Sulindac decreases basal-like mammary tumor burden and pro-inflammatory mediators in obese mice

by

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Honors Thesis

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2016

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ACKNOWLEDGEMENTS

I would like to thank Dr. Stephen Hursting for his tremendous guidance and support throughout my undergraduate studies. I would also like to thank Emily Rossi whose continuous support, dedication, and enthusiasm towards cancer research has inspired me to pursue a career in the field of cancer. Lastly, I would like to thank my family, in particular my parents, for their numerous years of support and encouragement.

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I. ABSTRACT

Background: A hallmark of the metabolic dysregulation associated with obesity is a pro-inflammatory environment perpetuated by pro-inflammatory mediators including adipokines and growth factor signaling. We previously showed that inflammation and basal-like breast cancer (BLBC) growth are increased in chronically obese mice and these effects persist following significant weight loss.

Hypothesis: We tested the hypothesis that Sulindac, a nonsteroidal anti-inflammatory drug (NSAID), can reduce chronic obesity-related inflammation and subsequent BLBC growth in mice.

Methods: Mice were administered a control diet (10 kcal % fat) or diet-induced obesity regimen (DIO, 60 kcal % fat). After 15 weeks on diet, DIO mice either continued on DIO diet or were switched to the low fat control diet to induce gradual weight loss, resulting in Formerly Obese (FOB) mice. Half of the mice in all three groups (CON, FOB, DIO, n=20/group) were randomized to receive Sulindac supplementation at 160 ppm in the diet, which remained constant across diets. Ten weeks after initiating Sulindac supplementation and weight loss in the FOB groups, serum was collected via submandibular bleed on a subset of fasted mice (n = 10-12/group). Thereafter, all mice were orthotopically injected with E0771 cells, a model of BLBC. Five mice/group were killed 4 weeks after tumor cell injection, while 12 mice/group continued in a survival study; these mice were killed when tumor size reached 1.2 cm in diameter. An NF-κB signaling targets PCR array (Qiagen) was completed on mammary fat pad harvested at end of study.

Results: Sulindac supplementation in DIO mice significantly reduced serum insulin and leptin to

levels statistically equivalent to both control and FOB mice. Interestingly, body weight and body fat percentage were unchanged between all supplemented and nonsupplemented counterparts (i.e. DIO vs. DIOSU). Sulindac supplementation in DIO mice (but not in CON or FOB mice) significantly reduced mean tumor volume relative to their nonsupplemented counterparts in the interim sac. Additionally, DIOSU had significantly increased percent survival compared to DIO, but no significant differences were observed in CON vs. CONSU or FOB vs. FOBSU. However, both FOB and FOBSU had significantly increased percent survival compared to DIO. The results of an NF-κB targets PCR array illustrated that Sulindac supplementation in DIO mice prevented losses in the expression of the NF-κB regulator TNF receptor-associated factor 2 (TRAF2) and also decreased expression of the apoptosis regulator X-linked inhibitor of apoptosis (XIAP) and two additional inflammation-related genes: tumor necrosis factor (TNF) and colony stimulating factor 1 (CSF1).

Conclusions: Sulindac supplementation significantly reduced the obesity-associated metabolic mediators insulin and leptin and decreased mammary tumor burden in DIO but not CON or FOB mice. Furthermore, Sulindac supplementation did not modulate body weight, and had no significant tumor effect in normal weight mice, suggesting that Sulindac indeed offsets some of the pro-tumorigeneic effects of obesity. Analysis of our PCR array of NF-κB targets suggests that Sulindac prevents altered expression of pro-inflammatory cytokines and anti-apoptosis genes. Ongoing analyses of inflammatory surrogates, including circulating prostaglandins, mammary gland crown-like structures and cyclooxygenase-2 levels, will help to determine if Sulindac's effects are mediated through its anti-inflammatory activity.

II. INTRODUCTION

The prevalence of obesity has risen dramatically within the past 30 years. Today nearly 40% of adults in the United States (U.S.) are considered obese [1]. The World Health Organization (WHO) estimates that 1.9 billion of the world's population is overweight and another 600 million are obese, and these numbers continue to increase [2]. Overweight and obesity are characterized by excessive fat accumulation and classified by a weight-for-height index, commonly known as body mass index (BMI), with obesity defined as BMI $\geq$ 30 kg/m^2. Must et al. [3] demonstrated a strong correlation between obesity and mortality risk that increases with advancing age. Given that life expectancy in the U.S. and other industrialized countries is on the rise, the now heavier and older population has a greater chance of experiencing the adverse health consequences of being overweight and obese [4].

Obesity engenders a state of chronic, low-grade inflammation characterized by excessive secretion of inflammatory mediators by adipocytes, macrophages, and other cells, including the gut microbiota. These pro-inflammatory factors disrupt metabolic homeostasis and thereby promote insulin resistance, type 2 diabetes, cardiovascular disease, genome instability and cancer [5]. The disparity in mortality between obese individuals and their lean counterparts is attributed, at least in part, to this aberrant pro-inflammatory signaling and the resulting metabolic dysfunction [6]. Unfortunately, significant and sustained weight loss is difficult to achieve in obese individuals. Thus, anti-inflammatory interventions such as prophylactic nonsteroidal anti-inflammatory (NSAID) use may be needed to reduce the inflammatory burden imposed with morbid adiposity levels.

Numerous clinical and epidemiological studies have shown beneficial health effects with increased supplementation of Sulindac, a nonsterodial anti-inflammatory drug (NSAID), including reductions in inflammation, hyperlipidemia and improved insulin signaling [7-9]. The anti-inflammatory and metabolic reprogramming properties of Sulindac have led to the

suggestion that Sulindac may reduce cancer risk and/or progression, although the effects of Sulindac in the context of obesity have not been evaluated.

Obesity promotes an increased risk of many cancers and a worse cancer outcome after diagnosis. Obesity is an established risk factor for endometrial, colorectal, breast (postmenopausal), esophageal (adenocarcinoma subtype), liver, kidney, gallbladder, pancreatic, uterine, and ovarian cancer [10]. Obesity also worsens the prognosis of each of these cancers as well several others, including prostate cancer, premenopausal breast cancer, thyroid cancer, and some leukemias. Morbid obesity (BMI > 40 kg/m^2) is associated with a markedly higher risk of dying from cancer, increasing rates by 52% in men and 62% in women [11]. The exact mechanisms underlying the obesity-cancer link remain unclear, but abundant evidence suggests that they involve increased adipose tissue inflammation and metabolic dysfunction.

