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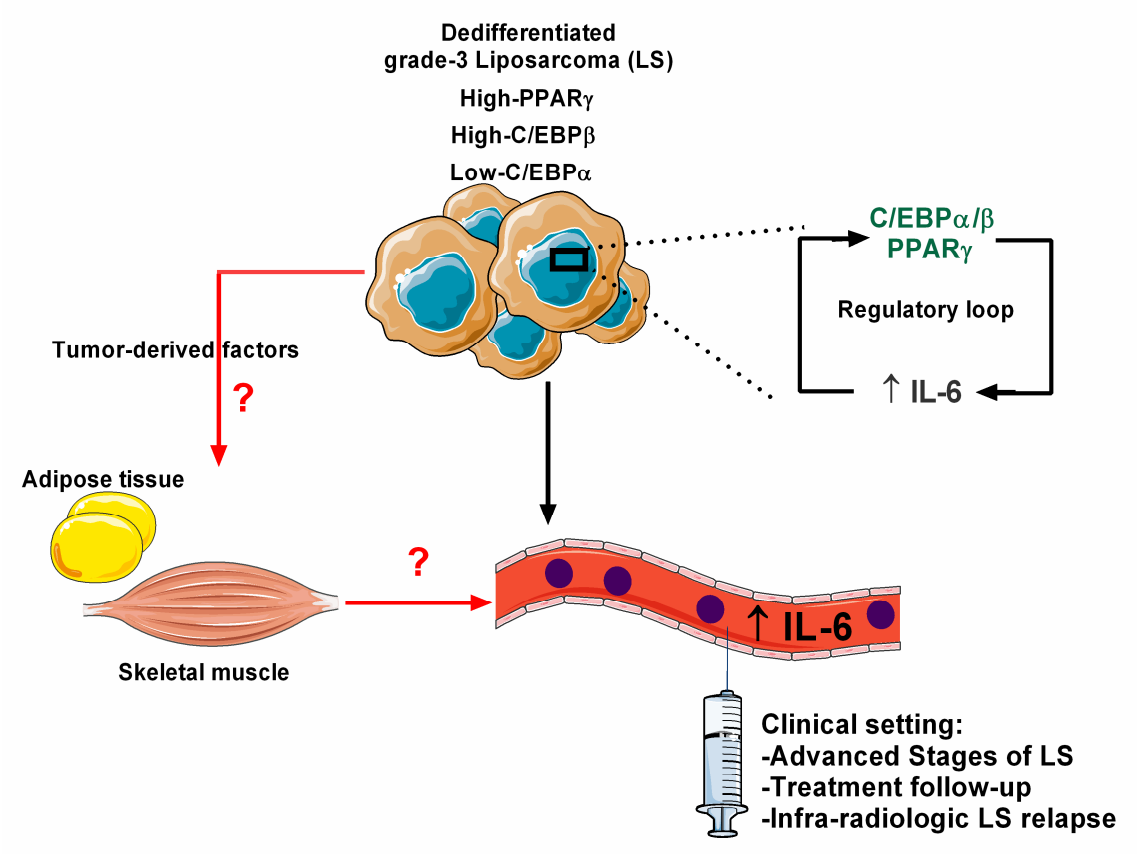

Interleukin-6, C/EBP-β and PPAR-γ expression correlates with intramuscular liposarcoma growth in mice: the impact of voluntary physical activity levels

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Abstract

IL-6 is an axial cytokine overexpressed in cancer to promote growth and increase resistance to anti-cancer therapies. As the application of IL-6-targeting therapies are still limited, alternative non-aggressive and adjuvant approaches, like physical activity (PA) could be useful to reverse IL-6 effects. To get more insights into liposarcoma (LS) pathophysiology, we investigated potential molecular links between IL-6 and LS growth and we tested the impact of PA on such mechanism in an orthotopic model of intramuscular LS. Initially active nude mice have received an intramuscular injection of either human SW872 cells or vehicle, then were respectively randomized into voluntary-active or inactive mice with open or restricted access to activity-wheels. We found that LS-bearing mice exhibited ~6 fold increase in circulating IL-6 comparing to controls, with a concomitant decrease in hepatic drug-metabolizing enzymes expression. Circulating IL-6 levels were positively correlated with intra-tumor *IL-6* expression ($r=0.85$, $P<0.01$). Interestingly, intra-tumor *IL-6*, *C/EBP-α/β* and *PPAR-γ* expression were correlated together and with greater tumor mass and autophagy markers, notably, *GABARAPL-1*. Intriguingly, we found that maintaining a spontaneous PA after tumor injection did not reduce the levels of IL-6, but even enhanced tumor growth, induced body weight loss and increased the risk of developing lung metastasis. Our findings suggest that (1) *IL-6*, *C/EBP-β* and *PPAR-γ* exert a potential role in promoting growth of dedifferentiated LS and (2) that PA failed to mechanistically interfere with these factors, but enhanced LS growth *via* other independent-mechanisms. The preclinical data reported here could be helpful in the sub-molecular classification of LS patients to improve diagnosis and design a low-risk treatment. Circulating IL-6 could serve as an indicator for treatment follow-up and, perhaps, for infra-radiologic LS relapses.

