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The consequences of PPARγ activation in melanoma cells on the tumour microenvironment

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1 Abstract

Melanoma is one of the most murderous tumours over the world. The capability of the tumour to invade all tissues in the body and the low-response to chemotherapies lead to a poor prognosis with high mortality rate. The metastatic stage has still no curative treatment and it is the reason why the investigation of new therapies is required. Tumour progression involves the activation of a tumour-promoting chronic inflammation, in part induced by the secretome of tumours cells with, as consequences, changes in the tumour microenvironment. This is one of the hallmarks permitting the tumour to grow, proliferate, survive and migrate.

Unpublished data from the group has shown that the activation of the Peroxisome proliferator-activated receptor gamma (PPARγ) induces a modification of gene expression in metastatic melanoma cells, thereby promoting a pro-inflammatory response with the expression of the cytokines IL6 and IL1β. Based on these data, the idea that PPARγ may be new target to treat metastatic melanoma emerged.

PPARγ is a nuclear receptor regulating transcription, already used as therapeutic target by drugs called Glitazone, used to treat Diabetic mellitus type II by decreasing Insulin resistance. However, the effect on tumours of PPARγ activation is still unclear, as well as the toxic side effect on cardiovascular system, causing the withdrawal of many of these drugs. Although some epidemiologic studies have reported a probable tumour protector effect of PPARγ activity by reducing inflammation on some types of cancer in human, a significant increase of bladder cancer after treatment with Pioglitazone (a Glitazone still used in our country) has been reported.

In this work, PPARγ was activated with the agonist Glitazone in melanoma cell lines that were in different stages of progression of the disease. The cell lines tested were in a non-metastatic stage of progression of the disease and they did not change their pro-inflammatory gene expression in the presence of PPARγ agonist.

Nevertheless, it has been demonstrated in the lab that metastatic melanoma cells respond to PPARγ agonist, changing the secretome of the tumour. In order to create an experimental model, this work analysed the effect of the secretome of human metastatic melanoma cells treated with PPARγ agonist on mouse fibroblasts. No change of gene expression was observed in normal mouse fibroblasts when they were directly treated with PPARγ ligands. However, the interesting results showed that murine fibroblasts increased the expression of pro-inflammatory markers after incubation with the secretome of melanoma cells treated with agonists of PPARγ.

Accordingly, PPARγ activation may have an effect on modifying the secretome of the tumour, inducing the microenvironment to change into a pro-inflammatory state. However, the recruitment of inflammatory cells can also have an anti-tumoral effect, the in vivo model may show if this inflammation may be deleterious or may be beneficial for the tumour growth and proliferation.

2 Introduction

2.1 Melanoma: a little epidemiology

Over the last 50 years, the incidence of malignant melanoma has risen to become one of the most lethal malignant tumours in Caucasian countries (1). In Switzerland, the incidence has grown to become the fourth most common cancer (after prostate/breast, colon and lung carcinomas) in men and women (Statistic from OFS 2012). It now represents 6-10% of the tumours which are diagnosed every year (2), that is one of the highest rates in Europe and in the world. Through the better recognition of the symptoms and the efficacy of the screening, 95% of the patients never develop metastasis. However, metastatic melanomas are associated with a poor prognosis and low long-lasting response to the treatment, showing 10% survival rates after 2 years with a median overall survival of 6 months (3). In fact, people of all ages are susceptible to develop melanomas, since sun exposure is the major (but not essential) risk factor, and death occurs in younger ages than in most of the other tumours. Other risk factors are described in Figure 1A.

A RISK FACTORS

ENVIRONMENTAL	-Sun exposure -White skin -Sunburn -Immunosuppression Iatrogenic or acquired
GENETICS	-BRAF (40-50%), NRAS (10-20%), PTEN (60%), CDNK2A (30%), c-KIT (1%), -DNA repair defect (xeroderma pigmentosum) -Family history -Tendency to burn and not to tan -Red hair colour
PHENOTYPIC	-Melanocytic nevi -personal history of cutaneous melanoma

B

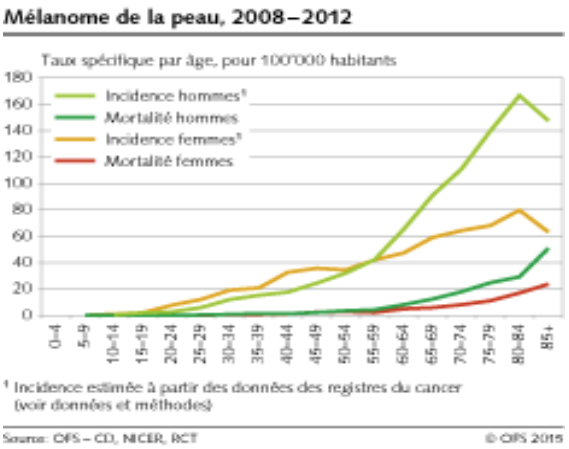

Figure 1: A: Risk factors correlated with malignant melanoma development. (4,5) B: Statistics of incidence and mortality of malignant melanoma in Switzerland (Office Fédéral de la Statistique, 2015). More likely old people and men are diagnosed with melanoma; but the death occurs at a higher level in women.

2.2 From melanocyte to melanoma

The statistics and prognostics are frightening, but what is a melanoma?

Basically, malignant melanoma emerges from the transformation of melanocytes, which are the pigmented cells of the skin, due to mutation in their DNA. In human skin, melanocytes are found in the basal layer of the epidermis, close to the dermal junction (3,6). The process of transformation is multi-stepped. There are more than a hundred genetic mutations in melanoma cells that accumulate with time. According to the Clark model of tumour formation, the transformation of melanocytes into malignant melanomas begins with the formation of a tumour of the radial type, which develops into vertical and then invasive type, as shown in figure 2. It has been described that melanoma cells are phenotypically different according to these three

main stages of transformation (figure 2; Clark's level of development), hypothesizing that mutations accumulate in the tumour, giving to the tumour cells the capacity to reach the metastatic stage.

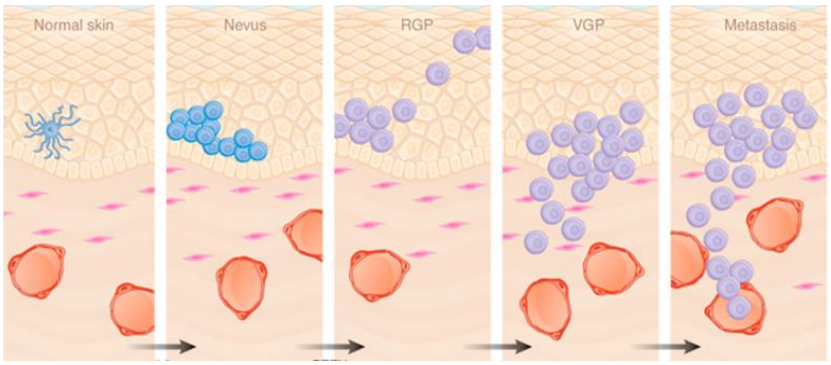

Figure 2: Different stages of malignant melanoma development.
A: Melanocytes arise from the neural crest and migrate into the basal layer of the epidermis during development. They produce melanin, the main pigment of the skin. There is about one melanocyte for ten keratinocytes (epidermal cells). They have cellular extensions (dendrites) between the keratinocytes permitting them to provide the keratinocyte with melanin. **B:** Naevi are proliferations of melanocytes; they are benign but can transform into malignant melanomas. **C:** The malignant non-metastatic melanomas radial phase of proliferation can emerge from naevi changes, but usually arises from *de novo* transformation. The cells grow horizontally on the epidermis (in situ). **D:** malignant non-metastatic melanoma vertical phase of proliferation, growing and invading the dermis and hypodermis. **E:** Metastatic malignant melanomas with transformed cells invading the blood and lymphatic vessels which permits them to invade distant organs. (7)

The thickness of the tumour according to Breslow is the principal prognostic factor and is linearly correlated with the 10-year-survival rate (5,6). The main reason for the tumour aggressiveness is the capacity to metastasize and to grow in distant organs due to an extraordinary ability to disseminate by lymphatic and hematogenous routes. The goal of therapy is early diagnosis, before the transformation of the tumour into the invasive form, followed by total excision (4).

The first step of melanomagenesis involves key driver mutations which can be induced by UVA and UVB (8). Thereby, the cells transform into malignant cells and develop biologic abilities permitting them to lose their differentiation, survive, proliferate and migrate. Once the key drivers are mutated, the cells accumulate mutations leading to a diversity in melanoma genetics, being one of the explanations of differences between the development and prognostic of the tumour (3). The eight hallmarks that explain tumour formation are acquired functional capabilities and involve sustaining proliferative signalling, evading growth suppressor, resisting cell senescence and apoptotic programs, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics, and finally avoiding immune destruction. Since 2011, it had been added that these hallmark capabilities are acquired due to two main characteristics of tumorous cells: the genome instability and the tumour-promoting inflammation (9).
In fact, the stroma of melanoma cells is important for tumour growth and survival. Due to the presence of growth factors, nutrients (e.g. glucose), oxygen, and cytokines secreted by surrounding fibroblasts and endothelial cells, the tumour can survive, proliferate and progress into a metastatic form (3). It has been described that fibroblasts can transform into Cancer-associated-fibroblast (CAF), which sustain the secretion of pro-inflammatory cytokines, leading to the promotion of angiogenesis, as well as immune cell attraction and activity (10). The effect of the microenvironment on tumour progression, and how the

microenvironment can be regulated by the tumour to stimulate growth and aggressivity, have to be better understood.

Moreover, environmental, genetic and host factors are interesting points that are required for the progression of the tumour which will not be developed in this work.

2.3 Inflammation

"Inflammation is a systemic and local reaction of tissues and microcirculation to pathogenic insult" (11). It describes the capability of the body to respond to cell injuries in order to heal the affected tissues. Many mechanisms are implicated: there are two distinct types of cells mediated inflammation: the innate immunity composed of granulocytes (regrouping of neutrophil, eosinophil and basophil), monocytes, which differentiate into macrophages or Antigen-presenting cell in tissues and mast cells (responsible to allergic reactions); and the adaptive immunity recruiting trigger-specific T- and B-lymphocytes (production of antibodies against pathogenic antigens). The innate immunity is associated with the complement system, which involves proteins that inhibit bacteria, virus, parasites and transformed cells by opsonisation, help the phagocytosis, kill by lysis of the pathogen, increase vascular permeability and stimulate inflammation.