The local secretion of inflammatory adipocytokines from adipose tissue, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), resistin, and monocyte chemotactic protein-1 (MCP-1), is increased in obese individuals compared to their normal weight counterparts [12-14]. These pro-inflammatory cascades stem from an overabundance of immature pre-adipocytes, which recruit activated macrophages to the adipose tissue [15]. These adipose tissue macrophages (ATMs) are a primary source of pro-inflammatory cytokines, which are involved in paracrine and endocrine signaling and often have potent pro-tumor effects [15]. Cytokines promote tumor growth in the microenvironment by increasing angiogenesis and fostering an immunosuppressive environment, which works against the body's anti-tumor immunity. IL-6 inhibits the maturation of dendritic cells, thus reducing the population of cytotoxic T-cells, which kill cancer cells [16]. IL-1β promotes tumor growth by inducing angiogenic factors, including vascular endothelial growth factor, which support the tumor with a nutrient rich blood supply [17]. These extracellular inflammatory and angiogenic signals can lead to intracellular activation of the transcription factor NF-κB through the phosphorylation of its upstream activator

IkB kinase-β (IKK-β), which induces increased gene expression of pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β. TNF-α is known to increase tumor cell proliferation, tumor stage, and systemic metastatic growth [18-19]. In addition, TNF-α and IKK-β activate c-Jun NH2-terminal kinase (JNK), which promotes proliferation and survival of tumor cells [20-21]. TNF-α also contributes to insulin resistance by increasing insulin receptor substrate 1 (IRS-1) phosphorylation at serine 307, which impairs its ability to initiate downstream signaling and consequently blocks the biological actions of insulin [22-23]. Like TNF-α, IL-6 also affects insulin signaling by inhibiting the gene transcription of IRS-1 and glucose transporter type 4 (GLUT-4), resulting in decreased insulin sensitivity [23]. Moreover, this increased secretion of cytokines from adipose tissue can further lead to genomic instability through the shortening of telomeres. Telomere shortening is widely accepted as a biological occurrence of accelerating aging and aging-related diseases such as cancer. This is mediated through increased oxidative stress, DNA damage, and telomere shortening activation of p53 and p21 that upregulates secretion of TNF-α and IL-6 further leading to insulin resistance.

The adipokines leptin and adiponectin are primarily thought of as regulators of calorie intake and energy expenditure, however they are also involved in modulating inflammation and insulin resistance. Leptin signaling promotes satiety, thereby decreasing food intake. However, the onset of obesity causes individuals to become leptin resistant despite excess leptin production by the adipose tissue. In many obese individuals, leptin acts directly on macrophages to stimulate the synthesis of pro-inflammatory cytokines like TNF-α and IL-6. Adiponectin acts as an antagonist to leptin's pro-inflammatory effects in part by activating AMP-dependent protein kinase (AMPK), which increases fatty acid oxidation and glucose uptake in skeletal muscle and decreased hepatic gluconeogenesis [24]. However, TNF-α, IL-6 and other pro-inflammatory mediators suppress adiponectin secretion from adipocytes, and the obese population typically has low adiponectin levels [24].

Leptin secretion from adipocytes has been shown to induce tumor growth as well as cancer cell invasion and angiogenesis, suggesting the role of adipose tissue as a catalyst to cancer development and progression. Interestingly, leptin antagonist treatment reduces the growth of triple negative breast tumors in mice through decreased VEGF, pSTAT3, and cyclin D1 levels, highlighting leptin's pro-carcinogenic potential [25]. Alternatively, clinical studies have shown circulating levels of adiponectin are inversely correlated with obesity-related malignancies such as breast cancer [25].. Adiponectin functions to decrease endothelial cell proliferation and migration, induce apoptosis, and decrease tumor vascularization. Clinically, the leptin:adiponectin ratio (L:A) is increasingly accepted as a biomarker for metabolic syndrome [25]. Additionally, in colorectal cancer patients the L:A ratio is as much as eight fold greater compared to cancer-free controls and also serves as an independent predictor for adverse outcomes in colorectal cancer [26].

High BMI levels are associated with increased insulin secretion, leading to hyperinsulinemia. This metabolic dysfunction contributes to the decreased insulin sensitivity that is also induced by obesity-associated chronic inflammation. Elevated levels of insulin have been associated with cancer progression through insulin-like growth factor-1 (IGF-1) signaling. Increased circulating levels of IGF-1 have been correlated with increased risk of prostate, breast, and colorectal cancer [27]. Insulin itself is thought to have anti-inflammatory effects in healthy individuals, as it can decrease reactive oxygen species (ROS) in mononuclear cells and suppress MCP-1 and plasminogen activator inhibitor-1 (PAI-1) levels and intranuclear NF-κB binding [28-32]. Thus, adequate insulin signaling is needed to repair the inflammatory state of obesity, otherwise continuous secretion of TNF-α, IL-6, and C-reactive protein (CRP) will occur [33].

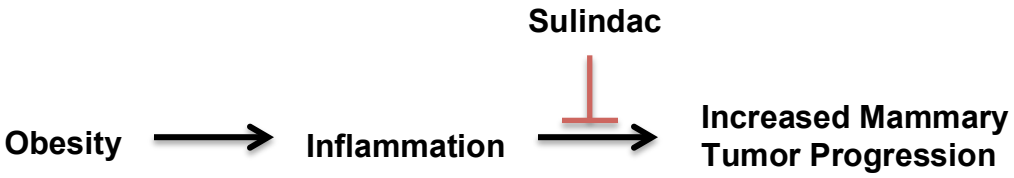
A hallmark of the metabolic dysregulation associated with obesity is a pro-inflammatory environment perpetuated by pro-inflammatory mediators including adipokines and growth factor signaling. Sulindac mediates inflammatory signaling through inhibition of cyclooxygenases

(COXs) specifically COX-2 which are known to induce secretion of prostaglandins and thromboxanes. The anti-cancer effect of Sulindac is illustrated through its ability to decrease activity of COX-2, which is often over-expressed in cancerous tissues and results in carcinogenesis as well as dysfunctional apoptosis and inhibition of tumor growth in various studies of breast, colon and, ovarian cancer [34-36]. Sulindac's chemopreventive effects are also regulated through its ability to inhibit the activation of cellular stress pathways such as the NF-κB pathway by decreasing IKKβ activity [37]. *In vitro* work has shown that stimulation of NF-κB pathway induces anti-apoptotic genes such as cellular inhibitor of apoptosis-1 and -2, which block regulation of apoptosis and further increases cell growth and proliferation [38-39]. Therefore it is likely Sulindac *in vivo* could provide its host with a wide range of metabolic benefits, including decreased systemic inflammation and sustained apoptosis, which would provide an anti-cancer benefit.