Keywords— Interleukine-6, Liposarcoma, C/EBP-α/β, PPAR-γ, Physical activity

1. Introduction

The evolution of tumors is highly affected by the presence of inflammatory cytokines and many anti-tumor agents like, trabectedin, reduced tumor growth *via* lowering the levels of pro-angiogenic and pro-inflammatory cytokines, such as interleukin-6 (IL-6)[1]. There is a substantial body of evidence showing that IL-6 has a special role in cancer pathogenesis and resistance to chemotherapy [2]; its intra-tumor expression and circulating levels were associated with poor prognosis and negative outcomes in numerous malignancies [3]. In this light, several IL-6 and IL-6 receptor-targeting antagonists have been developed, but their clinical application still limited due adverse side-effects and invasive routes of administration [4]. Hopefully, other low-cost, non-aggressive adjuvant therapies like hospital-supervised physical activity (PA) could be simple to apply and have shown effectiveness in reducing inflammation (i.e. IL-1ra, IL-6) in cancer [5]. There is even a dose-response relationship between PA levels and reduced breast, prostate and colon cancer growth/progression in animals and patients [6,7,8].

Liposarcoma (LS) is the most frequent type of soft tissue sarcoma (STS), mainly localized within thighs of patients [9]. Its intramuscular localization and the lack of alternative therapies, render complex the treatment of LS, as patients usually undergo heavy surgery or, even, limb amputation in some cases. Thus, there is a need to understand the underpinning molecular mechanisms, to improve diagnosis and treatment in this population. At the molecular level, fibrosarcoma and LS cell lines were able to express and produce IL-6 [1,10]. The expression of IL-6 is controlled by a number of transcriptional factors including: nuclear factor-kappaB (NF-κB), activator protein-1 (AP-1) and CCAAT/enhancer-binding proteins (C/EBPs) [11]. Particularly in adipose tissue, C/EBPs play a central role, as they regulate several biological responses like, proliferation, differentiation, adipocytes maturation and cytokines production [12]. Therefore, we suppose that IL-6 and C/EBPs could be involved in the pathogenesis of tumors from adipocytic origin, which is the case of LS. In this report, we aimed to get more insights about the existing molecular links between IL-6 and dedifferentiated high-grade LS growth and we determined the ability of regular PA to interact with such mechanisms.

2. Materials and methods

2.1. Ethics approval and study protocol

The study was performed in accordance with the recommendations of the European community (directive 2010/63/EU) and were approved by the French ministry of higher education and research, in accordance with the local committee of research ethics of Rennes (authorization no. APAFS#581-2015050411405743). As previously described [13], four-week old male nude mice (n=36) performed spontaneous PA on wheels. After six weeks, mice were divided into four groups (n=9/each), which have received an intramuscular injection of either human SW872 cells or vehicle (DMEM culture media) with access (2 groups: Control and Lipo Active mice) or not to wheels (2 groups: Control and Lipo Inactive mice). At eight weeks post-injection (PI), mice were euthanized, tumor, liver and lungs were recuperated and frozen in liquid nitrogen or fixed with 4% paraformaldehyde (PFA). Plasma was obtained from venous blood and collected in EDTA-coated tubes.

2.2. Histology analysis and tumor grading

Tumor sections of 4 μm were performed in the wide part of the tissue, then stained with hematoxylin and eosin (H & E). Tumors were graded according to the "world health organization (WHO) classification of tumors of soft tissue and bones" and the "French FNCLCC system". Three independent factors were used to define tumor grade: necrosis, mitosis and differentiation. Each prognostic factor had a score ranging from 1-to-3 for mitosis and differentiation or 0-to-2 for necrosis. The grade was obtained by adding the three independent scores. A well-differentiated LS grade 1 corresponds to a total score of 2-3, for myxoid LS grade 2 the score augments to 4-5 and reaches a total value of 6-to-8 in undifferentiated LS grade 3. The number of lung metastatic nodules was counted on three lung sections of 4 μm, separated each other by a distance of 40 μm, and stained with H & E.

