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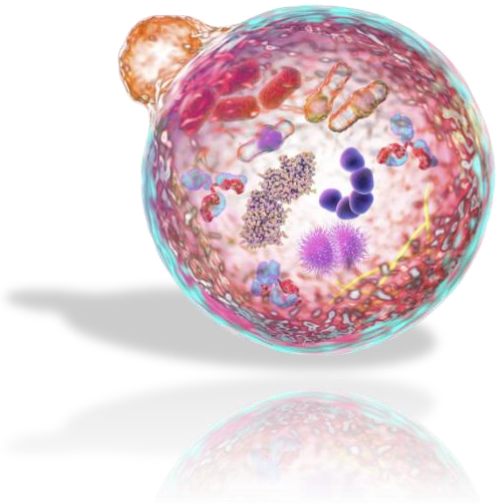
"ALEXANDER FLEMING"
Biomedical Sciences Research Center

Investigation of the role of Cancer Associated Fibroblasts Autophagy in shaping the anti-tumor immune response and tumor development

By Legaki Aigli-Ioanna, BSc



A Thesis for the Degree of International Master of Science

In Molecular Biomedicine

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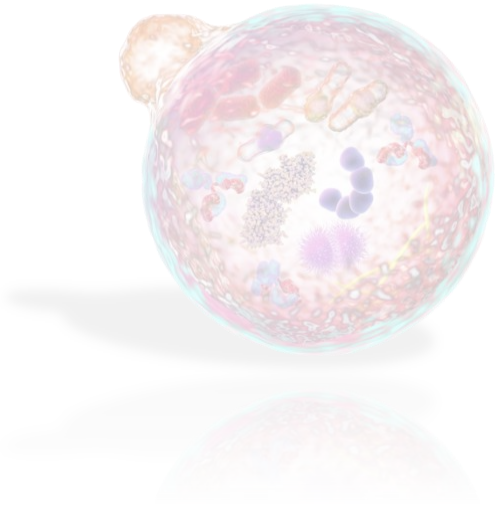

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Author,

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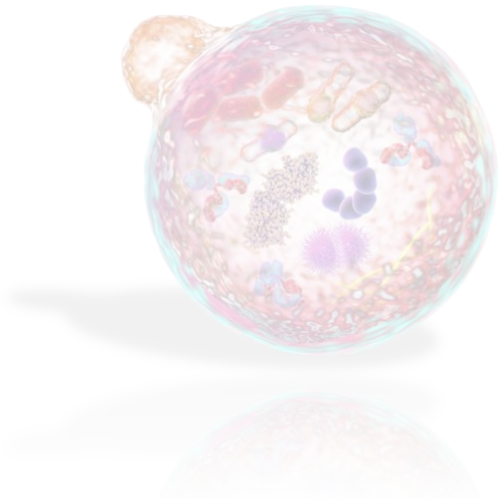

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Abstract

The advent of immune checkpoint inhibitors (ICI) has revolutionized cancer therapeutics, yet a sizable portion of patients is associated with low response rates. A major impediment in the effectiveness of ICI immunotherapy is the hypoxic conditions of the tumor microenvironment (TME). A mechanism that is induced by hypoxia and represents a cardinal feature of most tumors is autophagy. Even though most studies have focused on the role of autophagy in tumor cells, the involvement of autophagy in the stromal compartment, and in particular in cancer-associated fibroblasts (CAFs), has been investigated to a limited extend. CAFs represent a highly heterogeneous yet abundant cell population of the TME, quite resistant to chemo/radiotherapy. Although their tumor-promoting function is acknowledged, their role in shaping the anti-tumor immune response remains elusive.