III. SPECIFIC AIMS, HYPOTHESIS

The current world population has an unprecedented risk of dying from the consequences of being overweight and obese. Chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer are often accelerated because of excess adiposity. Various biological mechanisms are implicated in the obesity-cancer link, particularly local and systemic inflammation as well as altered growth factor signaling pathways. In order to combat obesity-induced inflammation and the resulting increases in cancer risk and progression, mechanistic anti-inflammatory targets are imperative. Notably, nonsteroidal anti-inflammatory drugs (NSAIDS) such as Sulindac, can modulate the secretion of pro-inflammatory cytokines such as tumor necrosis factor alpha and interleukin-6, restore insulin sensitivity, and can prevent or delay tumorigenesis. Delineating the precise mechanisms by which Sulindac suppress obesity-induced inflammation will help identify promising key targets for clinical application to break the obesity-cancer link.

We have previously shown that inflammation and basal-like breast cancer (BLBC) growth are increased in chronically obese mice and persist following weight normalization. Weight normalization to levels equivalent of mice never exposed to an obesity-inducing regimen (obesity reversal) failed to normalize obesity-associated tumor burden, methylation patterns, and mRNA expression of pro-inflammatory mediators such as IL-6 and TNF-α relative to control mice. Thus, significant weight loss was not sufficient to reverse the effects of chronic obesity on epigenetic reprogramming and inflammatory signals in the microenvironment in association with breast cancer progression [40]. This study seeks to complement weight loss with Sulindac supplementation and determine its mechanism in offsetting the procancer effects of obesity and subsequent inflammation.



Aim 1: Identify whether mechanistic anti-inflammatory interventions such as Sulindac supplementation can reduce chronic obesity-related inflammation and/or basal-like tumor growth.

Aim 2: Determine whether Sulindac's effects are exclusive to obesity or if it could complement weight loss in formerly obese mice to more effectively reduce residual inflammation and/or basal-like tumor growth.

Aim 3: Identify the effects of Sulindac supplementation on key proponents of pro-growth and inflammatory obesity-associated mediators including the targets of the nuclear-factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway.

IV. Methods

Animal Study Design: Housing, Diet, and Care

All animal protocols were approved and coordinated with all compliance and guidelines by the University of North Carolina at Chapel Hill Institutional Care and Use Committee (IACUC). One hundred and eighteen female 6-8 week old C57BL/6 mice (an obesity-responsive mouse model) were obtained from Charles River Laboratories International, Inc. (Wilmington, MA). Upon arrival mice were housed three per cage on a 12-hour light/dark cycle with food and water consumption *ad libitum*. Standard chow was given to all mice for one week in order to acclimate. Post-acclimation, mice were randomized to two groups, either receiving a control (CON; 10% kcal from fat) diet (n=40; Product # D12450J; Research Diets, Inc.) or a diet-induced obesity (DIO; 60% kcal from fat) regimen (n=99; Product # D12492; Research Diets, Inc.) in order to model chronic obesity. Body weight and food intake were measured weekly. After 15 weeks on diet, with the weights of both the CON and DIO mice statistically significant from each other ($p < 0.001$), a subset of control mice (n = 20) were supplemented with Sulindac (160 ppm, equivalent to 160 mg Sulindac per kg of food) while the rest (n=20) remained on the control diet alone. DIO mice either continued on the DIO diet with (n = 20) or without (n = 19) Sulindac supplementation or were switched to the low fat control diet with (n = 20) or without (n = 20) Sulindac to induce gradual weight loss, resulting in Formerly Obese mice (FOB) mice. The resulting groups of mice are as follows: control (CON), control + Sulindac (CONSU), formerly obese (FOB), formerly obese + Sulindac (FOBSU), diet induced obesity (DIO), and diet induced obesity + Sulindac (DIOSU). After 27 weeks on diet and when all weights were stabilized, all mice were orthotopically injected with 3.5×10^4 E0771 mammary tumor cells into the 4^{th} mammary fat pad (Figure 1). *In vivo* tumor growth was measured two times per week with skinfold calipers and *in vivo* tumor area was determined using the formula πr^2. Four weeks later, a subset of mice (n = 5/diet group) was killed due to tumor diameter reaching 1.0 cm in any direction (interim sac).

Blood was collected by cardiac puncture, allowed to clot at room temperature for 30 minutes and centrifuged for 10 minutes at 1000 x g to isolate serum, and stored at -80°C. Mammary tumors, tumor-adjacent and tumor-distal mammary fat pad were excised and sectioned to either be formalin fixed or flash frozen in liquid nitrogen and stored at -80°C until further analysis. All remaining mice, (n=12/diet group) were followed for tumor-free survival; non-mammary tumor-related deaths were censored. When the tumors reached 1.2 cm in any direction, mice were killed (week 50) and blood, mammary tumor, and tumor-adjacent and tumor-distal mammary fat pad were collected as previously described. *Ex vivo* tumor volume was calculated using the formula $1/6\pi \times D_1 \times D_2 \times D_3$ (where D is equal to *ex vivo* diameter of the tumor). End of study blood was collected by cardiac puncture, allowed to clot at room temperature for 30 minutes and centrifuged for 10 minutes at 1000 x g to isolate serum, and stored at -80°C.