2.3.1 Normal activation of inflammation

Acute inflammation (innate immunity and adaptive immunity) occurs directly after tissue damage or infection due to activation of affected cells leading to the secretion of eicosanoids, of cytokines such as IL1, IL6, and TNFα and presentation of pathogen antigens at their surface, that increase vascular permeability and recruit leucocytes. First neutrophils are attracted to try to restore homeostasis by fighting exogenous triggers, cleaning endogenous death cells and increasing leucocyte attraction and differentiation into a pro-inflammatory phenotype (12,13). In a second phase, monocytes, lymphocytes and stromal cells are brought into the lesion (oedema) and produce a pro-inflammatory microenvironment, with more cytokines, chemokines and proteases. This pro-inflammatory environment induces modification of the transcription of several genes in immune cells, leading to differentiation of macrophages (15,16), promoting a pro-inflammatory phenotype in fibroblasts (17) and inducing angiogenesis in endothelial cells (12,15). The clinical outcome of the vasodilatation, oedema and tissue damage is described by the cardinal signs rubor (redness), calor (heat), tumor (swelling) and dolor (pain).

The inflammatory reaction is regulated by proteins and cells which both try to focalize the reaction where the pathogenic trigger takes place and to reverse the acute phase after elimination of the pathogen, by eliminating neutrophils and inflammatory cytokines, restructuring the architecture of the tissue, changing the phenotype of immune cells into a non-inflammatory one (macrophages and monocytes become key for the restructuration of the tissue). Usually, it is solved in a relatively short period of time. If the inflammation trigger cannot be eliminated, or regulatory systems are not sufficient, it leads to a chronic inflammation,

where the extracellular matrix constantly regenerates and remodels, with the persistence of microenvironmental changes and immune cell attraction (14).

2.3.2 Inflammation and cancer: the good and bad effects

Rudolph Virchow was the first person to link inflammation and tumorigenesis by proposing the hypothesis that chronic inflammation can be the origin of cancer. However, how inflammation may help tumours to develop is still unclear (15,16).

The first hypothesis is that chronic inflammation preceding or following cancer may have a supporting effect on tumorigenesis, enhancing the cell capacity to survive, grow and proliferate, for instance patients with ulcerative colitis have a higher risk to develop a colorectal adenocarcinoma (17). Chronic inflammation or repeated injuries stimulate cell division, induce permanent cell destruction, and remodelling, being capable of destroying healthy tissues and supporting development of cancer (15,16). This prospective means that immune cells (e.g. macrophages, neutrophils and mast cells) produce tissue damage through reactive oxygen species and protease production (16), leading to cell transformation (16,18) and accumulation of genetic mutations. The infected or mutated cells, the stromal cells and the immune cells continue to secrete chemokines and cytokines that attract and activate more immune cells, which also respond by secreting more cytokines and chemokines into the microenvironment. Other examples are gastro-intestinal stromal tumour (GIST) where chronic inflammation due to ulceration of the intestinal epithelium increases the risk of a tumorous transformation of the stromal cell, or a chronic colonisation of *Helicobacter pylori* is also related with chronic inflammation and activation of lymphoid follicle increasing the risk to develop a mucosal lymphoid tumour (MALT). Another known predisposition to develop cancer is typically chronic hepatitis C infection of the liver, that increases the risk of having an hepatocellular carcinoma (13,19).

The second hypothesis is that tumour initiates the inflammation process and tries to repair the tissue damage but becomes chronic and more likely sustains the tumour development. The chronic changes in the microenvironment have a highly relevant effect on tumour pathogenesis, inducing the transcription of genes in malignant as well as in pre-malignant cells, thereby acting on the survival, growth, proliferation and migration of the tumorous cells (13,18,19). One described effect is that fibroblasts can transform into Cancer Associated Fibroblast (CAF), which secrete cytokines and other factors such as IL6 and VEGF increasing angiogenesis and immune cell attraction (10,20). The effect of pro-inflammatory cytokines secreted by the tumour cells, such as IL6, may induce this transformation of fibroblasts (21).

However, chronic inflammation is not always correlated with tumour formation. The second hypothesis of inflammation interacting with cancer suggests that inflammatory activity, mostly through adaptive immunity, may help the body to fight against malignant cells and to reduce tumour growth. NK and T cytotoxic cells can recognize transformed cells through their presentation of abnormal antigens and induce cell death by apoptosis, but tumorous cells are able to avert the process, through expression of host normal antigens for example. As observed, new therapies enhancing the inflammation activity show promising results on several malignant tumours, such as malignant melanomas (1,22,23). Indeed, monoclonal antibody therapies

targeting CTLA-4 (Cytotoxic T-Lymphocyte-Associated protein 4, known pharmaceutically as Ipilimumab, approved in 2011 by Swissmedic) and PD-1 (Programmed Death 1 known pharmaceutically as Nivolumab and Pembrolizumab, approved by Swissmedic in 2015) are used nowadays to treat metastatic melanoma. These drugs improve T-lymphocyte activity, which was reduced because of the capability of melanoma cells to protect themselves through inhibition of the adaptive immune response by expressing normal-cell-receptors (24,25). These immunotherapies have shown an augmentation of progression-free survival time in patients with advanced malignant melanoma, becoming the first therapy for these patients (24).

2.4 PPARγ: a nuclear receptor

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors important for the regulation of the expression of genes in several physiological processes, such as metabolism, cell proliferation, differentiation and apoptosis (26) but also immunity and wound healing (27,28).
There are three PPAR isotypes, alpha, beta/delta, and gamma, differing with respect to role, activation and tissue distribution. As a nuclear receptor, the activation of the receptors is complex and involves heterodimerization with the retinoic X receptor (RXR), activation by endogenous or/and exogenous ligands,

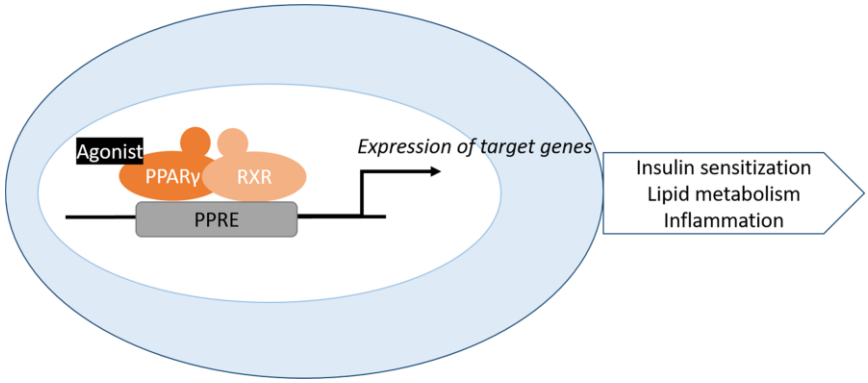

Figure 3: Activation of PPARγ nuclear receptor and effect of the transcription of the target genes. RXR: retinoic X receptor; PPRE: PPAR response element (22).

binding to the PPAR response element (PPRE) and modulating the expression of several target genes. The activity of the receptors implies the recruitment of co-activators to permit the transcription of target genes, and dissociation of co-activators and recruitment of co-repressors in the inactive state (29). PPARγ is especially localized in adipocytes but occurs in many other types of cells at lower expression levels (30,31). Natural ligands are fatty acids, mostly polyunsaturated, such as linoleic and linolenic acids, and eicosanoic have been reported to activate the receptor, having an impact on lipid and glucose balance (31). Synthetic molecules, such as thiazolidinediones (see next paragraph) also activate PPARγ.
In 2000, the PPARγ receptor has been described to be related to inflammation (32) and an effect of PPARγ activation in many different cancers has been reported (tab 2). Even if several contradictory ideas on inflammation have been proposed, the recent findings suggest that PPARγ activation may sustain chronic inflammation by increasing pro-inflammatory cytokines and chemokine expression, changing the polarisation of macrophage into a pro-tumorous form and modifying the microenvironment in order to support tumour growth, survive, proliferation and migration (results from C.Pich).

Tumour	Effects of PPARγ agonists
Glioblastoma	Reduce growth, malignancy and expansion of brain tumour stem cells
Lung carcinoma	Inhibition of growth.
Non-small cell lung carcinoma	Induce differentiation and apoptosis. Potentiate cisplatin and paclitaxel inhibitory effects inhibiting cell cycle
Gastric carcinoma	Induce apoptosis and G1 cell cycle arrest.
Colocarcinoma	Modest decrease of risk of development, but controversial acceptance of this action.
Hepatocellular carcinoma	Reduce cell growth, migration and adhesion to tumorous cells in the extracellular matrix.
Pancreatic carcinoma	Attenuate cell growth, migration and invasion. May have an effect on reduction of angiogenesis, but the effects are still controversial.
Testicular cancer	Antiproliferative effects
Osteosarcoma	Reduce proliferation. May have a beneficial role in the prevention of Osteosarcoma.
Haematological malignancy	Anti-proliferative, pro-differentiative, anti-metastatic and pro-apoptotic effects.
Breast-cell cancer	Reduce proliferation but controversial idea on the real benefit of the treatment Beneficial effects were observed in combination treatment with COX-2 inhibitors.
Bladder cancer	Increase grade and stage of carcinoma with Pioglitazone, not for Rosiglitazone

Tab 2: List of PPARγ agonist effects on some cancers based on meta-analysis and cohort studies. (19,20,26-32)

2.5 The Thiazolidinediones

The Thiazolidinediones, also called Glitazones, are molecules invented in the nineties that were shown to increase Insulin sensitivity and to reduce glucose in the blood of rodents (33,34). In 1995 a study demonstrated PPARγ implication as the molecular target of these new molecules, and suggested the possible use as a therapy against non-insulin dependent diabetes mellitus (type II) (35). Extensive investigations were done to show that thiazolidinediones have an impact on lowering plasma lipid levels potentiating their use against metabolic diseases (34). However, the general beneficial effects of these therapies are unsure because of numerous side effects. In cancer, whether PPARγ agonists are pro- or anti-tumorous is controversial (25,26,32–38). On the one hand, Thiazolidinediones may exhibit possible anti-tumour and anti-inflammatory effects (36,37) as a result of possible PPARγ dependent and independent effect, for example a mitochondrial stimulation (57). On the other hand, a first meta-analysis revealed a significant increase of

bladder cancer in patients treated with pioglitazone, inducing the retrieval of this drugs in 2010 in some countries (38).

Nowadays, Thiazolidinedione therapies generate apprehension in many countries in Europe, due to the high number of side effects. It is the reason for the proscription of some Glitazone listed in tab 3 (40,41).