2.3. RNA extraction and RT-qPCR

Experiments were performed on tumor tissues powder obtained after grinding in liquid nitrogen. Total RNA was extracted using Trizol® reagent according to the manufacturer's protocol (Sigma Aldrich). RNA amounts was determined by spectrophotometer and RNA quality was controlled on 1.2% agarose gel using the FlashGel system (Lonza). Reverse transcription

(RT) reaction, followed by Syber-green real time PCR were performed in CFX-real time machine. Relative expression was calculated using the ΔCt method. Primers are listed in **Table 1**.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Plasma IL-6 levels were determined by a specific ELISA kit (Biolegend, 431307). Briefly, 50µl of plasma were added to wells pre-coated with specific primary antibodies. Then, Detection antibody coupled to a peroxidase was added with a substrate solution and the color developed proportionally to the levels of IL-6. Optical density (OD) was measured at 450 nm respectively using a microplate reader.

2.5. Statistical analysis

Normality and equal variance were tested before statistical analysis. A two way ANOVA test was used to compare all four groups. Further interactions were analyzed with a Fisher LSD test. Significant difference between two groups was examined by a student *t*-test. A non-parametric Mann-Whitney rank sum test was used instead of *t*-test, when data failed to pass the normality test. Principal component analysis followed by Pearson's correlation were performed on tumor mass, plasma IL-6, intra-tumor *IL-6*, *C/EBPα/β*, *PPAR-γ*, and *FABP4* expression and lung metastasis. Hierarchical clustering analysis was performed to classify mice according to their tumor mass and/or intra-tumor molecular signatures. Results were considered statistically different at $P < 0.05$.

3. Results

3.1. Active mice developed greater intramuscular tumors and early cachexia symptoms

We first confirmed the impact of PA on SW872 tumor-bearing mice. We found that the total mass of *gastrocnemius* muscle-containing SW872 tumor (normalized to body weight) was significantly higher in active comparing to inactive mice (P=0.04; Figure 1**A**). At the end of the protocol, we observed that only active LS-mice manifested initial body weight loss, when comparing to the first week PI, while total body weight increased in inactive mice (P=0.01; Figure 1**B**). As SW872 are dedifferentiated LS cells, it was not surprising that both groups have developed a dedifferentiated tumor of grade-3 (Figure 1**C**). Nonetheless, the scores were more elevated in active group, perhaps indicating a more advanced stage of tumor growth. Hierarchical clustering analysis performed on active and inactive mice indicated the presence of three tumor

mass categories: <2.5; between 2.5 and 3.5g and >3.5g (Figure 1**D**). Importantly, ~67% of active mice were present in the >3.5g category comparing to only ~11% of inactive mice (Figure1**E**). At the molecular level, the mRNA expression of fatty acid-binding protein 4 (FABP4) tended to increase in tumors of active mice (*P*=0.08; Figure 1**F**) and its expression was positively correlated with lung metastasis (r=0.96, *P*<0.001; Figure 1**H**). Interestingly, when we classified mice according to their tumor mass, independently of PA level, we found that the expression of FABP4 tended to be greater in voluminous tumors (*P*=0.058; Figure 1**G**). These findings confirm our model, in which maintaining a regular PA aggravated LS growth [13], induced weight loss and increased the susceptibility to develop lung metastasis.

3.2. High circulating IL-6 levels are produced during intramuscular LS development, regardless the level of PA

As inflammation is an important factor promoting cancer progression [14], we measured the levels of plasma IL-6, well-known to be involved in cancer pathogenesis. We found that active and inactive LS mice exhibited ~6 fold increase in circulating IL-6 comparing to their respective controls (Figure 2**A**). Concomitantly with inflammation, the expression of hepatic drug-metabolizing enzymes was also lower in tumor-bearing mice, especially in active ones (Figure 2**B**). We must note that on average there was no change in circulating IL-6 amounts and intra-tumor *IL-6* mRNA expression between active and inactive tumor-bearing animals. RTqPCR experiments on LS tumor tissues have shown that the expression of *C/EBP-β* and *PPAR-γ* increased in tumors of active mice but only *C/EBP-α* had reached a statistical significance (*P*=0.02; Figure 2**C**). We then demonstrated the existence of an inter-relationship between C/EBPs and PPAR-γ network in LS tumors. Indeed, *C/EBP-β* positively correlated with *C/EBP-α* (*P*=0.01) and/or *PPAR-γ* (*P*<0.01) in the tumors of active and inactive mice, respectively (Figure 2**D**). We also found that *C/EBP-α* and *C/EBP-β* and *PPAR- γ* positively correlated with *IL-6* expression in tumors of active and inactive mice, respectively (Figure 2**E**, **F** and **G**). These data indicate that circulating IL-6 levels increase during LS development and that intra-tumor *IL-6* expression correlates with *C/EBP-α/β* and/or *PPAR-γ*. PA did not directly interfere with these factors.