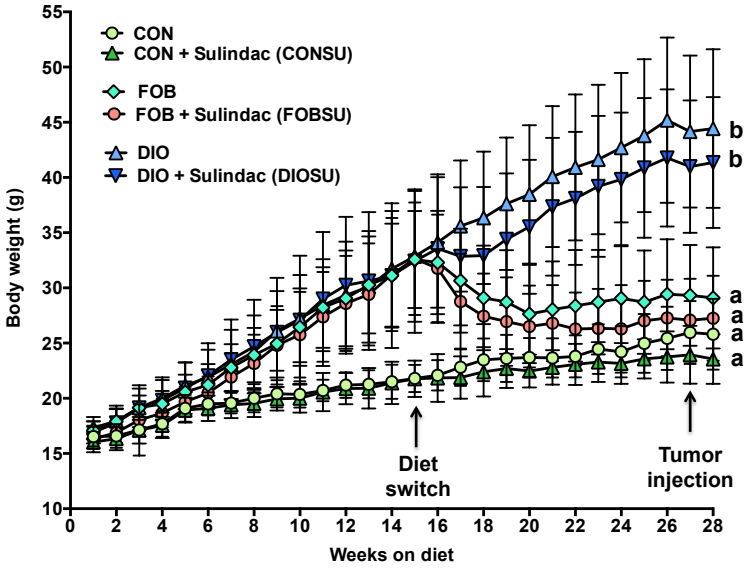


Figure 1. Dietary energy modulation affects body weight indiscriminant of Sulindac supplementation in CON, CONSU, FOB, and FOBSU vs. DIO and DIOSU. Differences in significance noted by different letters (a,b); p-value < 0.05.

E0771 Basal-like Mammary Tumor Cells

E0771 cells were purchased from CH_3 BioSystems (Catalog # 940001; Amherst, NY). These syngeneic mammary tumor cells model basal like breast cancer and were originally isolated from a spontaneous medullary breast adenocarcinoma in a C57BL/6 mouse [41] and adapted by Sirotnak et al. (1984) for chemotherapeutic experimentation [42].

Quantitative Magnetic Resonance Imaging Analysis

Quantitative magnetic resonance imaging (qMRI) (Echo Medical Systems, Houston, TX) was used to determine body composition for all groups (n = 9-10 mice/group) at end of study. The three components of total mass: lean, fat, and free water were quantified. Additionally, body weight was measured with a digital scale and used to calculate body fat percentage with qMRI data.

Metabolic Analysis of Serum Hormone, Cytokine, and Adipokine

Prior to tumor injections (week 27), serum was collected from mice fasted 4-6 hours (n = 10-12/group) via submandibular bleed. Serum hormones, adipokines, and cytokines including insulin, leptin, adiponectin, IL-6 and resistin, were measured using Bio-Plex Pro™ Mouse Diabetes Panel 8-Plex, Mouse Diabetes Adiponectin Assay, and Mouse Cytokine Panel A 6-Plex, respectively (Bio-Rad Laboratories; Hercules, California). Insulin-like growth factor 1 (IGF-1) concentrations were measured using R & D systems IGF-1 Bead-Based Single-plex Luminex assay (Minneapolis, MN).

Quantitative NF-κB Signaling Targets RT-PCR Array and RT-PCR of HIF-1α and Insulin Receptor (IR)

Total RNA was extracted from the homogenized flash-frozen distal mammary fat pad samples collected at end of study using TRI-Reagent (Sigma-Aldrich, St. Louis, MO) according to

manufacturer's instructions. RNA concentration was spectrophotometrically determined using a nanodrop (Thermo Scientific, Logan, UT). Samples were prepared and plated according to Qiagen's NF-κB Signaling Targets PCR Array protocol (Foster City, California). PCR reactions were completed using a ViiA^{TM}7 Real Time PCR system (Applied Biosciences, Carlsbad, CA). Gene expression was normalized to the housekeeping genes β-actin, β-2 microglobulin (β-2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-glucuronidase (β-GUS). Results were analyzed using Qiagen RT Profiler data analysis software (Catalog no. PAMM-225Z). For rt-PCR as a follow up to the array, additional RNA was reverse transcribed with Multiscribe RT (Applied Biosystems) and cDNA were assayed separately in triplicate for PCR using Taqman® Gene Expression Assays for hypoxia-inducible factor-1α (HIF-1 α) and insulin receptor (IR) (Applied Biosystems). PCR reactions were completed using a ViiA^{TM}7 Real Time PCR system (Applied Biosciences). Gene expression was normalized to the housekeeping gene β-actin and analyzed using the delta delta cycle threshold method [43].

Quantification of Adipocyte Infiltration in Tumor Tissue

A four micron-thick section was prepared from formalin-fixed, paraffin-embedded tumor tissue and stained with hematoxylin and eosin for all groups. Tumors were selected at random (n=5-6/group) and digitally imaged under 40X magnification using Aperio Digital Pathology Slide Scanners. The total area of adipocytes per section (3 representative sections, each 400 μm x 400 μm) was quantified using Adobe Photoshop 3.0.

Statistical Analysis

All values are represented as mean ± standard deviation (STDEV). One-way analysis of variance (ANOVA) using Tukey's multiple comparisons correction was used to assess the effects of diet regimen on body weight and fat percentage, mean tumor volume, serum hormone and cytokine concentrations and mammary fat pad gene expression. Log-rank (Mantel-Cox)

was utilized to determine the effect of Sulindac supplementation on survival. Results were analyzed using GraphPad Prism software (Graphpad Software Inc., La Jolla, CA) and $p \leq 0.05$ was considered statistically significant.

V. Results

Body Weight and Body Composition of CON, FOB, and DIO mice +/- Sulindac Supplementation

Mice were fed a low fat (control) or a diet-induced obesity (DIO) regimen for 15 weeks in order to establish a lean/control or obese phenotype. Body weights between CON and DIO mice achieved statistical significance at week 3 and continued throughout study ($p < 0.001$). At end of study, body fat percentage was assessed by quantitative magnetic resonance imaging analysis and was significantly higher in DIO (53.9 ± 4.18) and DIOSU (45.5 ± 5.65) mice compared to CON (20.1 ± 8.26), CONSU (17.6 ± 4.57), FOB (40.0 ± 7.08), and FOBSU (22.5 ± 7.47) mice (Table 1). FOB mice also had statistically higher body fat percentage relative to CON and CONSU mice and intermediate levels illustrated in FOBSU mice (Supplementary Figure 1A).

Table 1. qMRI body fat percentage results of significance across diet groups with or without Sulindac supplementation (NS = not significant).