Herein, we demonstrated that α-SMA^{+}CAFs were enriched in the TME of B16-F10 inoculated C57BL/6 mouse melanoma, while a α-SMA^{+} population was detected in circulation in later tumor stages. After validating that the phosphorylated levels of AKT, mTOR and S6 were downregulated upon tumor progression *in vivo*, we observed that tumor explants supernatant (TES)-treated fibroblastic cells exhibited deregulated autophagy compared to untreated cells *in vitro*. The phosphorylated levels of PI3K/AKT/mTOR pathway were also decreased upon administration of immune checkpoint inhibitor, anti-PD-L1 *in vivo*. To assess the functional role of autophagy in CAFs, we generated for the first time the transgenic mice α-SMA-Cre Atg5$^{fl/fl}$; the ablation of autophagy from CAFs led to earlier tumor development, with altered frequencies of tumor infiltrating immune cells (*P=0.064*), MDSCs (*P=0.044*) and α-SMA^{+} CAFs (*P=0.059*) in the TME.

Collectively, our data bring into focus CAFs as an important cell population, characterized by enhanced autophagy, yet deregulated. Also, upon anti-PD-L1 administration, autophagy is enhanced in CAFs as tumor progresses. Elucidation of how these processes unfold will provide a better mechanistic insight of how CAFs influence tumor immune response and ultimately lead to development of more efficacious immunotherapeutic approaches.

Περίληψη

Οι εξελίξεις της ανοσοθεραπείας έχουν φέρει επαναστατική πρόοδο στη θεραπευτική αγωγή του καρκίνου, όμως ένα σημαντικό ποσοστό ασθενών συνδέεται με χαμηλά ποσοστά ανταπόκρισης. Ένα σοβαρό εμπόδιο στην αποτελεσματικότητα της ανοσοθεραπείας είναι οι συνθήκες υποξίας του μικροπεριβάλλοντος του όγκου. Ένας μηχανισμός που προκαλείται από την υποξία και αντιπροσωπεύει ένα βασικό χαρακτηριστικό των περισσότερων όγκων είναι η αυτοφαγία. Παρόλο που οι περισσότερες μελέτες έχουν επικεντρωθεί στον ρόλο της αυτοφαγίας σε κύτταρα όγκου, η εμπλοκή της αυτοφαγίας στο στρωματικό διαμέρισμα και ειδικότερα σε ινοβλάστες που σχετίζονται με τον καρκίνο (CAF) έχει ερευνηθεί σε περιορισμένο βαθμό. Τα CAF αντιπροσωπεύουν έναν εξαιρετικά ετερογενή αλλά άφθονο κυτταρικό πληθυσμό του μικροπεριβάλλοντος του όγκου, ο οποίος είναι αρκετά ανθεκτικός στην χημειοθεραπεία / ακτινοθεραπεία. Αν και αναγνωρίζεται η λειτουργία τους να προάγουν τον όγκο, ο ρόλος τους στη διαμόρφωση της αντικαρκινικής ανοσοαπόκρισης παραμένει ασαφής.

Στην παρούσα εργασία δείξαμε ότι το μικροπεριβάλλον του B16-F10 εμβολιασμένου μελανώματος ποντικού C57BL/6 είναι εμπλουτισμένο με α-SMA^{+} CAFs, ενώ ένας πληθυσμός α-SMA^{+} CAFs ανιχνεύθηκε στην κυκλοφορία σε μεταγενέστερα στάδια όγκου. Μετά την διαπίστωση ότι τα φωσφορυλιωμένα επίπεδα των AKT, mTOR και S6 *in vivo* μειώθηκαν κατά την εξέλιξη του όγκου, παρατηρήσαμε ότι κύτταρα ινοβλαστών που υποβλήθηκαν σε αγωγή με υπερκείμενο έκφυσης όγκου (TES) εμφάνισαν απορυθμισμένη αυτοφαγία σε σύγκριση με κύτταρα που δεν υπέστησαν αγωγή *in vitro*. Τα φωσφορυλιωμένα επίπεδα του μονοπατιού PI3K/AKT/mTOR επίσης μειώθηκαν κατά τη χορήγηση αναστολέα ανοσοποιητικού σημείου ελέγχου, anti-PD-L1, *in vivo*. Για να αξιολογηθεί ο λειτουργικός ρόλος της αυτοφαγίας στα CAFs, δημιουργήσαμε τον πρώτο διαγονιδιακό ποντικό α-SMA-Cre Atg5$^{fl/fl}$; η αφαίρεση της αυτοφαγίας από τα CAFs οδήγησε σε γρήγορη ανάπτυξη όγκου, με μεταβαλλόμενες συχνότητες των κυττάρων ανοσοποίησης που έχουν διεισδύσει στο μικροπεριβάλλον του όγκου (*P = 0,064*), MDSCs (*P = 0,044*) και α-SMA^{+} CAFs (*P = 0,059*).