3.3. IL-6, C/EBP-β and PPAR-γ expression correlates with tumor mass and autophagy markers

Clustering analysis showed that molecular signatures including *IL-6*, *C/EBP-α/β* and *PPAR-γ* were associated with tumor mass, independently of PA levels (data not shown). Therefore, we focused the next part of the study to understand the relation between IL-6, C/EBP-α/β, and PPAR-γ with LS tumor growth. We classified mice into two groups based on tumor mass (<3.5g and >3.5g), regardless their historical PA levels. Interestingly, we found that the expression of *C/EBP-α* (*P*=0.09; Figure 3**A**), *C/EBP-β* (*P*=0.02; Figure 3**B**), *PPAR-γ* (*P*=0.03; Figure 3**C**) and *IL-6* (*P*=0.01; Figure 3**E**) was higher in tumors superior to 3.5g comparing to those less than 3.5g. Accordingly, the expression of *IL-6*, *C/EBP-β* and *PPAR-γ* was positively correlated with tumor mass (Figure 3**D** and **F**). Additionally, intra-tumor *IL-6* expression was highly associated with circulating IL-6 amounts (Figure 3**G**). Autophagy markers, *Beclin-1* and *GABARAPL-1* were expressed in tumors (data not shown) and their expression correlated positively with intra-tumor *IL-6* expression (Figure 3**H**). Particularly, *GABARAPL-1* was significantly correlated with tumor mass as well as *C/EBP-β* and *PPAR-γ* expression (Figure 3**I** and **J**). Therefore, the growth of LS tumor is naturally associated with increased *IL-6*, *C/EBP-α/β*, *PPAR-γ* and autophagy markers expression.

4. Discussion

In this report, we confirmed our previous findings that PA was deleterious in active LS-mice [13]. Additional experimental and statistical analysis indicated that active mice exhibited body weight loss and showed higher tendency to express FABP4 which was greatly correlated with lung metastasis. Indeed, FABP4 mRNA expression was up-regulated in human lung cancer and correlated with advanced tumor node metastasis [15]. Globally, LS-mice developed systemic inflammation and changes in liver metabolism, as evidenced by elevated circulating IL-6 levels and repressed expression of key hepatic drug-metabolizing enzymes including, *cytochrome P450 (CYP) 1A2 and 3A11*. Recently, Pedersen et al. demonstrated that voluntary wheel running decreases melanoma growth *via* lowering circulating IL-6 levels [16]. In our model, maintaining PA after tumor injection, failed to decrease circulating IL-6 amounts and enhanced tumor growth through other mechanisms, including the inhibition of p38-MAPK-p21 pathway, as reported in our previous study [13]. Also, PA seemed to exert a negative effect on *CYP1A2* and *3A11* expression. Particularly, *CYP1A2* negatively correlated with tumor mass (r=-0.65, *P*<0.01) and *IL-6* expression (r=-0.51, *P*=0.04), especially in active mice. These data are in keeping with other

studies showing that advanced cancer stages are associated with inflammation and decreased CYP1A and 3As expression and activity [17]. Here we describe, for the first time, similar mechanisms occurring in human dedifferentiated LS.

As LS is a tumor from adipocytic origin, it is believed that adipogenesis-regulating factors C/EBP-α/β and PPAR-γ play a pivotal role in controlling several aspects of LS biological response including, cytokine expression [18]. The expression of *C/EBP-α* was particularly up-regulated in active mice, perhaps reflecting the increase in LS growth. Although C/EBP-α has been mainly described as a tumor suppressor, some clinical data showed that its mRNA and/or protein expression was up-regulated in liver [19], breast [20] and epithelial-ovary tumors [21]. C/EBP-α correlated with clinicopathologic parameters and poorer survival [20,22]. These results emphasize a pro-oncogenic role for C/EBP-α in a context- and tissue-specific fashion. However, a prominent role for *C/EBP-β* and *PPAR-γ* in LS pathogenesis could be suggested as they were 25-to-40 fold more expressed than *C/EBP-α* in active/inactive animals. *C/EBP-β* was associated with *C/EBP-α* and *IL-6* expression in active mice and with *PPAR-γ* and *IL-6* expression in inactive counterparts. Previous studies have shown that, notably, C/EBP-β can regulate the expression of C/EBP-α and PPAR-γ [23]. Indeed, C/EBPs have homologous carboxyl-terminal domain that can bind to regulatory DNA regions to induce each other's expression and that of a battery of genes like, *cyclooxygenase-2* and *IL-6* [18,24]. Therefore, our results indicate that there is an interplay between *C/EBP-α/β*, *PPAR-γ* and *IL-6* expression in LS tumors, regardless PA status.