Body Fat (%)		CON	CONSU	FOB	FOBSU	DIO	DIOSU
	CON						
	CONSU	NS					
	FOB	NS	$p < 0.001$				
	FOBSU	NS	NS	NS			
	DIO	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$		
	DIOSU	$p < 0.0001$	$p < 0.0001$	$p < 0.001$	$p < 0.0001$	NS	

Effects of Sulindac on Serum Metabolic Hormones, Pro-Inflammatory Cytokines and Gene Expression in the Microenvironment

When weights were stabilized in FOB and FOBSU mice (week 24, 9 weeks after diet switch), FOB and FOBSU mice had statistically equivalent body weights compared to CON and CONSU mice, serum was collected from a subset of fasted mice via submandibular bleed (n=10-12/group). Insulin levels in CONSU were significantly lower compared to FOB, DIO, and DIOSU

(Table 2) with intermediate levels that were not significantly different in CON and FOBSU mice. DIOSU mice experienced a modest but not significant decrease in insulin levels compared to DIO mice. However, CON, CONSU, and FOBSU possessed statistically equivalent levels of insulin-like growth factor 1 (IGF-1), which were all significantly lower than DIO mice (Table 2). FOB and DIOSU mice illustrated intermediate IGF-1 levels in comparison to the rest of the groups. Moreover, leptin (Table 2) was significantly reduced in DIOSU vs. DIO mice and this was also correlated to a significant decrease in the leptin to adiponectin ratio in DIOSU vs. DIO mice (Supplementary Figure 1D). However, no significant relationships were illustrated in adiponectin levels between all diet groups (Table 2). IL-6 concentrations did not differ between diet groups, partially due to high variability within groups (data not shown). Lastly, levels of adipose tissue-specific secretory factor (ADSF) resistin were higher but not significant in DIO and DIOSU mice relative to CON and FOB mice with or without Sulindac supplementation (data not shown).

Moreover, RNA extracted from the distal mammary fad illustrated varied differences in the relative gene expression of HIF-1α and insulin receptor (IR). CONSU, FOB, and FOBSU had significant reductions in HIF-1α gene expression levels relative to DIO mice (Supplementary Figure 1E). CON and DIO displayed intermediate but reduced levels of HIF-1α in comparison to DIO mice. IR relative gene expression was modestly elevated but not significant in all of the Sulindac supplemented groups compared to their nonsupplemented counterpart (Supplementary Figure 1F).

Table 2. Summary results of serum metabolic hormone concentrations between diet groups with or without Sulindac supplementation. Data presented as mean ± STDEV; NS=not significant.

		Average ± STDEV	CON	CONSU	FOB	FOBSU	DIO	DIOSU
Insulin (ng / mL)	**CON**	189.9 ± 48.2						
	CONSU	118.3 ± 50.28	NS					
	FOB	255.7± 87.17	NS	p < 0.01				
	FOBSU	199.2 ± 64.4	NS	NS	NS			
	DIO	316.0 ± 109.8	p < 0.05	p < 0.0001	NS	p < 0.05		
	DIOSU	235.2 ± 50.4	NS	p < 0.05	NS	NS	NS	
		Average ± STDEV	**CON**	**CONSU**	**FOB**	**FOBSU**	**DIO**	**DIOSU**
IGF-1 (ng / mL)	**CON**	83.7 ± 26.7						
	CONSU	74.9 ± 27.7	NS					
	FOB	118.5 ± 38.9	NS	NS				
	FOBSU	80.1 ± 23.4	NS	NS	NS			
	DIO	134.3 ± 42.9	p < 0.05	p < 0.05	NS	p < 0.01		
	DIOSU	103.3 ± 17.5	NS	NS	NS	NS	NS	
		Average ± STDEV	**CON**	**CONSU**	**FOB**	**FOBSU**	**DIO**	**DIOSU**
Leptin (ng / mL)	**CON**	2.81 ± 2.11						
	CONSU	1.71 ± 1.29	NS					
	FOB	4.56 ± 2.85	NS	NS				
	FOBSU	1.87 ± 1.12	NS	NS	NS			
	DIO	17.1 ± 10.8	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001		
	DIOSU	9.52 ± 4.23	NS	p < 0.05	NS	p < 0.05	p < 0.05	
		Average ± STDEV	**CON**	**CONSU**	**FOB**	**FOBSU**	**DIO**	**DIOSU**
Adiponectin (ng / µL)	**CON**	10.6 ± 2.14						
	CONSU	12.2 ± 4.57	NS					
	FOB	10.9 ± 2.35	NS	NS				
	FOBSU	10.6 ± 1.69	NS	NS	NS			
	DIO	9.70 ± 2.28	NS	NS	NS	NS		
	DIOSU	12.2 ± 2.77	NS	NS	NS	NS	NS	

Sulindac Impacts *Ex Vivo* Tumor Volume in DIO Mice at Interim Sac and Tumor-free Survival

Sulindac supplementation in DIO mice blunted tumor growth compared to DIO mice. Tumor volume at interim sac illustrated a significant reduction in DIO mice supplemented with Sulindac

(Supplementary Figure 2A). DIO mice had the largest and fastest growing tumors, with ex vivo volume at the interim sac being significantly greater than CON, CONSU, FOB, FOBSU, and DIOSU. In the survival portion of the study, DIO mice supplemented with Sulindac experienced significantly ($p < 0.05$) prolonged time of death due to slower tumor growth in comparison to nonsupplemented DIO mice (Figure 2). FOBSU mice had moderately longer but not significant tumor-free survival than FOB mice (Table 3).

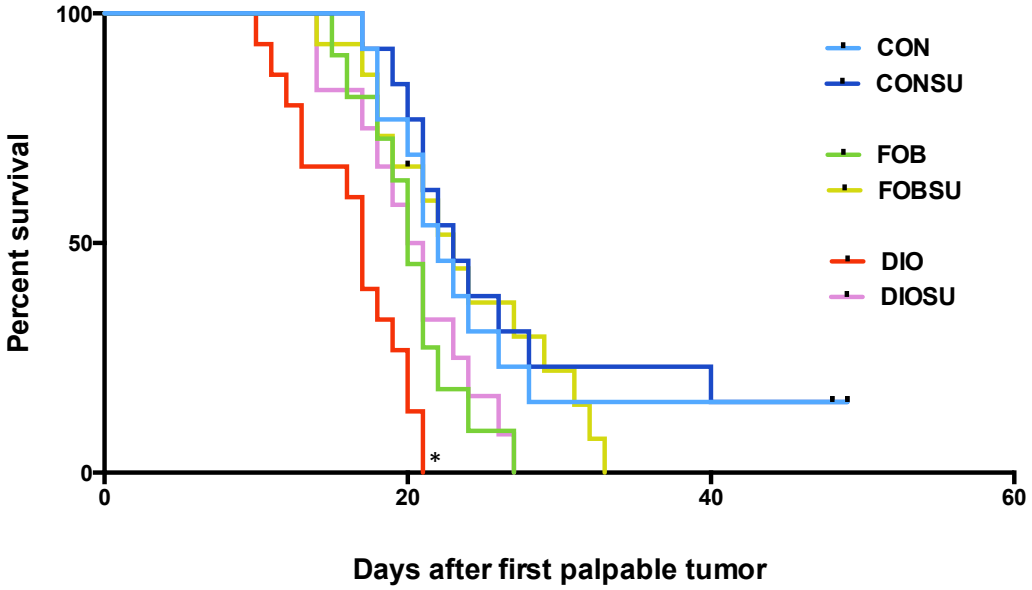


Figure 2. DIO mice supplemented with Sulindac experienced significantly ($p < 0.05$) prolonged time of death due to slower tumor growth in comparison to nonsupplemented DIO mice.

