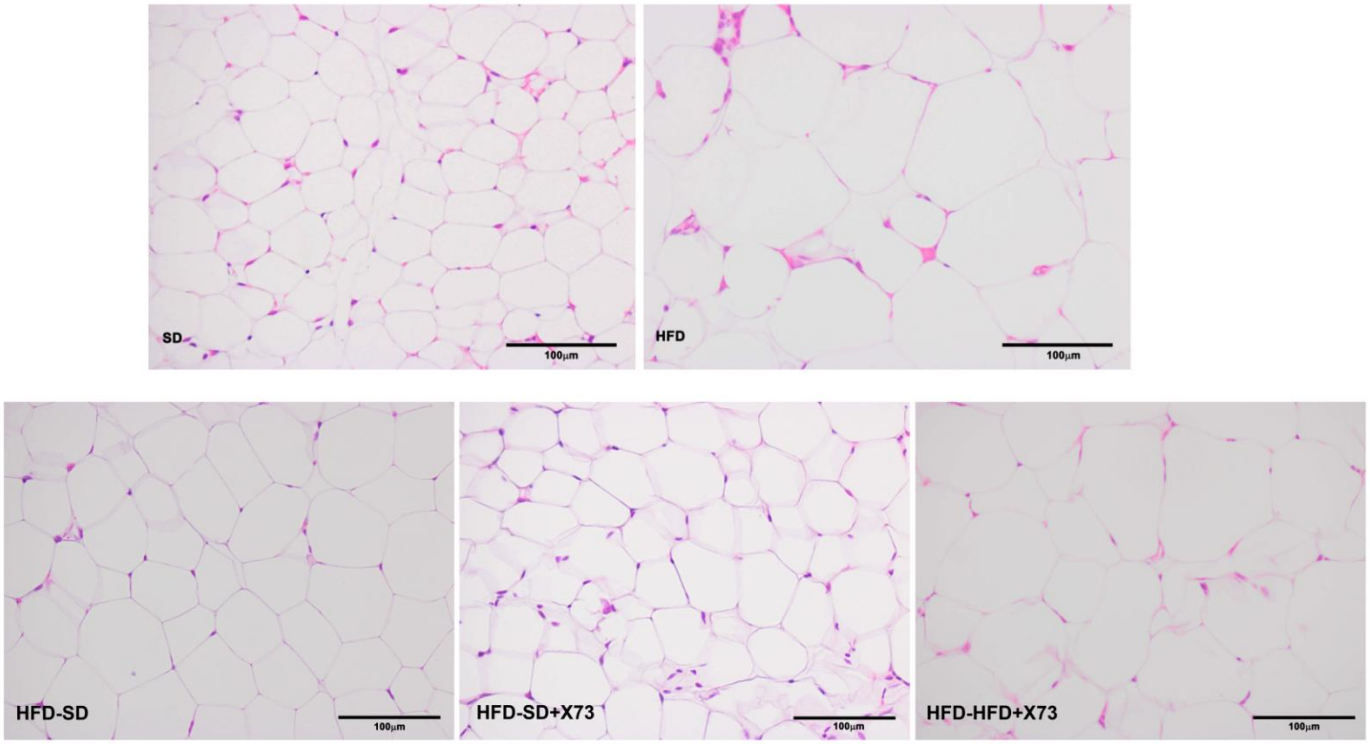