Since IL-6 was higher in LS-mice and, PA did not affect its levels, we aimed to determine the relation between IL-6 and late-stage LS growth, independently of PA. When mice were classified into two categories, according to their tumor mass (<3.5g and >3.5g), the expression of *C/EBP-α/β*, *PPAR-γ* and *IL-6* showed a trend-to-increase or was significantly increased in the >3.5g group. We must note that 80% of the >3.5g group were historically active mice; thus, even if PA did not mechanistically interfere with *C/EBP-β*, *PPAR-γ* and *IL-6* expression, its ability to promote LS growth will be reflected by elevated intra-tumoral levels of these factors. C/EBP-β and PPAR-γ mRNA/protein expression correlated with tumor invasiveness and metastasis in multiple cancer types, including myxoid LS [25,26,27,28]. In our model, *C/EBP-β*, *PPAR-γ* and *IL-6* were significantly associated with tumor mass, underscoring a potential role in promoting

LS progression. Additionally, intra-tumor *IL-6* showed a very high correlation with circulating IL-6 rates. This suggests that LS tumor is partly responsible for the high circulating IL-6 levels observed in animals. An elegant study have recently shown that autophagy promotes IL-6 expression and cancer progression *in vitro* and *in vivo* [29]. Accordingly, *Beclin-1* and *GABARAPL-1* were expressed in LS tissue and correlated with *IL-6* expression. Notably, *GABARAPL-1* was associated with tumor mass and was significantly correlated with *C/EBP-β* and *PPAR-γ* expression. The interactions between C/EBP-β and the autophagic machinery in cancer have been previously demonstrated. Recent studies reported the ability of C/EBP-β to regulate the expression of autophagy-related genes [30] and linked C/EBP-β-depend autophagy activation to proliferation and resistance in cancer cells [31]. Furthermore, PPAR-γ was able activate autophagy in cancer cells [32]. These observations let us suppose that C/EBP-β and PPAR-γ could activate autophagy, which in turn controls IL-6 expression in late-stages LS.

Our findings support a role for *C/EBP-β* and *PPAR-γ* in promoting LS growth, which could help in providing sub-molecular classification for LS patients to improve diagnosis and treatment. Given the high affinity of C/EBP-β to induce inflammation-related genes [33], it could constitute a promising therapeutic target. Helenalin acetate is a new emerging C/EBP-β inhibitor with efficacy to reduce leukemic cells proliferation [34]; therefore, its usefulness in dedifferentiated LS deserves to be the object of future investigations. *C/EBP-α* is particularly decreased in dedifferentiated LS comparing to well-differentiated or normal adipose tissue [35], this low expression may narrow its functions comparing to other transcriptional factors. We suggest IL-6 as an accurate indicator reflecting advanced stages of LS; thus, it could be used as a marker to follow-up treatment efficiency (its levels should decrease after successful tumor excision) or detect infra-radiologic relapse. Finally, as PA enhanced LS development and deteriorated the global health status in mice, we suggest that it may be avoided before LS treatment, as a preventive step against an unproven strategy that could be deleterious for patients.

Conflict of interest

None.