Substance	Receptor affinity	Date of approbation	Effect	Adverse effect	Suggestion from the guidelines
Rosiglitazone **-Avandia** **-Avandamet** **-Avaglim**	PPARγ	July 2000	-Antidiabetic (Insulin sensitizer)	-increase risk of congestive failure, myocardial infarction and cardiovascular disease -all-risk mortality	-US: use under restriction -EU: out since 2010 for the cardiovascular safety
Pioglitazone **-Actos**	PPARγ	US:1999 UE:2002	-Antidiabetic -Reduces liver steatoses in Non-alcoholic steato-hepatitis (NASH)	-increase risk of bladder cancer (>1 year of use) -congestive heart failure -Osteoporosis, water retention -suspicion of increasing melanoma?	-FR and DE: suspension since 2011 for elevated risk of bladder cancer. -CH, USA permitted usually not in monotherapy
Troglitazone **-Rezulin**	PPARγ PPARα (less)	March 1997	-Antidiabetic -Anti-inflammatory	-Hepatotoxic -Renal failure	Withdrawal in 2000 for important hepatotoxicity
Ciglitazone	PPARγ	1980	-antidiabetic	cell apoptosis	Never used as a therapeutic drug
Rivoglitazone	PPARγ	-	-antidiabetic	Still under study	-
Lobeglitazone **-Duvie**	PPARγ PPARα	2013 (still under surveillance until 2019	-Antidiabetic -Reduces liver steatose in Non-alcoholic steato-hepatitis NASH	?	accepted only in Korea

Tab 3: The diverse thiazolidinediones with their positive and negative effects, issued from la Revue Médicale Suisse and Swissmedic (42) and other guidelines from different countries: NICE (UK) (43), ANSM (FR) (44), NPS (AUS) (45) , NIH (US) (46–48)). Other sources from papers: Lobeglitazone: (41,49); Ciglitazone: (50); Troglitazone (51); Rivoglitazone (52).

Nonetheless, they are still used in special cases to treat diabetes mellitus type II patients, Pioglitazone being the last Thiazolidinedione used in Europe (the last data indicate that 5% diabetic patients use pioglitazone in UE (53)) It is also used to treat Non-Alcoholic Steatohepatitis in diabetics type 2 patients, showing a reduction of the progression of the disease (54). A cohort study reported an increase of malignant melanoma and Non-Hodgkin lymphoma in diabetic patients using Pioglitazone, but more studies must be done (55). Rosiglitazone (Rosi) is a Thiazolidinedione (TZD) and a specific agonist of PPARγ, that promotes the recruitment of co-activators by the receptor. The activation promotes an up-regulation of genes acting on

adipogenesis, decreasing insulin resistance and inflammation. Rosiglitazone is used in USA but is not permitted in Europe due to an augmentation of cardiovascular diseases. As mentioned above, the Thiazolidinediones were suggested to increase risk of bladder carcinoma. This pro-tumoral impact could be the results of PPARγ independent effect, as bladder carcinoma major risk factor are toxic substances (cigarettes, chemical product, arsenic)(55). However, in culture, PPARγ can be directly activated in human bladder carcinoma with the agonist Rosiglitazone and the response is linked with an augmentation of inflammation (Annexe C).

2.6 Purpose of my study:

Recently, a global analysis of the consequences of PPARγ activation on melanoma cells was performed in the group of L. Michalik. This study suggested that PPARγ activation induces the expression of several pro-inflammatory cytokines such as IL1α, IL1β, IL6 and IL8 in metastatic melanoma cells, which can have a beneficial or detrimental effect depending on the amplitude of the response. Interestingly, the same study showed that this response to PPARγ activation was less important in normal human melanocytes. These data suggest that PPARγ can have an important role on the development of the tumour, and raise two captivating points:

- First, based on the Clark model of melanoma transformation, at which stage of malignant melanoma progression does the activation of PPARγ induce this pro-inflammatory response?
- Second, does this pro-inflammatory response, that has been analysed on malignant melanoma secretome, have an impact on the microenvironment surrounding the tumour?

These two questions are at the origin of my study, the goal of which was to address the following issues:

-Is the pro-inflammatory response induced by PPARγ agonists on metastatic melanomas cells also present in early stages of melanoma tumours or is it specific to end-stage melanomas?

-Does the secretome produced by metastatic malignant melanoma cell influence protein expression in mouse fibroblasts?

3 Methods

3.1 Cell culture

The following four cell lines were cultured: WM35 (passage 60; non-metastatic radial human cells line); WM115 (passage 72; non-metastatic vertical human cell line); tdTomato-labelled A375 (A375tdT; passage 15-18 and 38; human metastatic cell line of melanoma constructed in the lab from A375 purchased from Promocell); and NIH3T3 (passage 20-29; mouse fibroblasts purchased from DSMZ in Germany). The cells were stored at -130°C, then thawed rapidly at 37°C and re-suspended in specific media.
The human non-metastatic melanoma cells were grown in 2%Tü medium containing 80% MCDB 153, 20% Leibovitz'sL-15, 5 ug/ml Insulin (bovine), 2% fetal bovine serum, and 1.68mM calcium chloride,
The mouse fibroblast NIH-3T3 and human melanoma A375tdT cell were grown in DMEM-glutaMAX medium (Gibco Life Technology) containing glucose, glutamine, pyruvate with the addition of 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics.

All cells were cultivated in a humidified incubator at 37°C with 5% CO2.
To detach the cells, 2ml Trypsin 0,05% was added to the flask for 5 minutes, followed by 5 ml of complete medium to inhibit the trypsin. The detached cells were taken with the medium out of the flask and centrifuged (4 min at 1200rcf). After re-suspension of the cells in 5 ml of fresh complete medium, the cells were counted (Invitrogen Countess) and separated keeping 1/5 (WM35 and WM115 cell lines) or 1/50 (A375tdT and NIH3T3) of the total volume for another passage.

3.2 Activation of PPARγ:

For treatments, a specific number of cells were seeded into 6-well-plates: WM35 and WM115 were seeded at $3x10^5$ cells per well; A375 tdT were seeded at $2x10^5$ cells per well; NIH3T3 were seeded at $2x10^4$ or $2x10^5$ cells per well.
PPARγ activity was modulated using a final concentration of 10 µM and 5 µM of the agonist Rosiglitazone (Lucernachem, Avandia drug in powder form), 2µM of the antagonist T0070907 (Enzo Life Science), or 1 µM of the antagonist GW9662 (Focus biomolecule). The experiments were done in triplicate for WM35, A375 and NIH3T3 and in duplicate for WM115. All compounds were dissolved in DMSO (a solvent that permits to dilute salts and hydrophobic substances), which was used as the vehicle control. All cell lines were seeded for 24 hours then the treatments were added for 24 hours.
T0070907 is an antagonist of the PPARγ receptor. It binds and reduces the activity of the receptor blocking the recruitment of co-activator and increasing the recruitment of co-inhibitor (NcoR).

GW9662 is a potent irreversible selective antagonist of PPARγ.

3.3 Preparation of conditioned medium:

Metastatic melanoma cells were cultivated in complete medium DMEM+ 10%FBS and treated with 5 µM Rosiglitazone and antagonist GW9662 1 µM. After 24 hours, cells were washed with PBS and the medium was replaced by a fresh medium without FBS. After 72 hours, the medium incubated with A375tdT was filtered (0.2 um filter) and collected (conditioned medium). Then, mouse fibroblasts NIH3T3 were incubated with a 1:1 mixture of conditioned medium and complete DMEM medium for 72 hours (Figure 4).

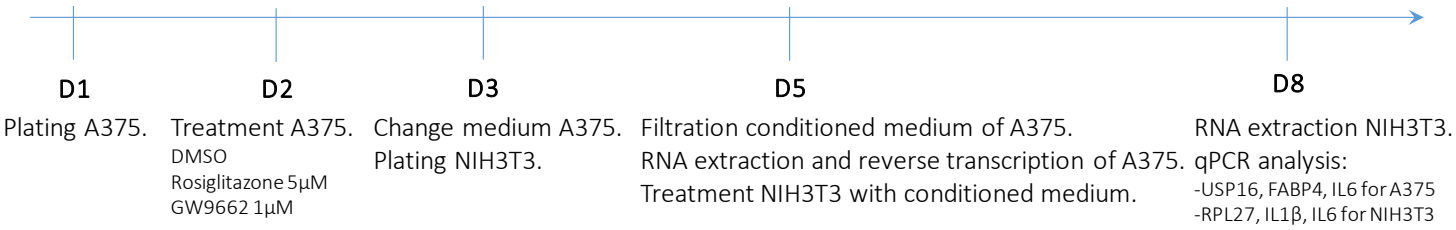

Figure 4: Diagram showing the time line of the conditioned medium experiment. D: Day

3.4 RNA extraction:

Cell samples were scrapped with 1 ml Trizol or TriFast (Invitrogen Life Technologies) and 150 µl 1,3-Bromochloropropane were added. The samples were centrifuged for 30 min at 9400 g (TRIzol) or 15 min at 12000 g (TriFast). RNA contained in 400 µl of the aqueous phase (supernatant) were precipitated with 600 µl Isopropanol overnight or for three days into -20°C.

The precipitated RNAs were centrifuged 25 min at 11000g, isopropanol was removed gently, replaced with 1 ml of 75% ethanol and vortexed to wash the pellet of RNA. The samples were centrifuged again 10 min at 7400g, to reform a pellet of RNA. After two cycles of washing with 75% ethanol, vortex and centrifugation, the RNAs were left to dry at RT for 10-20 min and re-suspended in nuclease-free water. The concentration and purity of RNA in solution was measured (NanoDrop 8000, Thermo Scientific). Total RNA was then diluted between 200 ng/µl and 500 ng/µl in order to have the same concentration for the future experiments.

The RNA was stored at -20°C for one day or at -80°C for long-time conservation.

3.5 Reverse-transcription:

The RNA obtained were transformed into cDNA which were used for the qPCR with the following protocols: For WM35 and WM115, a kit in two steps (Promega) was used. First step, the RNA (1 µg/sample) was mixed with random primers 0,25 µg, dNTP mix 0,5 µM and nuclease-free water (qsp 20 µL) and heated for 5 min at 70°C to melt RNA permitting primers to attach. Second step, a mix of 1x M-MLV Buffer (containing 250 mM Tris-HCl [pH 8.3 at 25°C], 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT), M-MLV Reverse Transcriptase H+ (100U) and water RNAse-free were added to the samples and were heated 10 min at 25°C, 60 min at 42°C for the reverse transcription, 15 min at 70°C for the inactivation of the Reverse transcriptase (RT) to be finally kept at 4°C.