Table 3. Comparison of tumor-free survival across all diet groups with or without Sulindac supplementation (NS=not significant).

Survival Curve		CON	CONSU	FOB	FOBSU	DIO	DIOSU
	CON						
	CONSU	NS					
	FOB	NS	$p < 0.05$				
	FOBSU	NS	NS	NS			
	DIO	$p < 0.001$	$p < 0.0001$	$p < 0.05$	$p < 0.001$		
	DIOSU	NS	$p < 0.05$	NS	NS	$p < 0.05$	

Sulindac Decreases Infiltrated Adipocytes into the Mammary Tumor

Sulindac effects adipocyte infiltration into the mammary tumor. CON, CONSU, FOB, and FOBSU average adipocyte area was significantly reduced compared to DIO mice (Supplementary Figure 2B). DIOSU mice portrayed intermediate levels of adipocyte area in the mammary tumor that was not significantly different from the remaining groups. H & E staining of mammary tumors also illustrated larger adipocytes in the DIO mice not supplemented with Sulindac (Supplementary Figure 2C) compared to DIOSU mice.

Sulindac Modulates Altered Gene Expression as Depicted in an NF-κB Signaling Targets PCR Array

Heat map representation of all diet groups compared to CON mice and altered inflammatory gene signaling was seen in the Sulindac supplemented groups where differences in color represents gene upregulation (green) and gene downregulation (red). Significant upregulated and downregulated genes in DIOSU were entered into Qiagen's Ingenuity® Pathway Analysis (IPA) to determine pathological signaling pathways modulated by Sulindac supplementation in DIO mice. Upregulation of apoptotic genes and downregulation of inflammatory genes such as IL-1β and NF-κB2 were illustrated in DIOSU mice relative to DIO mice (Supplementary Figure 3).

VI. DISCUSSION

The results of this study illustrate the ability of Sulindac supplementation to be an effective strategy to offset the pro-inflammatory environment induced by obesity, which in turn can reduce subsequent mammary tumor development. Interestingly, body weight and body fat percentage were unchanged between all supplemented and nonsupplemented counterparts (i.e. DIO vs. DIOSU). We observed Sulindac's effects in blunting obesity-associated metabolic dysfunction in the serum and microenvironment through a significant reduction in serum insulin and leptin in DIOSU to levels statistically equivalent to both CON and FOB mice with or without Sulindac. CON, FOB, and DIO mice supplemented with Sulindac experienced modest decreases in IGF-1 levels. CON, FOB, DIO mice supplemented with Sulindac illustrated reductions in Leptin:Adiponectin with a significant reduction in DIOSU vs. DIO. DIOSU mice also had decreased HIF-1α and increased insulin receptor (IR) relative gene expression in the mammary fat pad compared to its nonsupplemented counterpart.

Furthermore, DIO mice supplemented with Sulindac illustrated decreases in obesity-induced mammary tumor burden through reductions in *ex vivo* tumor volume and increased percent survival, indicative of slower growing tumors. Sulindac supplementation in DIO mice (but not in CON or FOB mice) significantly reduced mean tumor volume relative to their nonsupplemented counterparts. Quantified adipocyte infiltration into the mammary tumor portrayed Sulindac's ability in DIO mice to decrease average adipocyte area to levels seen in CON or FOB mice with or without Sulindac supplementation. Moreover, NF-κB signaling targets array results suggests that Sulindac prevents altered expression of cytokines and anti-apoptosis genes. Sulindac supplementation in DIO mice prevented losses in the expression of NF-κB regulator TNF receptor-associated factor 2 (TRAF2) and also decreased expression of apoptosis regulator X-linked inhibitor of apoptosis (XIAP) and inflammatory related genes, tumor necrosis factor (TNF) and colony stimulating factor (CSF1). In the survival arm of the study,

Sulindac significantly increased percent survival defined as time until one dimension of the tumor was 1.2 cm, requiring euthanization in DIO and FOB groups (but not CON and CONSU) in comparison to their nonsupplemented counterparts.

The anti-inflammatory properties of Sulindac was profoundly pronounced in DIO mice with reductions in IGF-1 and insulin levels, decreased tumor burden and tumor adipocyte infiltration, altered NF-κB pro-inflammatory signaling, and prolonged tumor-free survival. Future directions will consist of ongoing analysis of circulating prostaglandins, mammary gland crown-like structures and cyclooxgease-2 levels in order to further determine if Sulindac's effects are mediated through its anti-inflammatory activity in reducing the procancer effects of obesity. Furthermore, in collaboration with Dr. Andrew Dannenberg, we will determine the intersection between mouse to human concordance between obese women and mice supplemented with Sulindac and whether Sulindac can negate the pro-inflammatory factors that lead to local and systemic inflammation in promoting subsequent tumor burden. In summary, delineating the role and precise mechanisms of Sulindac in suppressing obesity-induced inflammation will help identify promising key targets to break the obesity-cancer link.