Συγκεντρικά, τα δεδομένα μας φέρνουν στο προσκήνιο τα CAFs ως ένα σημαντικό κυτταρικό πληθυσμό, που χαρακτηρίζεται από αυξημένη αυτοφαγία, αλλά απορυθμισμένη. Επίσης, κατά την χορήγηση anti-PD-L1, η αυτοφαγία ενισχύεται στα CAFs καθώς ο όγκος εξελίσσεται. Η διαλεύκανση του τρόπου με τον οποίο αυτές οι διαδικασίες διαδραματίζονται θα παρέχει μια καλύτερη μηχανιστική εικόνα της μεθόδου με την οποία τα CAFs επηρεάζουν την ανοσοαπόκριση του όγκου, καταλήγοντας με αυτόν τον τρόπο στην ανάπτυξη πιο αποτελεσματικών ανοσοθεραπευτικών προσεγγίσεων.

Introduction

Cancer is the second leading cause of death globally and is responsible for an estimated 9.6 million deaths in 2018. Even though therapeutic advancements in immunotherapy have led to long-lasting responses for some patients, a sizable portion of patients do not respond to these agents (Borcoman et al., 2019; Hanahan et al., 2011). Despite the operation of immune surveillance mechanisms, tumors form endowed immunosuppressive networks that impede the elicitation of potent anti-tumor immune responses and impair the success of immunotherapy (Chen et al., 2014; Costa et al., 2018; Ziani et al., 2018). As a result, a better understanding of the biology of cancer is necessary for improving these dismal outcomes.

In the past decade, the tumor microenvironment's (TME) role in promoting cancer progression and resistance to therapy has gathered great attention (Hanahan et al., 2011).Tumor progression and metastasis formation do not only depend on cancer cell genetic and epigenetic defects, but are also controlled by the TME or stroma (Chen et al., 2014; Costa et al., 2018; Ziani et al., 2018). The stroma is composed of cells from endothelial, mesenchymal, and hematopoietic origins embedded in a complex extracellular matrix, which enter a dynamic crosstalk with tumor cells, suitable for tumor growth (Chen et al., 2014; Costa et al., 2018; Ziani et al., 2018). Different elements such as angiogenesis, hypoxia, ECM remodeling, metabolism changes have recently received attention as key determinants of the TME, altering cancer cell behavior and disease progression, with potential clinical applications (Ziani et al., 2018).

Among cells present in the tumor microenvironment, fibroblasts are found in various proportions across the spectrum of carcinomas, constituting in many cases the predominant cell population of the tumor stroma (Kalluri, 2016; Kalluri et al., 2006; LeBleu et al., 2018). Activated fibroblasts, also known as cancer-associated fibroblasts (CAFs), although not by themselves malignant, play a critical role in the complex process of tumor cell-stroma interaction and have emerged as important regulators of the anti-tumor immune response on various levels, including immunosuppressive properties (Augsten et al., 2014; Capparelli et al., 2012; Kalluri, 2016; Kalluri et al., 2006; Kumar et al., 2017; LeBleu et al., 2018; Ziani et al., 2018). CAFs, one of the prominent stromal cell populations within a solid tumor mass in most types of human carcinomas (Augsten, 2014; Kalluri, 2016; LeBleu et al., 2018), have been involved in tumor growth, angiogenesis, cancer stemness, extracellular matrix remodeling, tissue invasion, metastasis, and even chemo-resistance (Castells et al., 2012; Hammer et al., 2017; Tommelein et al., 2015; Trimboli et al., 2009; Sun, 2015; Ziani et al., 2018).