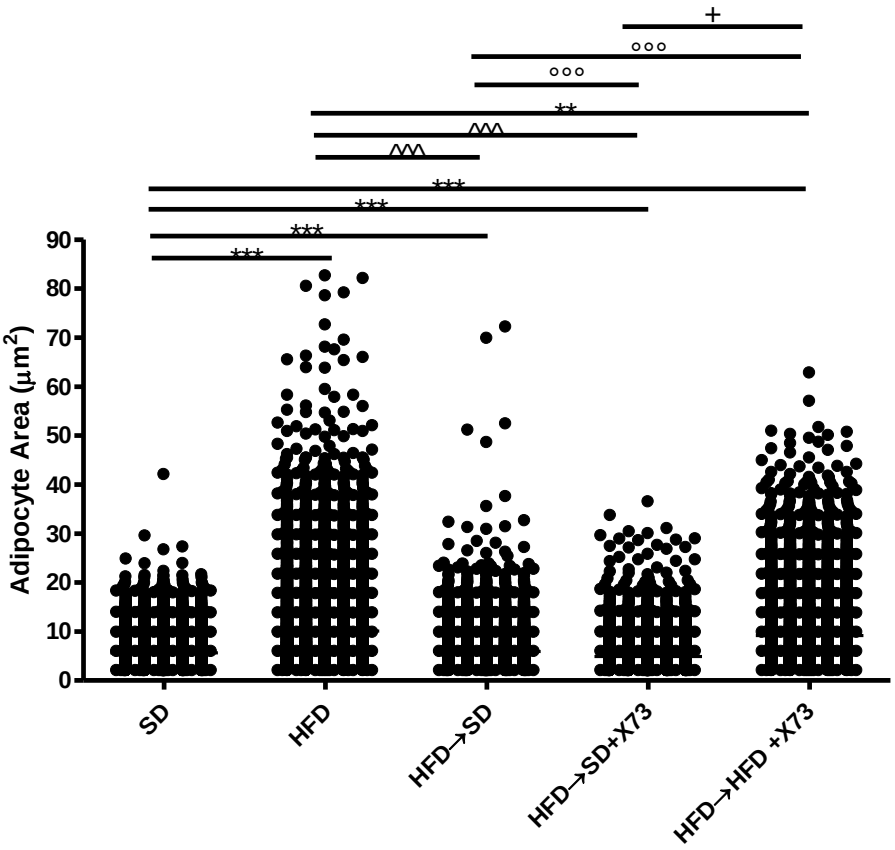