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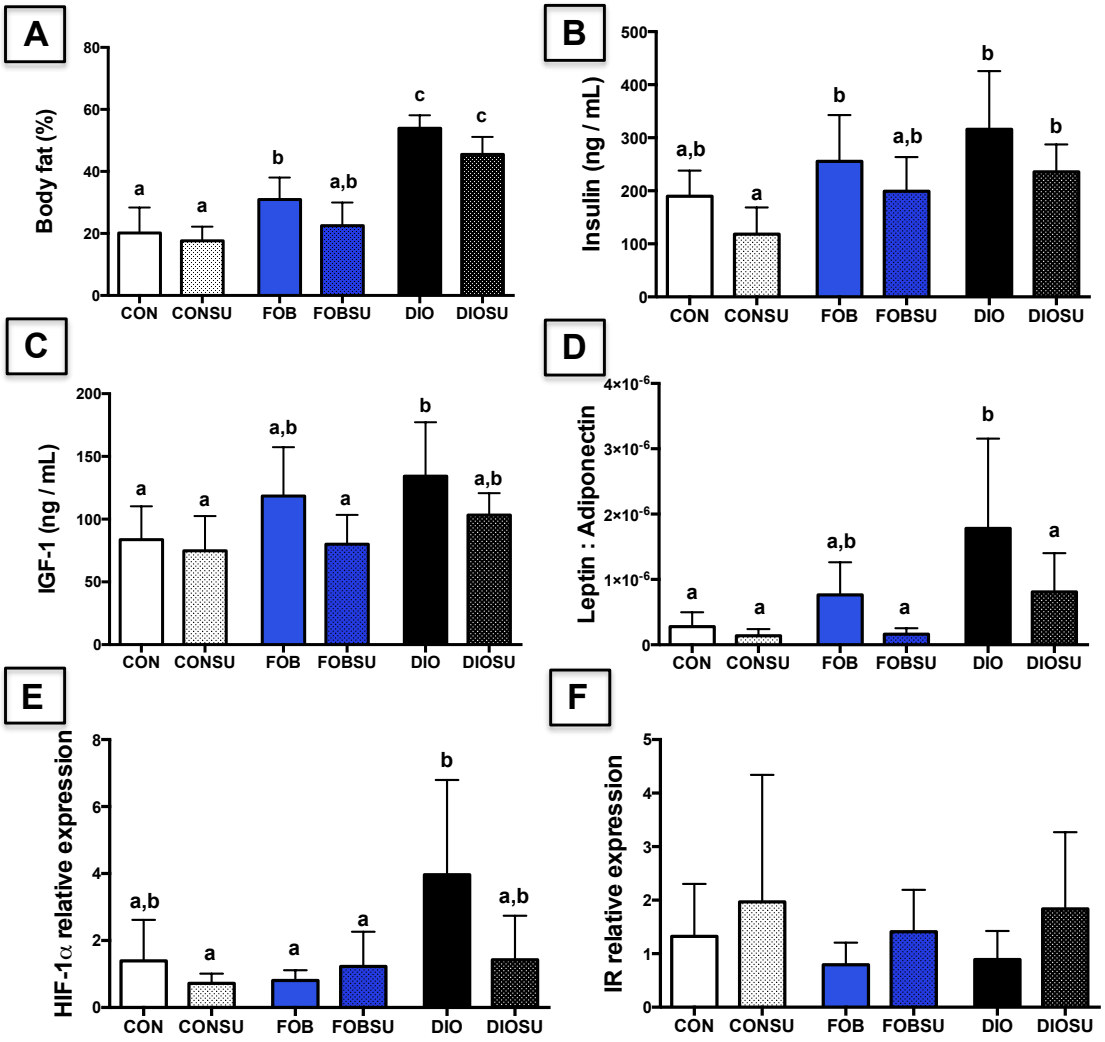
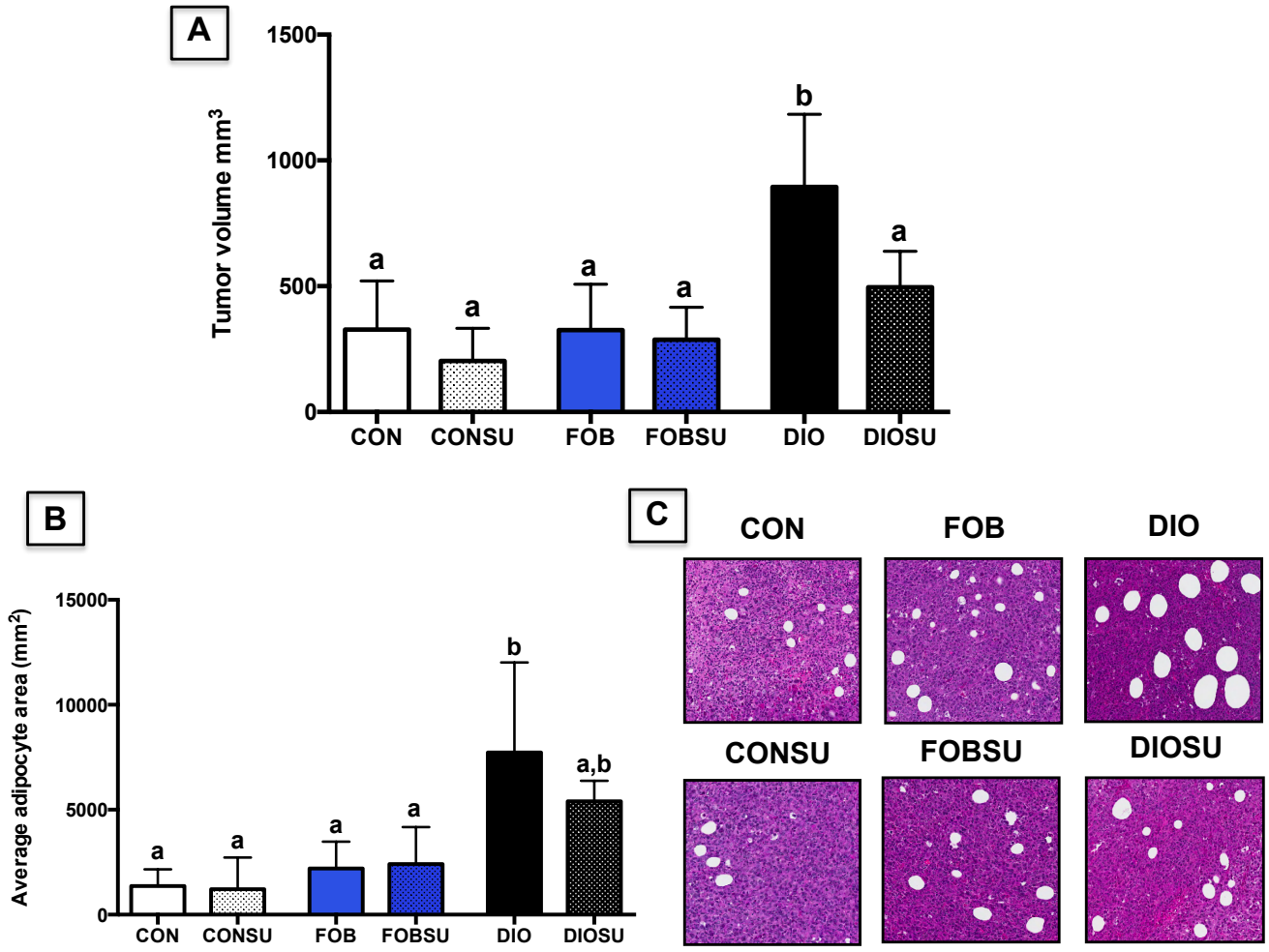
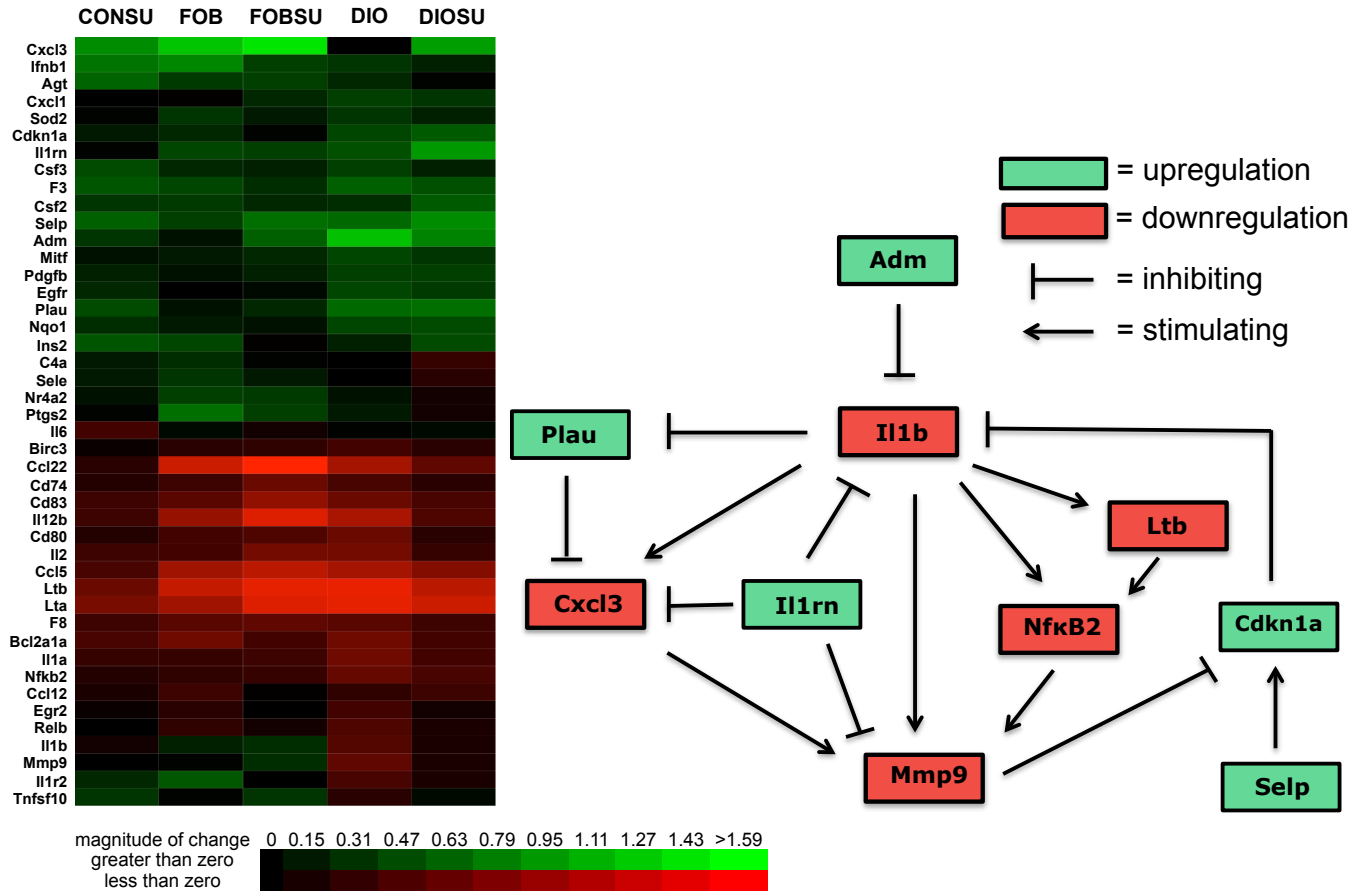
Supplementary Figures:



Supplementary Figure 1: Sulindac blunts obesity-associated metabolic dysregulation in the serum and microenvironment. CON, FOB, and DIO mice supplemented with Sulindac experienced modest decreases in (A) **Body fat %**, (B) **Insulin** and (C) **IGF-1**. CON and FOB, but not DIO mice experienced reductions in (D) **Leptin: Adiponectin**. DIOSU mice also had decreased (E) **HIF-1α** and increased (F) **Insulin receptor (IR)** relative gene expression in the mammary fat fad vs. DIO. For Luminex serum analysis (A-D; n = 10-12/group) and for PCR analysis of mammary fat pad (E-F; n = 5-7/group). Data presented as mean ± STDEV. Differences in significance noted by different letters (a,b); p-value < 0.05.



Supplementary Figure 2: Sulindac supplementation decreases obesity-associated increases in mammary tumor growth in obese mice. (A) Sulindac supplementation blunts *ex vivo* tumor burden and (B) decreases average adipocyte area infiltrated in the mammary tumor to non-obese levels compared to its nonsupplemented counterpart. (C) Representative images of adipocyte infiltration into the tumors of mice with or without Sulindac supplementation. Data presented as mean ± STDEV. Differences in significance noted by different letters (a,b); p-value < 0.05; Magnification: 400 µm.



Supplementary Figure 3: Heat map representation of gene expression in distal mammary fat pad (n= 4-6 mice/group). Differences in colors represents gene upregulation (green) and/or downregulation (red) relative to control. Sulindac supplementation in obese mice increases expression of regulators of apoptosis and inflammatory cytokines to decrease activation of the NF-κB signaling pathway. Regulation of growth and inflammatory gene signaling in DIOSU mice, expression quantified by NF-κB signaling targets array (Qiagen). Relationships predicted by IPA software.