For NIH3T3 and A375 tdT cells, a kit in one step was used (iScript cDNA Synthesis kit from Biorad). The RNA (1 µg/sample) was mixed with a reaction volume (20 µl; containing 1x iScript reaction mix, iScript Reverse transcriptase RNAse H+ and nuclease-free water) and heated for 5 min at 25°C for the priming of the primers, 30 min at 42°C for the reverse transcription, 5 minutes at 85°C for the inactivation of the reverse transcriptase, then finally kept at 4°C.

The retro-transcribed cDNA were diluted in 400 µl final volume with nuclease-free water and kept at -20°C.

3.6 Real time quantitative PCR (qPCR):

Gene Name	Full name	Sense sequence (forward 5'->3')	Antisense sequence (reverse 3'->5')	Final concentration	Acquisition
HUMAN					
FABP4 (aP2)	Fatty acid-binding protein, adipocyte	GATGATAAACTGGTGGTGGAATG	ATGCGAACTTCAGTCCAGGT	200 nM	Microsynth
CPT1A	Carnitil palmitoyltransferase 1A	CCGTAGCTGACTCGGTACTC	TCTAAGAGCTTCATGGCTCAG	400 nM	Microsynth
PPARγ	Peroxisome proliferator actived receptor gamma	AAGGCCATTTTCTCAAACGA	AGGAGTGGGAGTGGTCTTCC	200 nM	Microsynth
IL-1α Hu	Interleukin 1 alpha	ACCAACGGGAAGGTTCTGAAG	CTAGGCTTGATGATTTCTTCCTCTG	300 nM	Microsynth
IL-1β Hu	Interleukin 1 beta	GCACGATGCACCTGTACGA	AGAACACCACTTGTTGCTCCATATC	300 nM	Eurofins MWG Operon
IL-6 Hu	Interleukin 6	GGATTCAATGAGGAGACTTGCCTG	GACTTTTGTACTCATCTGCACAGC	300 nM	Eurofins MWG Operon
USP16	Ubiquitin specific peptidase 16	TGGGCTCTGTCGCCGTGGATTG	TGTCCGTTTCTTTCCCATGTTGGCAC		Microsynth
MOUSE					
ANGPTL4	Angiopoietin 4	Quote: QT00003661		300 nM	QIAGEN
IL-6	Interleukin 6	TTCCAGAAACCGCTATGAAGTTCC	TTGTGAAGTAGGGAAGGCCGTG	200 nM	Microsynth
IL-1β	Interleukin 1 beta	TGCCACCTTTTGACAGTGATGAG	TCATCTTTTGGGGTCCGTCAAC	300 nm	Microsynth
PPARγ	Peroxisome proliferator actived receptor gamma	TGATTACAAATATGACCTGAAGC	TTGTAGAGCTGGGTCTTTTCAGAAT	200nM	Microsynth
RPL27	Ribosomal protein L27	CTGGCCTTGCGCTTCAA	TCATGCCCACAAGGTACTCTGT	200 nM	Microsynth

Tab 4: Primers sequences used for the experiment.

The experiment is realized with the Stratagene Mx3005P machine. Amplification mixtures contained cDNA, specific sense and antisense primers (200 nM), SYBR GREEN master mix, containing dNTPs, MgCl2, and DNA polymerase (Qiagen) and nuclease-free water. The specific targeted cDNAs were amplified by temperature transition: 5 min at 95°, 40 cycles (10 sec at 95°C, 45 sec at 60°C), 1 min at 95°C, 30 sec at 55°C and 30 sec at 95°C. Primers for human cDNA: FABP4, CPT1A (direct target gene of PPARα/γ), PPARG (nuclear hormone receptor), IL1A, IL1B, IL6 (inflammation factors), USP16 (human housekeeping gene). Primers for mouse cDNA: Angptl4 (direct target gene of PPARα/γ), Il1b, IL6, Pparγ, Rpl27 (mouse housekeeping gene) as mouse primers (tab 4).

3.7 Statistical analyses:

Each experiment was carried out in duplicate or triplicate. The data are expressed as geometric mean values +/- standard deviation. The data were analysed using a one-way ANOVA test (non-parametric) when three independent experiment analyses were done (Figures 5 to 7). For Figure 7B, 8 and 10, a student T-test (non-parametric) was used because of an experiment comparing two independent experiments analyses.

4 Results and discussion

4.1 Non-metastatic malignant melanomas' response to PPARγ agonist

We know from previous work in the lab that metastatic malignant melanoma cell lines show a very strong response to PPARγ agonist, which is inducing a pro-inflammatory response (Annexe A). But whether this is also the case in non-metastatic melanoma is still unclear. In order to understand the effect of the activation of the nuclear receptor in the different stages of melanoma progression (Clark model, figure 2), the expression of inflammation marker genes in two human non-metastatic malignant melanoma cells were studied: WM35 as a representative model of the radial-growth stage and WM115 as a representative model of the vertical-growth stage.

4.1.1 PPARγ is poorly expressed in human radial growing melanoma cells

In the first experiment, WM35 human malignant melanoma cells were tested. After culture of the cells and 24-hour treatment with the PPARγ agonist (Rosiglitazone; 10 µM) or antagonist (T0070907; 2 µM), total RNAs were extracted from the cells and the genes of interests were analysed by RT-qPCR. To be sure that the treatment with PPARγ ligands was efficient, the expression of a PPARγ known target gene, Carnitil palmytoyl transferase-1A (CPT1A) was tested. A second target gene of PPARγ was also tested, the fatty acid binding protein 4 (FABP4), however, this gene was not expressed in these cells. Inflammation was studied by analysing the expression of Interleukins 6, 1 beta and 1 alpha (IL6, IL1B and IL1A).

The results show that PPARγ is almost undetectable in WM35 cells, which explains the variability between the independent experiments (Figure 5A). Surprisingly, the agonist Rosiglitazone and antagonist T0070907 both increase the expression of CPT1A. Since PPARγ expression is very low, this is probably a PPARγ-independent effect. Indeed, Rosiglitazone has the potential to bind other PPARs with a lower affinity, as PPARα, that is known to expressed in malignant melanoma (56). In order to check this hypothesis, the cells were treated with the two molecules together. The results (Figure 5C) show an increase in the expression of the PPARγ target gene CPT1A, confirming a PPARγ-independent effect. GW9662 was used as another antagonist, no significant modification compared to T0070907 treatment was observed.

Interestingly, the pro-inflammatory cytokines IL6 and IL1B have a low constitutive expression in non-metastatic melanoma cells WM35 (Figure 5B). However, no significant modulation after treatment with the PPARγ agonist and the PPARγ antagonist was observed (Figure 5B). When the cells were treated with the agonist and antagonist at the same time, similar mRNA expression levels of IL6 were observed compared to the Rosiglitazone, which suggests that Rosiglitazone has no influence on the transcription of this cytokine in these cells, dependent or independent of PPARγ.

These results and the variability of the response can be explained by the low expression of the nuclear receptor and the inflammatory markers in these cells but also by PPARγ-independent effect of Rosiglitazone. As a conclusion, the modulation of PPARγ activity by pharmacological agonist or antagonist does not have an influence on cytokine expression on the early-stage radial growing melanoma cells WM35.