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Figure Legends

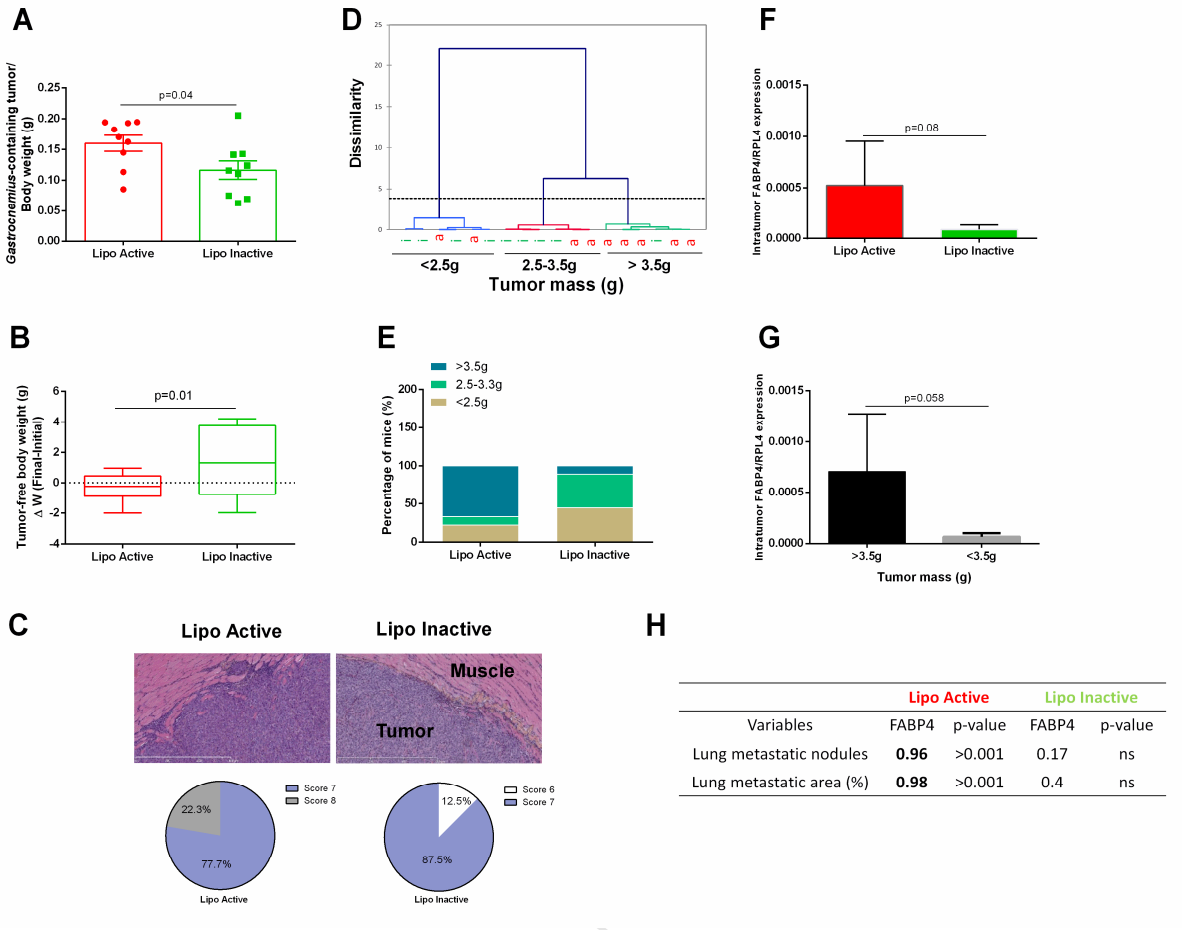
Table 1. List of primers used in RTqPCR analysis.

Figure 1. Impact of PA on LS-mice. (A) *Gastrocnemius* muscle-containing tumor mass normalized to body weight, (B) body weight loss between the first and last week PI, (C) H&E staining of grade-3 dedifferentiated LS and the scores of WHO classification of tumors of soft tissues, (D) hierarchical clustering analysis of active/inactive mice, based on tumor mass (a: active; i: inactive), (E) distribution of active/inactive mice according to their tumor mass, (F) RTqPCR analysis of FABP4 expression, (G) results from (F) were classified and analyzed according to tumor mass and (H) Pearson's correlation between FABP4 and metastasis. Results are expressed as mean ± SEM (Lipo Active: n=9; Lipo Inactive: n=9); Results were considered significant at $P < 0.05$.

Figure 2. Expression of *IL-6, C/EBP-α/β* and *PPAR-γ* in LS. (A) Circulating IL-6 levels in LS and control mice measured by ELISA, (B) transcript levels of hepatic drug-metabolizing enzymes and transcriptional factors determined by RTqPCR, (C) transcript levels of *C/EBP-α/β*, *PPAR-γ* and *IL-6* in tumor, (D-G) Pearson's correlations between *C/EBP-α/β*, *PPAR-γ* and *IL-6* (values in bold are statistically significant; values in italic are with high tendency). Results are expressed as mean ± SEM (CTL Active: n=9; Lipo Active: n=9; CTL Inactive: n=8; Lipo Inactive: n=9); Results were considered significant at $P < 0.05$. CTL: Control.

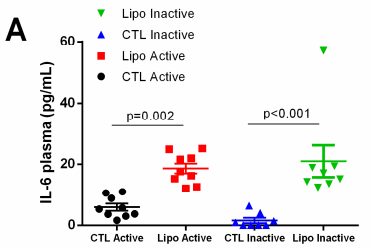
Figure 3. Correlation of *IL-6, C/EBP-β* and *PPAR-γ* with LS growth. (A-C) transcript levels of *C/EBP-α/β*, *PPAR-γ* in mice classified according to tumor mass, (D) Pearson's correlation between *C/EBP-β*, *PPAR-γ* and tumor, (E) transcript levels of *IL-6*. Pearson's correlation of *IL-6* with (F) tumor and (G) IL-6 plasma levels, (H-J) Pearson's correlation of GABARAPL-1 with tumor mass, *IL-6*, *C/EBP-β* and *PPAR-γ* expression. Results are expressed as mean ± SEM (Lipo Active: n=8; Lipo Inactive: n=8); Results were considered significant at $P < 0.05$.