Fig.10: Hematoxylin and eosin staining of adipose tissue samples.



***p< 0,001 SD vs HFD; HFD→SD;HFD→SD+X73;HFD→HFD+X73
^^^p< 0,001 HFD vs HFD→HFD+X73; HFD→SD
**p< 0,001HFD→HFD+X73
°°°p< 0,0001 HFD→SD vs HFD→SD+X73; HFD→HFD+X73
+p< 0,0001HFD→SD+X73;HFD→HFD+X73

Fig.11: Statistical analysis of adipocytes size: X73 treatment reduce adipocytes hypertrophy

4.6 Effect of HFD and X73 treatment on the inflammatory markers

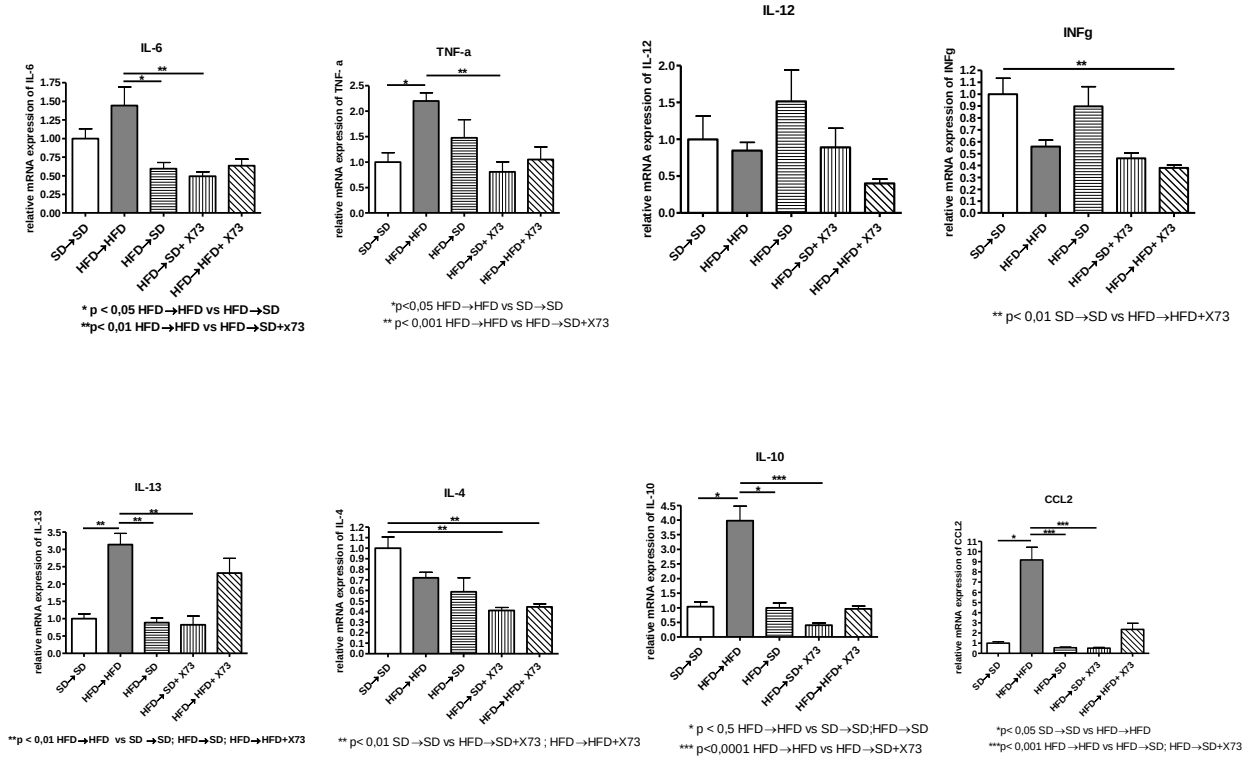
Obesity is characterized by a chronic, low-grade inflammation status. In order to evaluate the effect of X73 treatment on inflammatory state in mesenteric adipose tissue we performed quantitative RT-PCR on tissue samples (Fig.12). In particular we evaluated the changes in the gene expression profiles of the pro- and anti-inflammatory cytokines: IL-6, TNF-α, IL-13, IL-4,IL-12,INF-γ,IL-1, and the expression of macrophage and lymphocyte markers F4-80, CD3, CD4 and Foxp3.

HFD led to an increase in macrophages accumulation and a significant increase of pro inflammatory markers only for TNF-α and a trend for IL-6, that had a significant trend for reduction after treatment with X73 (Fig:12A). The level of the anti-inflammatory cytokine IL-10 was also significantly increased in mice fed with HFD compared with mice fed with SD, with a significant

trend for decrease after X73 treatment. It is noteworthy that there was also a trend for reduction of Th1 cytokines (IL-12 and INF-γ) in mice fed with HFD compared with mice fed with SD. Among Th2 cytokines, which are thought to mediate long-term adipose tissue inflammation (79) , there was a significant increase in IL-13 in mice fed with HFD compared with other groups, while the level of IL-4 was significantly higher only in SD mice (Fig:12A). This finding is novel, and sheds light into the differential involvement of these cytokines in adipose tissue inflammation.

Analysis of systemic inflammation, assed by ELISA of IL-6, revealed no differences between all groups of mice (data not shown).