Research has lately focused on the evaluation of drugs, which deplete CAFs (Duluk et al., 2015). However, subsequent clinical trials demonstrated that reducing fibrosis in addition to a first-line therapy was not beneficial for patients (Guet al., 2018). This suggests that reduction of CAFs is not a good option for cancer therapy. A better option may be to modify specific aspects of interactions between CAFs and carcinoma cells (Zhang et al., 2018).

A mechanism that is involved in the interaction between tumor and CAFs and has a crucial role in the tumor microenvironment is autophagy, which has recently been shown to be important for multiple aspects of cancer biology (Kimmelman, 2011; Santana-codina et al., 2017; Zhang et al., 2018). The role of autophagy in cancer is complex, as demonstrated by studies, describing situations in which autophagy can either promote or inhibit tumorigenesis (Kimmelman 2011; Levy et al., 2017; Santana-codina et al., 2017;Wang et al., 2016; Wang et al., 2017).

As a survival mechanism, autophagy plays a crucial role in the TME and although most studies have focused on the role of autophagy in tumor cells, the involvement of autophagy in the stromal compartment, and in particular in CAFs, has been investigated to a limited extend (Capparelli et al., 2012; Chaudhri et al., 2013; Levy et al., 2017; Liang et al., 2018). Autophagy is a fundamental lysosomal catabolic pathway, whereby cytoplasmic organelles and macromolecules are enveloped in autophagosomes and degraded by fusion with lysosomes for energy recycling (Wang et al., 2016; Wang et al., 2017). Autophagy maintains cell homeostasis (Alissafi et al, 2017; Alissafi et al., 2018; New et al., 2017) and controls the differentiation of fibroblasts into CAFs(Capparelli et al., 2012). Therefore, it is considered a central piece of the immune response in the tumor microenvironment.

Recent evidence proved that CAFs promote tumor growth through autophagy, which is responsible for providing nutrients to carcinoma cells (Sousa et al., 2016). New et al. assessed the role of autophagy in mouse xenograft models of Head and Neck Cell Squamous Carcinoma (HNSSC) and by inhibiting autophagy in the entire animal observed a significant reduction in tumor volume (New et al., 2017). Katheder et al. studied the role of autophagy in a *Drosophila melanogaster* malignant tumor model and showed that ablation of autophagy in the tumor had only a modest effect on tumor growth, but inhibition of autophagy in the non-tumor epithelia surrounding the tumor, had a marked effect on both tumor growth and invasiveness (Katheder et al., 2017). Moreover, Zhao et al. 2017 found increased CAFs autophagy in human luminal breast cancer tissues, which was correlated with poor prognosis. They also knocked down *ATG5* in CAFs and transplanted both CAFs and MCF-7, a breast cancer cell line, in NOD-SCID mice to study the effect of CAFs autophagy on breast cancer progression. Their research showed that CAFs promoted the tumorigenicity of MCF-7 cells in

mice and *ATG5* depletion in CAFs significantly decreased tumor growth, demonstrating that CAFs autophagy increase the tumorigenicity of luminal breast cancer cells (Zhao et al., 2017). However, it remains elusive what impact has the specific ablation of autophagy in CAFs on tumor immune profile.

The aim of this study is to evaluate how CAFs autophagy shapes the tumor growth, anti-tumor immune response and response to immunotherapy.

Cancer cells co-evolve with their tumor microenvironment and the role of autophagy in modulating how the cancer cell interacts with other cell types in the surrounding milieu is emerging as a key topic in determining whether autophagy inhibition is likely to be effective in cancer treatment (Mowers et al., 2018). The mechanisms underlying the effects of CAFs on cancer progression, along with the mechanisms behind the interaction between autophagy and the components of the tumor environment remain unclear (Ngabire et al. 2017). By considering the role of autophagy in CAFs, we explore how cancer therapy can be maximized by autophagy manipulation (Levy et al., 2017). Thus, elucidation of these processes is likely to lead to a better mechanistic understanding of how CAFs autophagy influences tumor immune response and tumor development. Furthermore, many studies have shown that the microenvironment is capable of normalizing tumor cells, suggesting that re-education of stromal cells, rather than targeted ablation per se, may be an effective strategy for treating cancer (Quail et al., 2013).