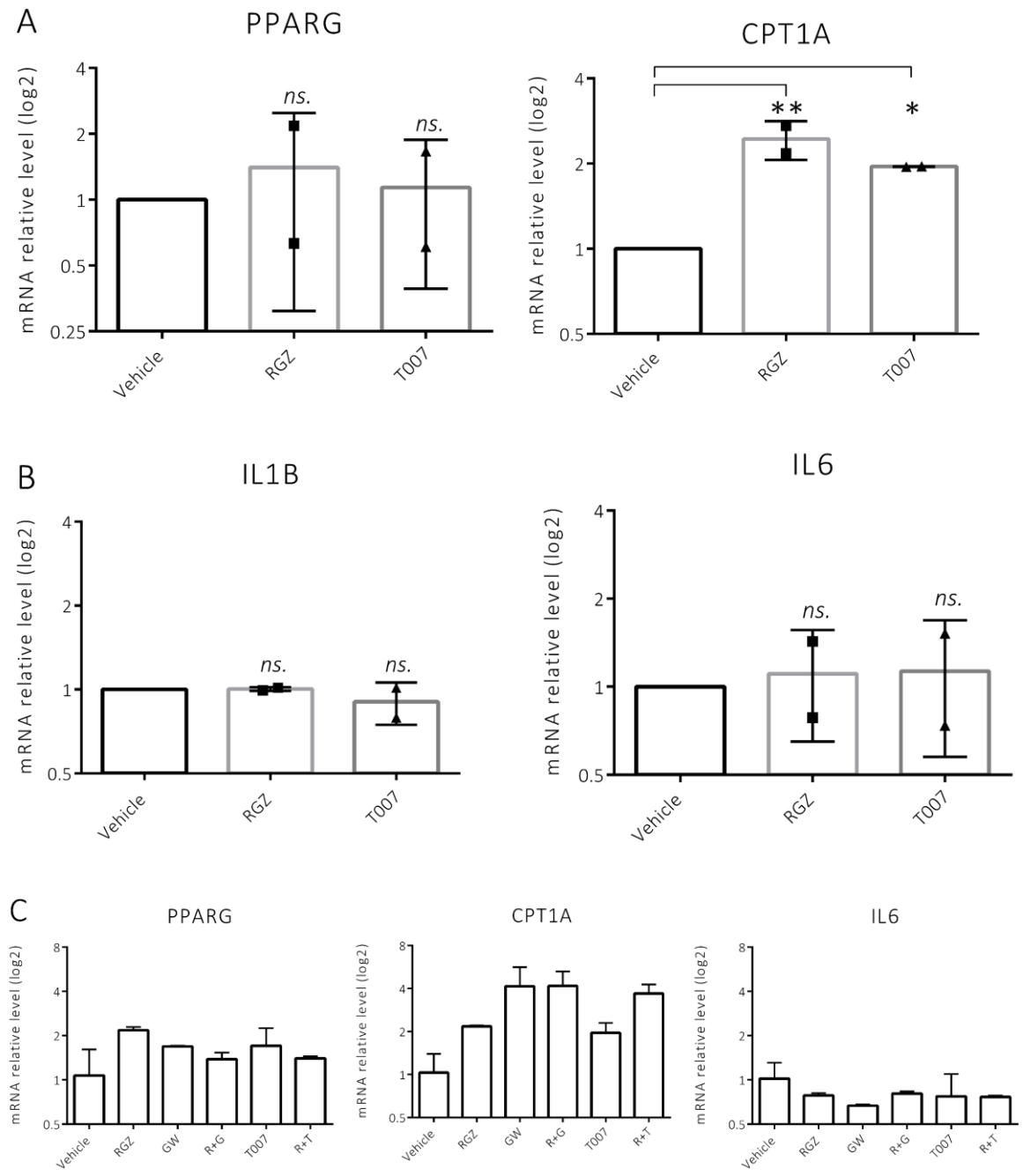

Figure 5: PPARγ agonist and antagonist have no impact on the expression of inflammation markers in WM35 early-stage melanoma cells. A: RT-PCR analysis of the expression of PPARγ (left) and its target gene CPT1A (right) in WM35 after treatment with vehicle (DMSO), the PPARγ agonist Rosiglitazone (RGZ; 10µM) or the PPARγ antagonist T0070907 (T; 2µM). **B:** RT-PCR analysis of the expression of IL1B (left) and IL6 (right) in WM35 after treatment with vehicle (DMSO), the PPARγ agonist Rosiglitazone (RGZ; 10µM) or the PPARγ antagonist T0070907 (T007; 2µM). **C:** RT-PCR analysis of the expression of PPARγ (left), CPT1A (middle) and IL6 (right) after treatment with vehicle (DMSO), PPARγ agonist Rosiglitazone (RGZ; 10µM), Rosiglitazone and antagonist GW9662 together (R+G; 10µM and 1µM), PPARγ antagonist T0070907 (T; 1µM), Rosiglitazone and antagonist T0070907 together (R+T; 10µM and 1µM) as a control of cell specific receptor response. The data are expressed as relative mRNA level using USP16 as House-Keeping-Gene (+/- SD.). Each point represents an independent experiment performed in triplicate. Statistical analysis: *: p-value: RGZ=0,00068; T0070907=0,0103 ns.: non-significant, one-way ANOVA test.

4.1.2 The agonist does not activate PPARγ in human malignant melanoma cells in vertical growing stage.

Another cell line was tested to observe the effect of PPARγ activation on more advanced melanoma cells: the WM115, which are on the vertical stage of tumour transformation. Two independent experiments were performed. After 24hours of treatment with the agonist Rosiglitazone 5µM and antagonist T0070907 2µM, the genes of interest were analysed by RT-qPCR (IL6, IL1B, ILA for the inflammation, CPT1A as PPARγ target gene).

First, PPARG was expressed at a higher level in WM115 cells compared to non-metastatic radial growth melanoma cells WM35. The level of expression of the nuclear receptor was not modulated in the presence of the agonist and the antagonist (Figure 6A). The target gene CPT1A expression was very low or not expressed in these cells, but was significantly up-regulated with Rosiglitazone. As for WM35, CPT1A is a target gene of PPARγ activation and it would be expected to decrease with the antagonist treatment. However, it is not the case, probably because of the very low expression which cannot be further decreased. The level of gene expression after treatment with the agonist and the antagonist (Rosiglitazone and T0070907, respectively) at the same time were also tested in a single experiment and showed that CPT1A was more highly expressed with the antagonist than with the agonist of PPARγ, probably due to a PPARG off-target effect (data not shown).

The inflammation genes IL1A, IL1B and IL6 were not significantly modulated after treatments with the agonist Rosiglitazone or the antagonist T0070907, but were expressed in WM115 cells (Figure 6B). The results show a variability of responses for IL6 with the antagonist,

Since it has been reported that high concentrations of PPAR ligands can have several PPARγ-independent effects, the cells were treated with 10 µM and 5 µM of Rosiglitazone for 24 hours to test the possibility of reducing the agonist concentration in future experiments. The expression of CPT1A was increased to a higher extent with 5 µM than with 10 µM of Rosiglitazone (Figure 6C), showing that the concentration may be reduced for the subsequent experiments. The expression of the inflammation markers IL1B and IL6 was not modulated after treatment with the two different concentrations of Rosiglitazone and with the antagonist T0070907.

These data show that PPARγ modulators did not influence the modification of the inflammatory cytokines in the vertical melanoma cell line WM115.

Altogether, these results suggest that the PPARγ agonist Rosiglitazone does not induce a modification of the expression of pro-inflammatory cytokines on early-stage of malignant melanoma development, in contrast to the observation of the same treatment on metastatic malignant melanoma cells previously shown by C. Pich in L. Michalik's Lab. It could result from the level of expression of PPARG on the different types of cell line or to the stage of transformation of the tumour (Annexe B). If the inflammation phenotype of melanoma cells induced by PPARγ agonist is specific to advanced malignant melanoma transformation, the idea that this mechanism could be an advantage for the tumour to survive may also be suggested. The level of expression of PPARG in the different types of cell may bring an interesting suggestion of the role of the receptor on

tumorigenesis. In fact, it has been shown in the lab that the A375 metastatic cell line used in this work express more PPARG than the normal human melanocyte or the WM35 and WM115. However, not all the metastatic melanoma cell lines express the same amount of PPARγ (data from the lab). Other cell lines, that can have a role on tumour progression, have been tested in the lab: activation of PPARγ with the agonist in human endothelial cells and in human fibroblasts did not have an effect on IL1A, IL1B and IL6 modification of expression (Annexe C).

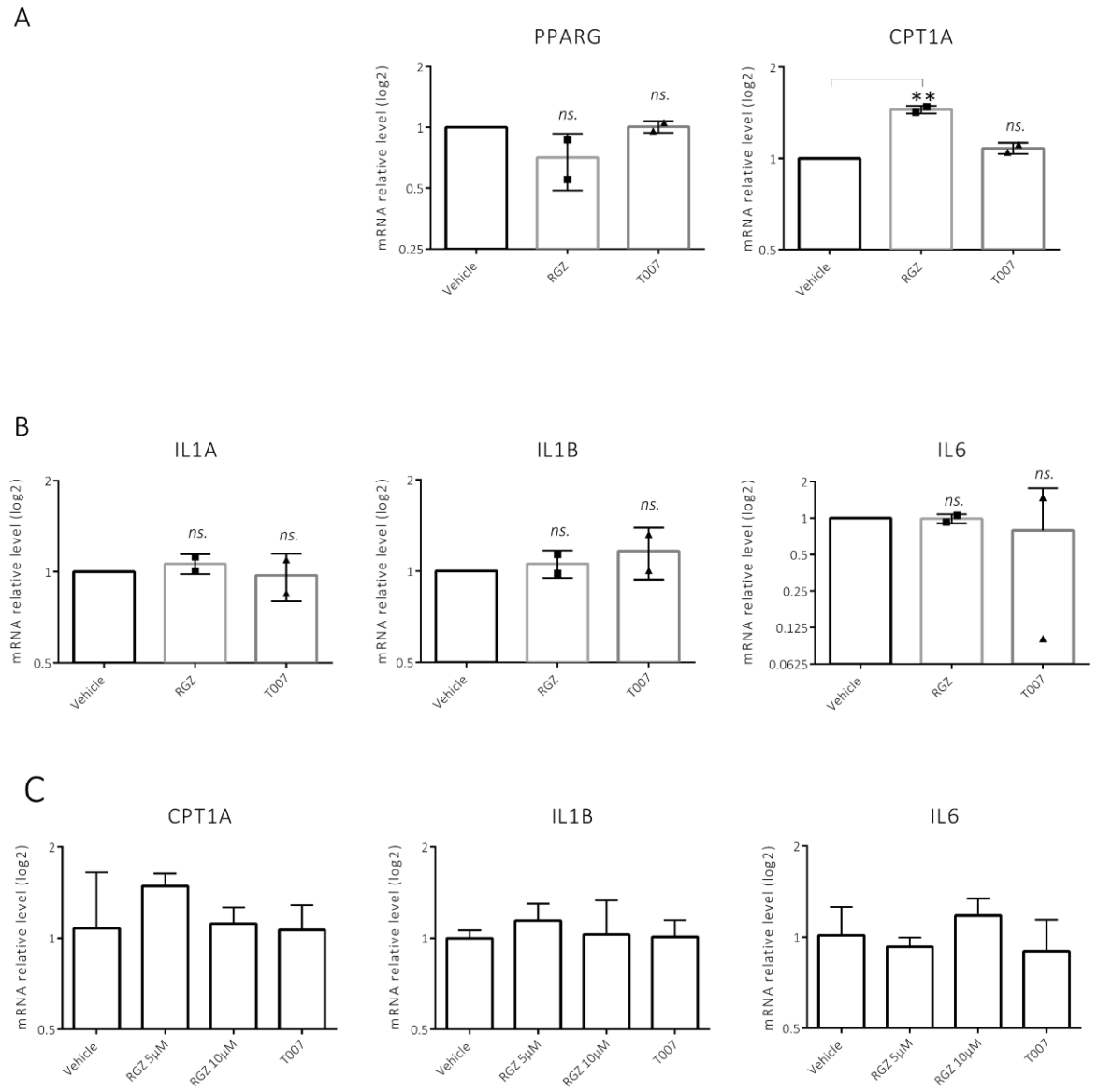

Figure 6: PPARγ activation in non-metastatic vertical growth malignant melanoma cells. A: RT-PCR analysis of the expression of PPARγ (left) and CPT1A (right) in WM115 after treatment with vehicle (DMSO), the PPARγ agonist Rosiglitazone (RGZ; 5µM) or the PPARγ antagonist T0070907 (T; 2µM). **B:** RT-PCR analysis of the expression of IL1A (left), IL1B (middle) and IL6 (right) in WM115 after treatment with vehicle (DMSO), the PPARγ agonist Rosiglitazone (RGZ; 5µM) or the PPARγ antagonist T0070907 (T; 2µM). **C:** One independent experiment RT-PCR analysis of the expression of CPT1A (left), IL1B (middle) and IL6 (right) after treatment with vehicle (DMSO), two different concentrations of PPARγ agonist Rosiglitazone (RGZ; 10µM and 5µM) and PPARγ antagonist T0070907 (T; 2µM). The data are expressed as relative mRNA levels using USP16 as House-Keeping-Gene (+/- SD.). Each point represents an independent experiment performed in triplicate. Statistical analysis: one-way Anova test, RGZ: p-value=0,0033; ns.: Non-significant.

4.2 Microenvironment: Malignant melanoma and its capacity of changing the cell secretome.

The activation of PPARγ induces a pro-inflammatory response in metastatic melanoma cells, as well as other melanoma and cancer cell lines respond to PPARγ activation culture (Annexe C). An important next step to study the role of PPARγ in cancer and inflammation will be to investigate the effect of PPARγ agonists and antagonists on the development of the tumour *in vivo*. Work is currently under way to explore the effect of PPARγ activation *in vivo* by injecting melanoma cells into Nude mice, and feeding them with PPARγ agonists or antagonists. However, the constitutive activity and the response of mouse cells to PPARγ agonists and antagonists is not yet known. Therefore, the effect of melanoma cell PPARγ activation on mouse fibroblasts, which is a major component of the tumour microenvironment in mouse, was analysed.