A



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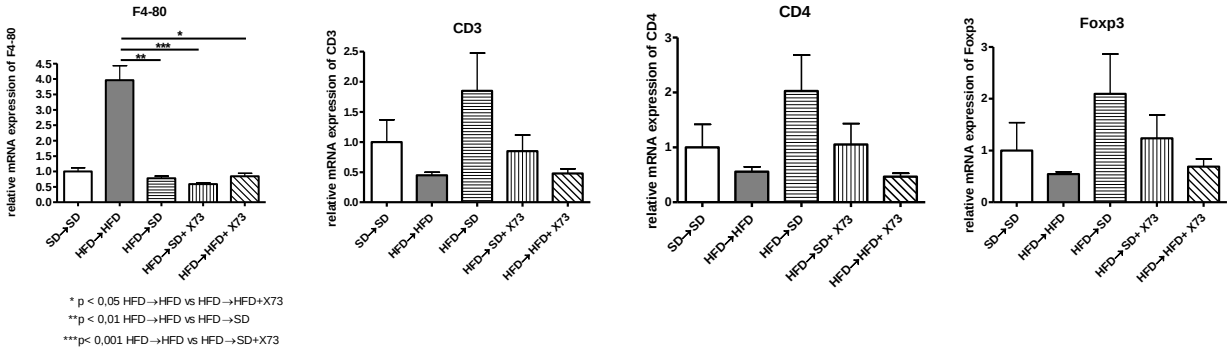

Fig.12: Effects of X73 treatment on the inflammatory markers. A: pro- and anti-inflammatory cytokines. B: macrophage and lymphocyte markers. Gene expression analysis by real-time qPCR of inflammatory markers and of T cell subsets in mesenteric adipose tissue. Data were normalized according to 18S mRNA level and presented as a value relative to that for SD fed mice.

5 DISCUSSION

The intestinal mucosa, composed by a monolayer of polarized intestinal epithelial cells (IECs) represents an important interface between the host and the microbiota. This interaction is crucial to the maintenance of gut homeostasis and it is mediated by TLR/NLR signaling. Any alterations of this equilibrium, such as dysbiosis, could induce an aberrant immune response and increase susceptibility to intestinal inflammation, even intestinal cancers (1,6,7). The Western diet is recognized as one of the main factors responsible for modifications in the gut microbiota. Although several studies reported that long-term feeding with WD causes intestinal neoplastic lesions in wild-type mice, there are no studies regarding TLR/NLR KO mice. The importance of TLR/NLR signaling in intestinal inflammation has been clearly shown by studies using chemically induced mouse models of intestinal inflammation, though their role is not well defined (69). Some evidence have shown a protective role of PRRs and MyD88 in intestinal inflammation, whilst others reported that MyD88 signaling was required for inflammation (70;71). Additionally, most of the studies have investigated the link between WD and colon tumors, and thus little is known about what occurs the small intestine (5-7).

Here, we examined the role of TLR signaling in WD-induced small intestinal inflammation. Our results demonstrate that WD, after only three months, is able to induce alterations in the small intestinal epithelium in KO mice and PRR-deficient mice for different TLR/NLRs respond differentially to dietary intake. Histological analyses showed small differences in KO mice fed with WD.

The group showing more significant alterations, as manifested by villus atrophy and crypt disorganization were the TLR9 KO mice. We demonstrate that 3 months of WD are sufficient for affect crypt cell proliferation in all TLR/NLRKO mice, with the difference being more marked in TLR9KO. Immunohistochemistry for Ki-67 shows that in TLR9 KO mice, Ki-67 positive cells were not restricted to the crypt region, but migrated inside the normal epithelium of the villus, which is normally occupied by differentiated cells. This abnormal migration may be due to defects in intestinal stem cell niche and could be conducive to inflammation.

It is well known that Wnt/β-Catenin is the central cell-fate determinant along the crypt-villus axis, and aberrant activation of this pathway is strongly associated with the development of intestinal cancer (72,73). Recenty, Schwitalla et al demonstrated that inflammatory Nf-kB pathway can modulate Wnt-signaling leading to the differentiation of epithelial non-stem cells into tumor-initiating cells (74). Wang et al recently showed an aberrant expression pattern for Fzd5, a Wnt receptor involved in Paneth cell differentiation, in wt mice fed with WD for 12 months (21). In contrast to the Wang study, we observed this alteration as early as only three months of WD feeding

in TLR9 KO mice. This finding suggests that in TLR9 KO, WD can induce either a defect in the localization of Paneth cells or an expression of this receptor in inappropriate epithelial cell types, substantially earlier than in wt animals. Our results demonstrate a critical role for TLR9 signaling in the maintenance of the small intestinal mucosa homeostasis.