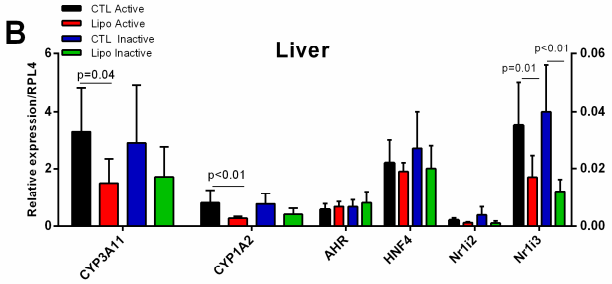
Gene (Human)	Forward (5'→3')	Reverse (5'→3')
IL-6	AGTGAGGAACAAGCCAGAGC	AGCTGCGCAGAATGAGATGA
C/EBPA	GCAAACTCACCGCTCCAATG	TTCTCTCATGGGGGTCTGCT
C/EBPB	ACCCACGTGTAACTGTCAGC	TGCCCCCAAAAGGCTTTGTA
PPARG	CCGTGGCCGCAGATTTGAA	CCACGGAGCTGATCCCAAAG
FABP4	TGGGCCAGGAATTTGACGAA	GCGAACTTCAGTCCAGGTCA
ADPOQ	GCCATCTCCTCCTCACTTCC	CATGACCGGGCAGAGCTAAT
BECN-1	AAGAGCATCGGGGGCTGA	CATCCTGGCGAGGAGTTTCA
GABARAPL-1	ACCATGGGCCAACTGTATGA	GGCTTCCAACCACTCATTTCC
CYP1A2	AAACTGTCCAGGAGCACTACC	ACCGTGTCCAGCTCCTCAT
CYP3A11	CTTGGTGCTCCTCTACCGATATG	TGGGTCTGTGACAGCAAGGA
AHR	AGCACAAATCAGAGACTGGCA	ATGACATCAGACTGCTGAAAGC
HNF4	ACACCACCCTGGAGTTTGAA	ACATTGTCGGCTAAACCTGC
Nr1i2	CCAACAAAAGCAGTGGCCC	GGCCCTTCTGAAAAACCCCT
Nr1i3	GCAGGGTTCCAGTACGAGT	GGCCCATCAGCTTTGCATAC
RPL4	TTCCTTTTCCTGTGGCAGCA	TTTTGCCAGATGACTCCCCC

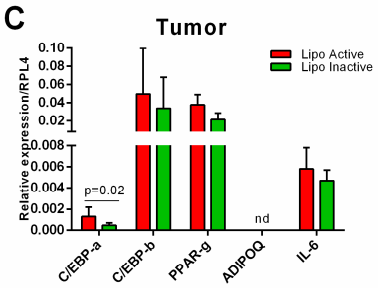


H

	Lipo Active		Lipo Inactive	
Variables	FABP4	p-value	FABP4	p-value
Lung metastatic nodules	**0.96**	>0.001	0.17	ns
Lung metastatic area (%)	**0.98**	>0.001	0.4	ns