4.2.1 PPARγ ligands have no influence on inducing an inflammatory phenotype in mouse fibroblasts.

It was decided to use the GW9662 antagonist instead of T0070907 because of a better characterization in the literature about GW9662 as a PPARγ antagonist used *in vivo* with same effect on cell in culture (Figure 5C), and Rosiglitazone 5µM as agonist. The mouse fibroblasts NIH-3T3 were incubated with agonist and antagonist of PPARγ for 24 hours. Three independent experiments were done, each in triplicate. PPARγ was poorly/not expressed in mouse fibroblasts and the activation or inactivation of the nuclear receptor with the agonist and antagonist, respectively, had no significant effect on the expression of PPARγ itself (Figure 7A). Moreover, the target gene of PPARγ Angptl4 was expressed and varied independently from PPARγ activation or inhibition, which can be explained by the low expression of the receptor. The antagonist induced highly variable responses between the three experiments. The variations of gene expression seen in Figure 7A may be due to the compounds and not to the activity of the molecules binding the receptor (interpreted as background effect).

The expression of the inflammatory genes Il1b and Il6 was variable and these genes were poorly or not expressed (Figure 7B). Il1b was not detected in the third experiment, explaining why there is only the two first independent experiments shown in the graph. The response to a combination of the two molecules Rosiglitazone and T00709707 was not significantly modified. At this time, it is not possible to interpret these results due to the large errors bar. The results showed no effect of PPARγ agonist and antagonist on the expression of genes of interest Il1b and Il6 in mouse fibroblast. This is comparable to the results obtained in the lab in a similar experiment on human fibroblasts (NHDF and HDF, Annexe C). The expression of PPARγ is low on mouse fibroblasts and the agonist treatment with Rosiglitazone has the same effect on both cells: it does not activate the nuclear receptor and no inflammation cytokine expression was induced.

This latter point suggests the possibility to analyse the activation of PPARγ activation in melanoma cells, independently of fibroblast in the *in vivo* experiments on mouse model, mouse fibroblasts not being influenced by PPARγ agonist.

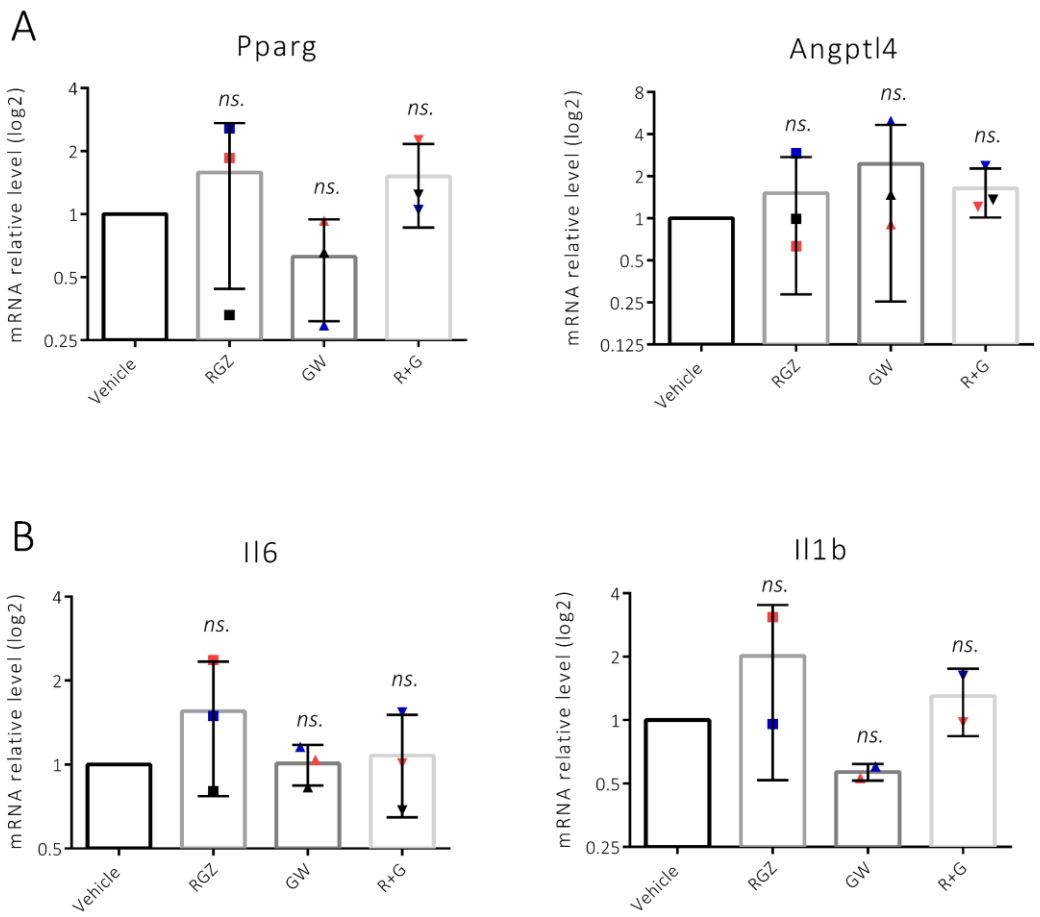

Figure 7: Response to agonist and antagonist of PPARγ in mouse fibroblasts NIH3T3. A: RT-PCR analysis of the expression of PPARg (left) and its target gene Angptl4 (right) in NIH-3T3 after treatment with vehicle (DMSO), the PPARγ agonist Rosiglitazone (RGZ; 5µM) or the PPARγ antagonist GW9662 (GW; 1µM). **B:** RT-PCR analysis of the expression of Il6 (left) and Il1b (right) in NIH-3T3 after treatment with vehicle (DMSO), the PPARγ agonist Rosiglitazone (RGZ; 5µM) or the PPARγ antagonist GW9662 (GW; 1µM). The data are expressed as relative mRNA levels using Rpl27 as House-Keeping-Gene (+/- SD.). Each point represents an independent experiment performed in triplicate: blue: first experiment; red: second experiment; black: third experiment. The data are normalized relative to the Housekeeping-gene Rpl27. RGZ: Rosiglitazone; GW: GW9662; R+G: Rosiglitazone combined with GW9662; ns.: non-significant. Statistical analysis: one-way Anova test (for PPARg, Angptl4), t-test (IL6, Il1b).

4.2.3 Mouse fibroblasts are sensitive to malignant melanoma secretome.

It had been described in the lab that Rosiglitazone induces the modification of gene expression in metastatic melanoma cells. Moreover, an RNA and a proteomic analysis done in the lab on the metastatic melanoma cells A375 confirmed that Rosiglitazone induces modifications on the melanoma cell gene expression, thereby having an impact on the secretion of proteins such as cytokines and chemokines, changing in culture the secretome of these tumorous cells in most advanced stages of melanoma transformation (Annexe D).

To test the effect of the melanoma cell secretome on mouse normal fibroblasts, the metastatic melanomas cells A375 tdT that are a human melanoma cell line, were treated with agonist and antagonist of PPARγ for

24 hours, then the medium was removed and a fresh medium was added for three days. This conditioned medium was filtered and incubated with murine fibroblasts (NIH3T3). The NIH3T3 were analysed by RT-qPCR 72 hours after.

The level of expression of PPARG and its activation with agonist and antagonist in A375 tdT cells were first tested. The results show a higher expression of PPARG in A375 tdT than in the two non-metastatic primary cell lines tested (WM35 and WM115, Figure 5 and 6). Then, A375 tdT were very sensitive to PPARγ agonist (Figure 8). After incubation with Rosiglitazone, the mRNA relative level of IL6 is significantly higher than in the vehicle control (DMSO). The treatments were efficient since the PPARγ target gene FABP4 was up-regulated when incubated with Rosiglitazone. Moreover, the activation of PPARγ is correlated with a significant increase of IL6 mRNA level. The three independent experiments show similar results, despite the variability between them. An interesting point that will not be explored in this work is that IL6 level of expression is increased when the cells are treated with agonist of PPARγ, but it seems that IL6 expression does not go beyond a certain level of expression, even if FABP4 is more expressed.

The treatment with the antagonist GW9662 did not generate significant modifications compared to the vehicle. No difference of IL6 and FABP4 level of expression were observed in the two first independent experiments (not performed in the third experiment).

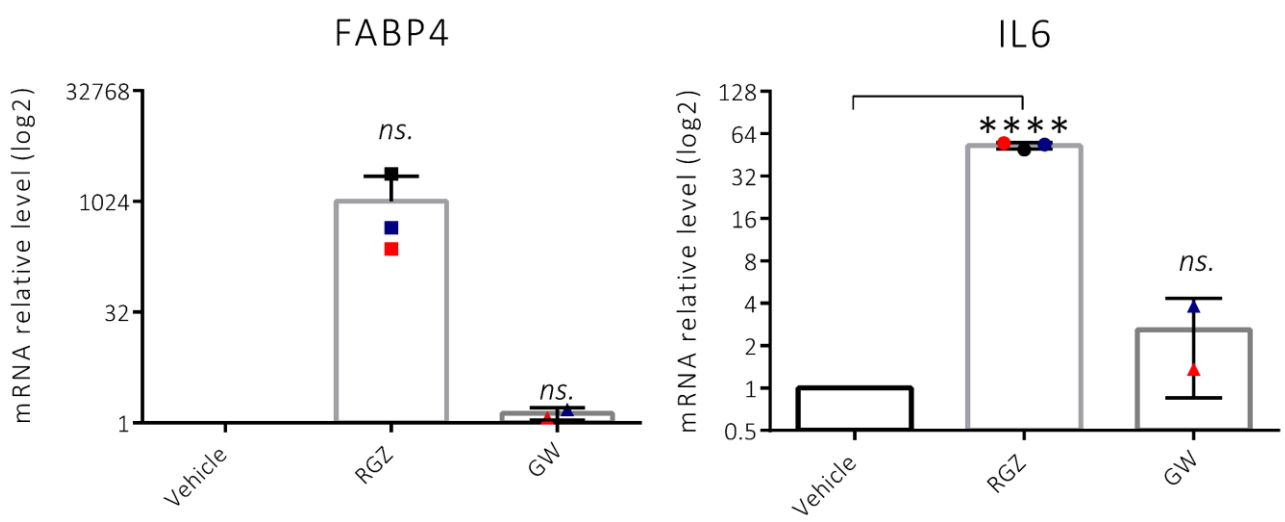

Figure 8: A375 tdT melanoma cells treated with agonist and antagonist of PPARγ: RT-PCR analysis of the expression of FABP4 (left) and IL6 (right) in A375 after treatment with vehicle (DMSO), the PPARγ agonist Rosiglitazone (RGZ; 5µM) or the PPARγ antagonist GW9662 (GW; 1µM). The data are expressed as relative mRNA levels using USP16 as House-Keeping-Gene (+/- SD.). Each point represents an independent experiment performed in triplicate: red: first experiment; blue: second experiment; black: third experiment. Two independent experiment were done for the antagonist. The data are expressed as relative mRNA level using USP16 as House-Keeping-Gene (+/- SD.). Statistical analysis: ****: p-value: <0,0001; ns.: non-significant, t-test