Our results also indicate a possible role for TLR9 in the regulation of intestinal stem cell function, given the effects we observed on proliferation and localization of Paneth cells in TLR9KO mice. Previous studies show that Paneth cells express TLR9, which is critically important in detecting microbial pathogens (28). Impairment of TLR9 function may facilitate the entry of bacteria into epithelial cells due to a defective regulation of antimicrobial production resulting in impaired bactericidal capacity (29). Gut microbiota have been recently shown to impact intestinal stem cells (75). Given that the intestinal stem cells exist in close association with the microbiota, it stands to reason that signaling receptors that recognize components of the microbiota, as TLR9, may be present on and have effects on intestinal stem cells.

In addition to the molecular defects restricted to the intestinal epithelium, most studies of IBD focus on the nature of the inflammatory response and in particular on the role of T cells (41-46).

The intestinal epithelium is populated by a large number of T cells that perform regulatory as well as pro-inflammatory functions (76). Here we examined the contribution of T cells, in particular Treg and Th17, to the alterations in the small intestinal mucosa associated to consumption of WD.

Treg have a role in the suppression of immune reactions and prevention of autoimmune responses, The Th17 subset is a recently-described proinflammatory T helper cell lineage that produces IL-17 and IL-22 to eliminate specific types of pathogens that require a strong inflammatory response. However, an exaggerated Th17 response can give rise to chronic inflammation and autoimmune disorders (41;47). Unexpectedly, our RT-PCR results reveal a decrease of the total number of CD3 in all WD-fed mice, as judged by the levels of their signature gene, CD3, compared with SD-fed mice. Interestingly, our results demonstrate a significant relative increase of Treg and Th17 with respect the total T cell population in WD-fed TLR2 and TLR4 KO mice. The Treg/Th17 ratio shows a relative reduction of the Treg subset, which was significant only in WD-fed TLR2 KO mice. Recent evidence demonstrates that composition of the microbiota regulates the Th17:Treg balance (49). Moreover, Sutmuller et al demonstrate that TLR2, but not TLR4, controls the expansion and function of regulatory T cells, establishing a direct link between TLR and the modulation of immune response through Treg (77). Our data add support for a possible role for TLR2 and TLR4 signaling in the balance of anti-inflammatory versus inflammatory response in the gut. In the absence of absolute T cell quantification and functional experiments (suppression assays), it would be difficult to further define the exact nature of the effect of these two receptors.

Interestingly, NOD2-deficient mice fed with WD not show notable alterations. Strong evidence associates mutations in NOD2 gene with an increased susceptibility to intestinal inflammation, as seen in patients with Crohn's disease (32). Probably, in our case, dysregulation of the NOD2 pathway alone or three months of WD feeding are not sufficient to induce small intestinal inflammation in NOD2 KO mice.

In summary, it is becoming increasingly evident that signalling through TLR/NLR can generate differential responses depending on the inflammatory status of the tissue. Our results support the notion that WD promotes intestinal inflammation. They also demonstrate that individual TLRs have differential effects in the small intestine, regulating the mucosa homeostasis and inducing inflammatory responses. Our data contribute to the understanding of the factors by which the intestinal mucosal barrier is regulated in response to external stimuli, such as WD. This may be useful in developing novel strategies for managing and preventing intestinal inflammation.

In the second part of my work we evaluated the effects of X73, a commercial small-molecule anti-inflammatory drug (the structure of which we cannot disclose in this dissertation for Intellectual Property reasons) in a mouse model of diet-induced obesity (DIO). Several studies have demonstrated that obesity modifies the metabolic and endocrine functions of adipose tissue and is closely associated with chronic, low grade inflammation (11,60-62).