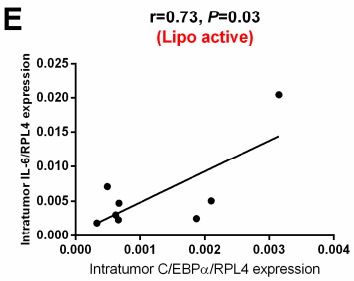


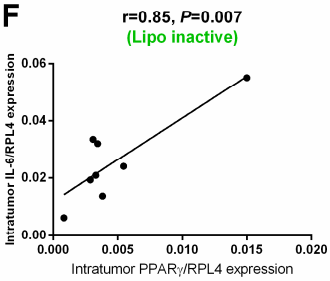


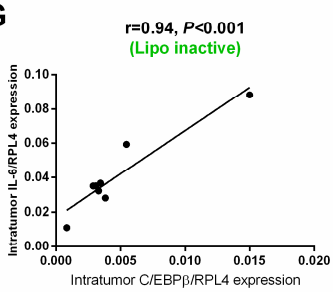


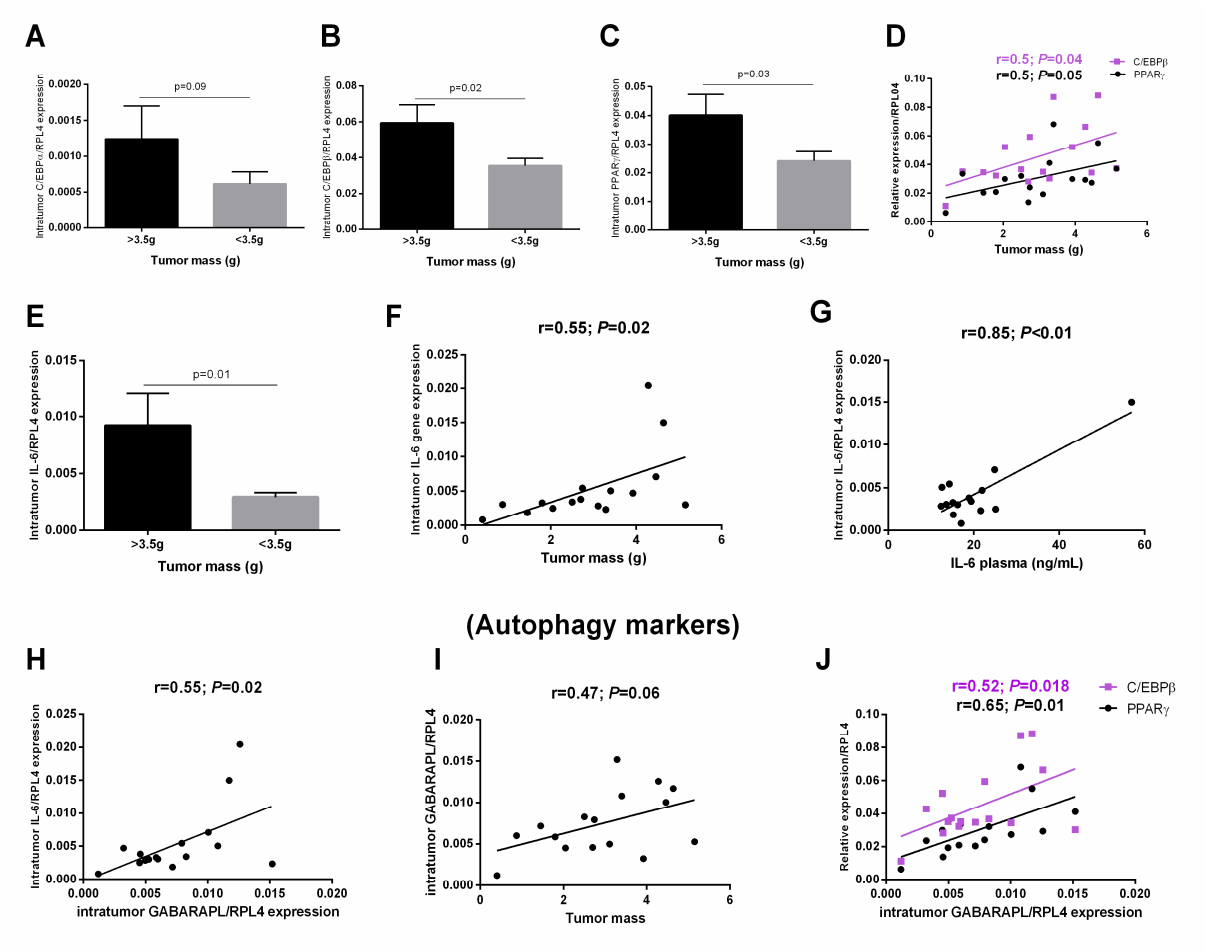
D

	Lipo Active			Lipo Inactive		
	C/EBPα	C/EBPβ	p-value	C/EBPα	C/EBPβ	p-value
C/EBPα	1	**0.81**	0.01	1	0.49	ns
PPARγ	0.32	*0.67*	0.06	0.17	**0.86**	0.005








A
p=0.09
>3.5g
<3.5g
Tumor mass (g)
B
p=0.02
>3.5g
<3.5g
Tumor mass (g)
C
p=0.03
>3.5g
<3.5g
Tumor mass (g)
D
r=0.5; P=0.04
r=0.5; P=0.05
C/EBPβ
PPARγ
Relative expression/RPL04
Tumor mass (g)
E
p=0.01
Intratumor IL-6/RPL4 expression
>3.5g
<3.5g
Tumor mass (g)
F
r=0.55; P=0.02
Intratumor IL-6 gene expression
Tumor mass (g)
G
r=0.85; P<0.01
Intratumor IL-6/RPL4 expression
IL-6 plasma (ng/mL)
(Autophagy markers)
H
r=0.55; P=0.02
Intratumor IL-6/RPL4 expression
intratumor GABARAPL/RPL4 expression
I
r=0.47; P=0.06
intratumor GABARAPL/RPL4
Tumor mass
J
r=0.52; P=0.018
r=0.65; P=0.01
C/EBPβ
PPARγ
Relative expression/RPL4
intratumor GABARAPL/RPL4 expression

Highlights

- **Circulating IL-6 were higher in liposarcoma bearing mice**
- **Intra-tumor *IL-6* expression correlated with circulating IL-6 levels**
- **Intra-tumor *IL-6, C/EBP-β* and *PPAR-γ* correlated with tumor mass and autophagy**
- **Regular PA failed to decrease IL-6 levels**
- **Active liposarcoma-bearing mice exhibited greater tumors and significant weight loss**