These results correlate with the data obtained in the lab, showing a high effect of Rosiglitazone on the expression of IL6 on the human metastatic melanoma cells A375. Melanoma is the result of an accumulation of DNA mutations in melanocytes but the genetic mutations are heterogenous between the malignant melanoma that are diagnosed in patients. The action of Rosiglitazone may be different depending on the

type of the tumour genetic. Other human metastatic melanoma cell lines were analysed in the lab (mutated for BRAF, p53 or CDKN2A) with the same results as those obtained in A375 and A375 tdT cells: the expression of inflammatory cytokines was increased. Previous work in the lab showed that the up-regulation of cytokine RNA expression Is always correlated with a higher secretion by the melanoma cells. We can assume that if the treatment increases cytokine RNA expression, it also increases its secretion.

After having the certitude that A375 tdT had a pro-inflammatory response to PPARγ ligands, the murine fibroblasts were exposed in culture to the filtered medium issued from metastatic melanoma A375 tdT cells treated with DMSO, Rosiglitazone as agonist or GW9662 as antagonist.

The NIH3T3 mouse fibroblasts grew well in culture, no morphologic difference was observed between cells grown in different conditions (Figure 9). Moreover, the mouse fibroblast shape was similar to the mouse fibroblasts directly treated with agonist and antagonist of PPARγ: a heterogenic spindle form and a branched cytoplasm, with smaller circular cells in the middle of the colonies. In comparison, malignant melanoma cells A375 were smaller and rounder at the first day of culture than fibroblasts and became also spindle formed after treatment with PPARγ agonist and antagonist.

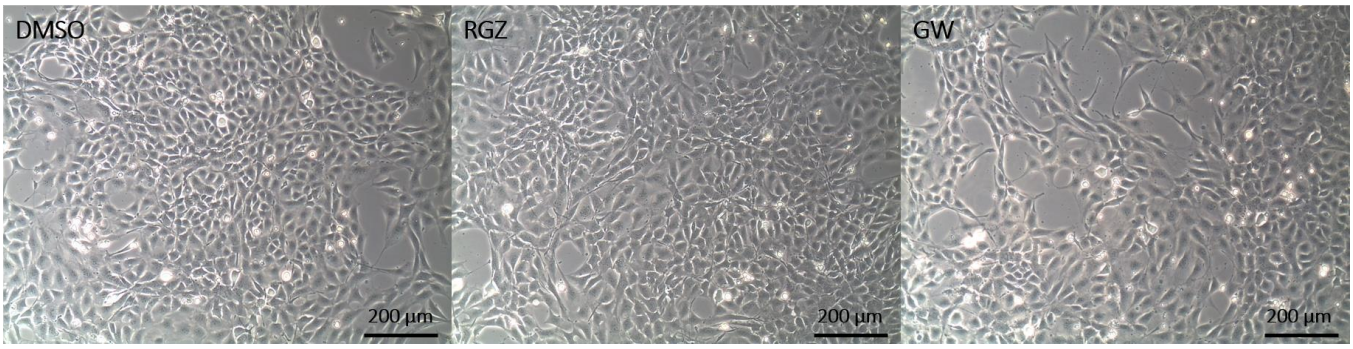

Figure 9: Picture of mouse fibroblasts in culture. The cells were incubated five days with medium from the melanoma cell A375 tdT, which were conditioned with DMSO, Rosiglitazone (RGZ, 5µM) and GW9662 (GW, 1µM) for three days. (Leica microsystem microscope).

In order to test if the secretome of melanoma cells modifies gene expression in mouse fibroblasts, the expression of Il1b, IL6 was analysed. First, Il1b was not expressed in NIH3T3 murine fibroblasts after incubation with the conditioned medium (for all the different conditions). Interestingly, IL6 was significantly up-regulated when the murine fibroblasts were incubated with conditioned medium issued from A375 tdT treated with Rosiglitazone (Figure 10).

According to these results, the secretome from A375 tdT cells may have the potency to change gene expression of IL6 in mouse NIH3T3 fibroblasts, inducing a pro-inflammatory state, an effect that was not induced by adding directly the PPARγ agonist on the murine fibroblasts. The influence of malignant melanoma secretome, which was incubated with normal fibroblasts, changed fibroblast gene expression, inducing the secretion of cytokines. This interesting result suggests that PPARγ has an effect on the microenvironment through activation of metastatic malignant melanoma cells (Figure 11).

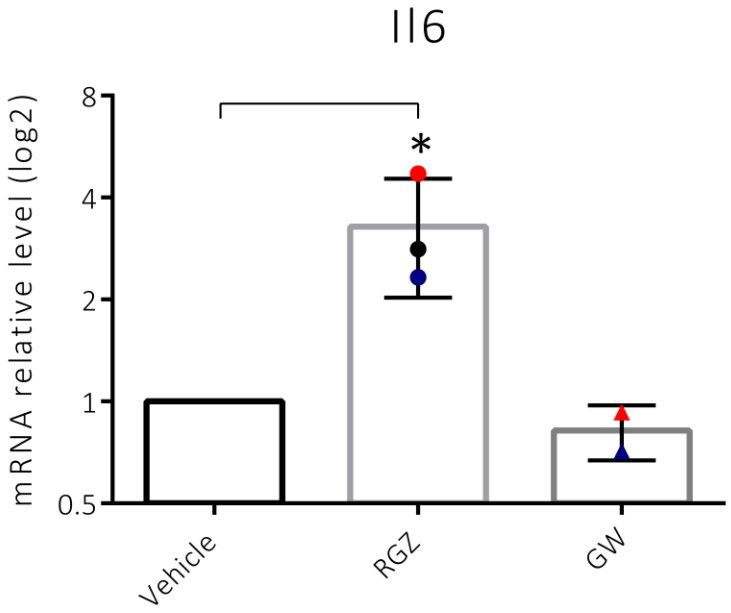

Figure 10: NIH3T3 respond to medium produced by melanoma by up-regulating IL6: RT-PCR analysis of the expression of IL6 after treatment with medium from A375 dtT conditioned with vehicle (DMSO), Rosiglitazone (RGZ; 5µM) and GW9662 (GW; 1µM). Each point represents an independent experiment performed in triplicate: red: first experiment; blue: second experiment; black: third experiment. The antagonist was not used for the third experiment. The data are expressed as relative mRNA levels using RPL27 as house-keeping-gene (+/- SD.). Statistical analysis: *: p-value: 0,0323, t-test between Vehicle and RGZ condition.

5. Conclusion and perspectives

The data obtained in this work show that PPARγ activation in malignant melanoma leads to a pro-inflammatory expression of genes. Interestingly, the findings suggest that this effect of PPARγ activation was not present in the WM35 and WM115 analysed after culture and incubation with Rosiglitazone (agonist). It may be the result of a lower expression of the receptor on these cells and/or an effect of progression of the tumour. Thus, the results on non-metastatic melanoma cells were similar to the ones obtained in normal human melanocytes , rather than those obtained in metastatic melanoma cells. Moreover, the data from this work shows that human normal melanocytes and non-metastatic melanoma WM35 express the same level of PPARG.

Rosiglitazone is a specific activator of PPARγ receptor, however the molecule is known to have lower affinity for other PPARs, inducing responses of these receptors acting on other pathways (55). The concentration of Rosiglitazone used in the experiment and the low expression of PPARγ can explain the diversity of the responses measured, suggesting that Rosiglitazone pro-inflammatory effect depend on PPARγ expression.

More investigations to differentiate the effect of Rosiglitazone on tissues with higher expression of PPARγ have to be done.

This study has also shown that human malignant metastatic melanoma cells are capable to change the microenvironment in response to a PPARγ agonist, inducing the expression of pro-inflammatory cytokines

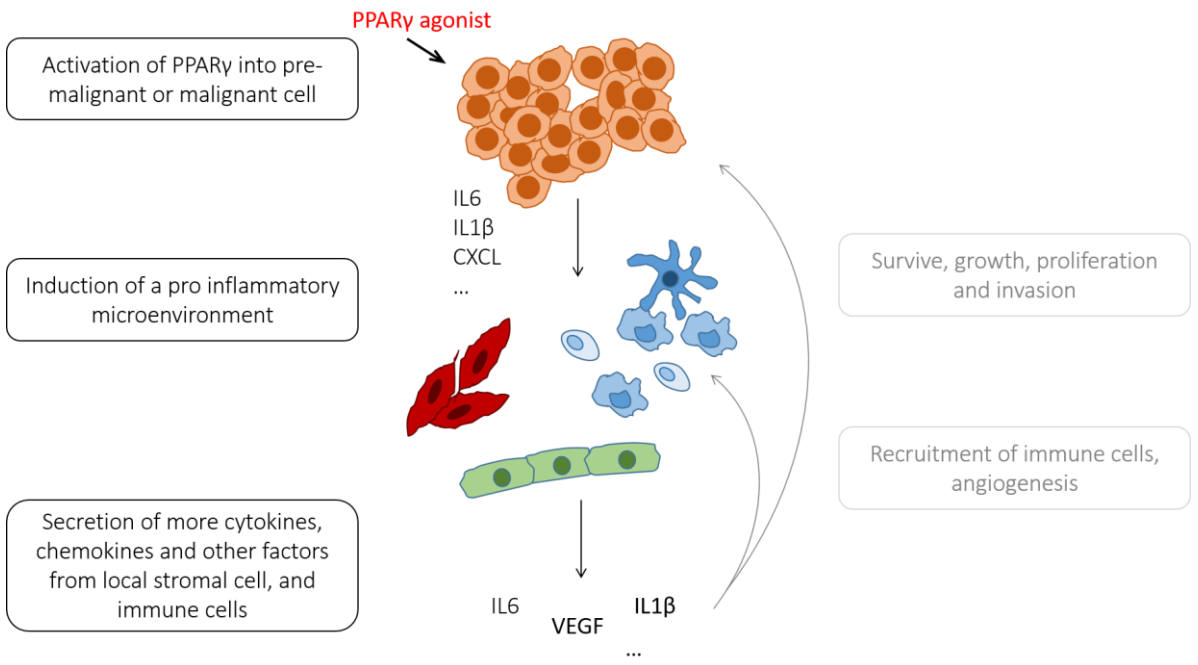

Figure 11: Hypothesis of the possible influence of the secretome secreted by malignant melanoma cell on the microenvironment. This was elaborated with the informations and the ideas brought from this work. Only the fibroblasts were incubated with the secretome from melanoma cells, the murine endothelial and immune cells have to be investigate. Murine endothlelial cells and Human endothelial cells: the effects of their activation are under investigation. These results are issued from cultivated cells, the effect of stromal cell secretome (right) was not analysed. Orange: melanoma cell ; red: fibroblast ; green: endothelial cell ; blue: immune cells (dentritic cell, macrophages and lymphocytes.)

and producing a microenvironment which can change mouse fibroblast IL6 expression. This effect proves that factors secreted by melanoma cells are capable to modify the activity of non-tumorous cells in melanoma microenvironment. IL6 and IL1β are not the only modification of expression induced with PPARγ activity, other chemokines and unknown proteins are involved and must be investigated with large proteomic analysis. To continue this experiment, other cells of the tumor microenvironment must be explored as murine endothelial cells (possibility of inducing angiogenesis) and immune cells (activation of pro-tumoral macrophage).