In our experiments, after three months of HFD, mice showed a significant increase in serum levels of glucose and insulin compared with the normal control group, indicating development of insulin resistance. This chronic secretion of insulin is due to tissue insensitivity to insulin signalling and a decreased capacity of circulating insulin to regulate glucose and lipid metabolism. It is interesting to note that X73 treatment reversed insulin resistance in mice that continued to be fed with HFD.

The biological events leading to obesity are characterized by changes in cell properties of adipocytes and include an increase of size (60). Morphological analysis on adipose tissue sections stained with H/E reveal large adipocytes in mice fed with HFD compared with mice fed with SD. X73 reduced adipocyte hypertrophy in mice fed with HFD. This data suggests that X73 treatment can exert anti-adiposity effects, even though no biologically relevant change in weight was observed.

In the context of insulin resistance, it is now well accepted that TNF-α, a proinflammatory cytokine, has a critical role in the regulation of insulin action in obesity. TNF-α blocks insulin-receptor signaling through inhibition of serine phosphorylation of insulin receptor substrate 1 (IRS-1), via the activity of the serine kinase inhibitor of nuclear factor-κβ kinase (IKK-β) (57,78). In addition to

TNF-α, several other inflammatory mediators are also overexpressed in adipose tissue (53). Our results show an increase of pro inflammatory marker TNF-α and IL-6 in mesenteric adipose tissue of HFD-fed mice, that is significantly reduced after treatment with X73.

Analysis of systemic inflammation, assed by ELISA of IL-6, revealed no differences between all groups of mice. We thus conclude that the inflammatory response that emerges in this mouse model of obesity resides predominantly in adipose tissue.

Similar to other studies (58,59), HFD induced an increase of the macrophage gene expression marker F4-80, which was significantly decreased in X73-treated HFD-fed mice. In particular, several lines of evidence reported that diet-induced obesity (DIO) leads to increased macrophage infiltration and shifts toward the classical pro-inflammatory (M1) state. M1 macrophages aggregate around necrotic adipocytes, forming "crown-like structures" and produce pro-inflammatory cytokines perpetuating WAT inflammation and dysfunction contributing to the induction of insulin resistance (60). However, Fujisaka et al have demonstrated that the number of M2 macrophages was also increased in mice fed with HFD (61). We found that the expression of the anti-inflammatory cytokine IL-10 is also increased significantly in adipose tissue of HFD-fed mice compared with SD-fed mice.

It is noteworthy that there was a trend for the reduction of Th1 cytokines (IL-12 and INF-γ) in mice fed with HFD compared with mice fed with SD. On the other hand, Th2 cytokine IL-13 was increased in mice fed with HFD compared with other groups, while the level of IL-4 was high only in SD mice. This novel evidence supports and further clarifies the findings of Fujisaka et al.

Recent data by Kwon and coworkers demonstrated that although IKKβ is a central signal transducer that controls the expression of proinflammatory mediators, it also induces the expression of IL-13, which may be limiting adipose tissue inflammation (79). Our findings are compatible with these results; the emerging conclusion is that long-term adipose tissue inflammation may be switching to a Th2-polarized phenotype, rather than Th1, as originally proposed by several studies. This would be more compatible with the increased tumor susceptibility associated with high calorie diet, given the pro-tumoral associations of type 2.

In conclusion our results demonstrate that X73 not only displays anti-adiposity effects by improving reducing adipocyte hypertrophy and reversing insulin resistance, but it also modulate adipose tissue inflammation. In the future, this project shall be extended in order to find the mechanism of action of X73 and improve the understanding of the immunological pathways and factors that initiate, regulate, and amplify the inflammatory cascade during obesity. This, we hope, will aid the optimization of clinical interventions and lifestyle changes aimed at improving human health.

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