Does the inflammation have a beneficial effect by decreasing tumor malignancy or does it rather improve the capacity to survive, grow, proliferate and invade? Bladder cancer cells have shown the same response to PPARγ agonist, but not all the tumours tested increased pro-inflammatory cytokine expression after treatment with Rosiglitazone in culture (Annexe C): some specific cell types or tissues could be more sensitive to the activation of PPARγ.

These observations are issued from cell culture models; further investigation in mouse models *in vivo* (already on- going in the lab) may allow to see if the secretome of malignant melanoma cells conditioned

with PPARγ agonist have an effect on the modification of the whole microenvironment, which contains fibroblasts, endothelial cells and immune cells. What is the influence of fibroblasts on the development of the tumor? Which other cells respond to the metastatic melanoma cells secretome under PPARγ agonist? Which proteins are secreted in the microenvironment and what are the effects on the different systems that could help the tumor to develop and to be so aggressive? These are open questions that remain to be investigated.

6. Acknowledgments

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Key words: Melanoma, PPARgamma, Inflammation, microenvironment, culture, qPCR

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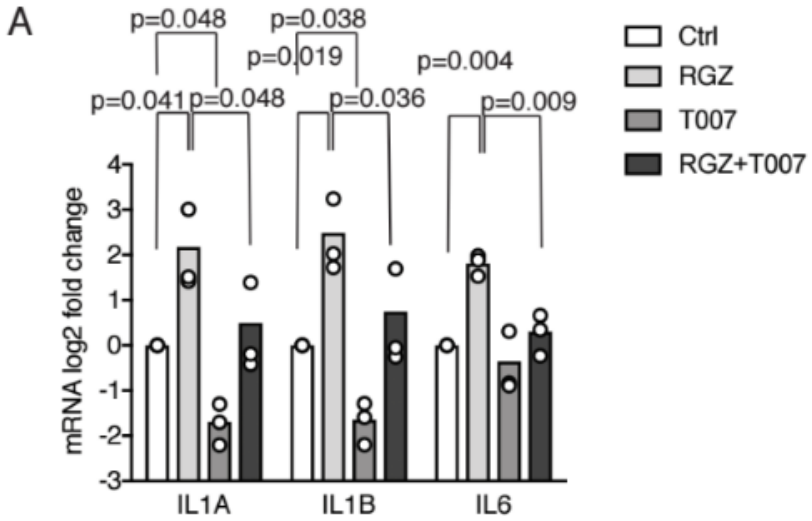
8 Annexes

Annexe A: Malignant metastatic melanoma cell A375 show a strong response to PPARγ agonist by inducing an increase of pro-inflammatory cytokines expression

RT-PCR analysis of the expression of IL1A, IL1B and IL6 in A375 after treatment with vehicle (Ctrl), the PPARγ agonist Rosiglitazone (RGZ; 10 µM), the PPARγ antagonist T0070907 (T007; 2 µM) and Rosiglitazone with T0070907 together (RGZ+T007; 10 µM and 2 µM).



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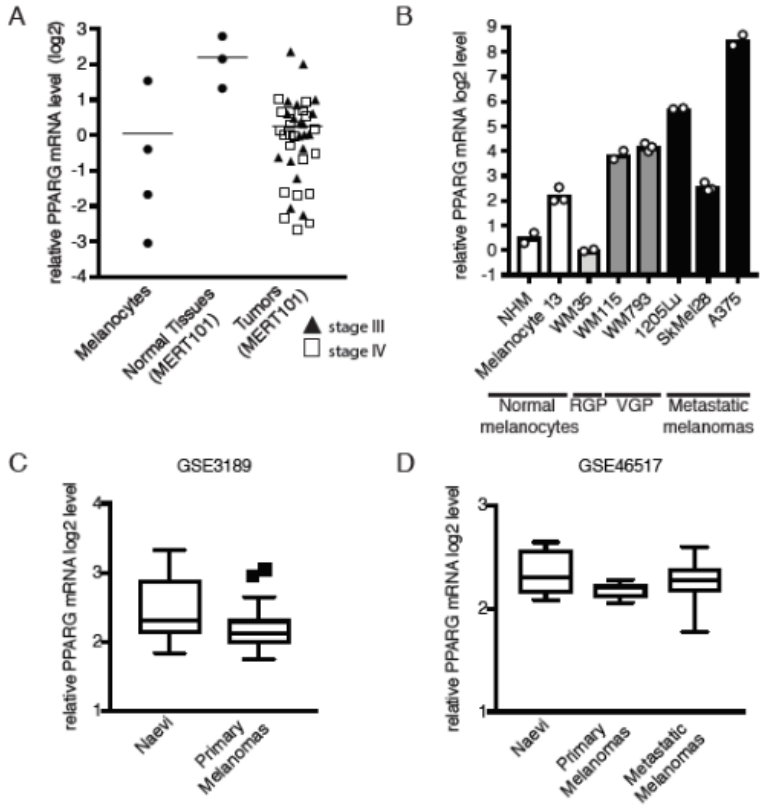
Annexe B: PPARG expression on different cell lines

A. Tissue Scan array analyse the PPARG RNA expression level between different type of cell line. black triangles and white squares represent respectively stage III and stage IV Melanomas **B.** PPARG expression on different cell lines issued from the progression of melanoma. **C, D**. Two microarray datasets were analysed for PPAR expression in patient's samples. Results are represented as box plot with Tukey whiskers. NHM: Normal human melanocyte; Radial-growth melanoma cells line; VGP: vertical-growth melanoma cells line



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Annexe C: Different types of cells that were tested for PPARγ-dependent increased inflammation. RGP: Radial-growth melanoma cells line; VGP: vertical-growth melanoma cells line; MET: Metastatic melanoma cell line; Mel: melanoma; SCC: squamous cell carcinoma; Mut: mutation; Del: deletion; HPV: Herpes papillomavirus; PTEN: phosphatase and tensin homologue; Hem del: heterozygote deletion; F: female; M: Man; +: expressed; -: not expressed;

Cell line	Type	Mutations		Sex / strain	PPARγ activation	IL-1α, β, 6 up
NHM	human normal melanocyte			pool	+ low	+ low
Melanocyte 2	human normal melanocyte					
Melanocyte 13	human normal melanocyte					
WM-35	RGP Mel	BRAF V600E		F	-	-
WM-115	VGP Mel	BRAF V600E	PTEN Hem Del	F	-	-
WM-793	VGP Mel	BRAF V600E		M	+	+
1205Lu	Xenograph MET from WM-793	BRAF V600E	PTEN Mut / Hem Del	M	+ low	+ low
A375	MET Mel	BRAF V600E	CDKN2A Mut	F	+ high	+ high
A375-M2	MET from A375	BRAF V600E		F	+	+
C8161	MET Mel	p53 Mut		F	+	+
B16	murine MET Mel	p16 Del	p19 Del	C57BL/6J	-	-
YUMM	murine MET Mel	BRAF V600E	PTEN Del	C57BL/6J	-	-
SCC13	primary SCC	p53 Mut		F	+ low	+ low
HeLa	cervical adenocarcinoma	HPV-18 virus	low p53	F	-	-
T24	bladder carcinoma grade3	H-Ras	p53 Mut	F	+	+
NHDF	human normal fibroblast			pool	+ (72h)	-
HDF	human normal fibroblast					
NIH-3T3	murine embryonic fibroblast			NIH/Swiss	-	-
HUVEC	human umbilical vein endothelial cell			pool	-	- (IL6)
	murine endothelial cell					

Annexe D: Proteomic analysis of A375 secretome after treatment with Rosiglitazone (C.Pich). FC: Fold change; bdl: below detection limit; n.d: not detected.

Gene	Protein	Major attributed function (Uniprot)	RNA FC	Protein secretion FC
ANGPTL4	Angiopoietin-related protein 4	Regulator of angiogenesis	73,04	46,02
AREG	Amphoregulin	Growth factor, EGFR ligand	20,02	16,1
FABP4	Fatty acid-binding protein 4	Lipid transport	1943,8	17,81
IL1A	Interleukine 1 alpha	Inflammatory response	4,83	Bdl
IL1B	Interleukine 1 beta	Inflammatory response	4,09	Bdl
IL6	Interleukine 6	Inflammatory response	3,02	Bdl
IL1-RL1	Interleukine-1 receptore-like 1	IL-33 receptor, immune response	4,21	16,63
CXCL1	C-X-C motif chemokine 1	Neutrophils attractant, inflammation	n.d	2,33
CXCL2	C-X-C motif chemokine 2 / GRO-2	Hematoregulatory chemokine, inflammation	2,46	Bdl
CXCL3	C-X-C motif chemokine 3 / GRO-3	Neutrophils attractant, inflammation	3,23	5,78
CXCL8	C-X-C motif chemokine 8 / Interleukine 8	Immune cells attractant, inflammation	n.d	4,1
CCL2	C-C motif chemokine 2 / MCP-1	Monocyte attractant, inflammation	-2,13	Bdl
CSF2	Granulocyte-macrophage colony-stimulating factor	Growth factor, hematopoietic lineage	18,18	15,25