1.1. Cancer Immunotherapy

Cancer immunotherapy is an artificial stimulation of the immune system to treat cancer, improving its natural ability to fight cancer; it exploits the machinery of the immune system that halted the immune system to restore or induce the capacity of effector cells to indentify, target and destroy tumor cells (Chen et al., 2012; Topalian et al., 2011).

Monoclonal antibodies targeting immune checkpoints are able to reverse immune escape or evasion and promote tumor cell death. Such antibodies include those targeting the cytotoxic T lymphocyte antigen 4 (CTLA-4)–CD28 and programmed cell death 1 (PD-1)–programmed cell death 1 ligand 1 (PD- L1) axes (**Figure 1.1.1.**).

PD-1 is a cell surface receptor, a member of the immunoglobulin super-family, which recognizes and binds to the endogenous ligands PD- L1 and PD- L2. It is expressed on T cells and B cells, but also on cells involved in innate immunity, like natural killer cells and myeloid cells (Martins et al, 2019).

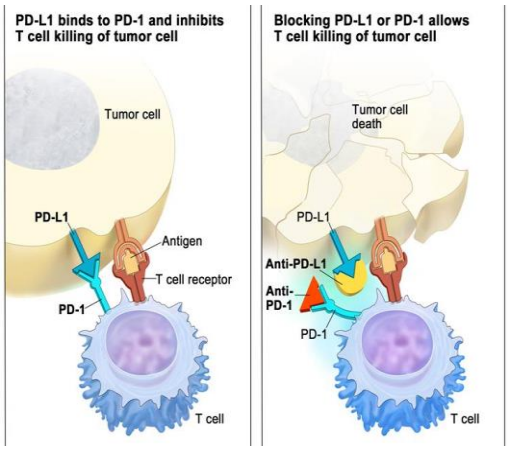


Figure 1.1.1.: Monoclonal antibodies that target either PD-1 or PD-L1 can block this binding and boost the immune response against cancer cells (National Cancer Institute).

In the absence of a malignancy, activation of either or both of these receptors has an inhibitory effect on the T cell response, thus inducing immune tolerance and preventing autoimmunity. However, both immune checkpoints can also be hijacked by tumor cells in order to develop a microenvironment that is tolerant of tumor growth (Martins et al., 2019).

The advent of checkpoint blockade immunotherapy has revolutionized cancer therapeutics; yet such regimens are associated with low response rates in a sizable portion of cancer patients (**Figure 1.1.2.**) (Borcoman et al., 2019; Hanahan et al., 2011). Despite the operation of immune surveillance mechanisms, tumors form endowed immunosuppressive networks that impede the elicitation of potent antitumor immune responses and impair the success of

immunotherapy (Chen et al., 2014; Costa et al., 2018; Ziani et al., 2018). As a result, a better understanding of the biology of cancer is necessary for improving these dismal outcomes.

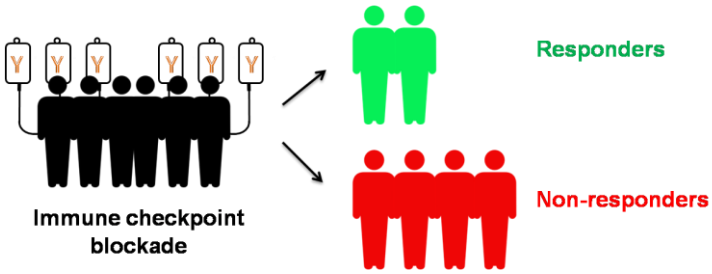


Figure **1.1.2.**: Even though immunotherapy has led to long-lasting responses for some patients, a sizable portion of patients do not respond to these agents.

1.1.1. Cancer Immunotherapy and Melanoma

Malignant melanoma, a fatal form of skin cancer with high rates of genomic mutations, has shown an effective response to immunotherapy options compared to other conventional treatments, like chemotherapy. Melanoma cancer cells represent upregulated expression of PD-L1 compared to inflammatory signaling activators, such as IFN-γ, which, in turn, upregulate PD-1 on T cells and mediate inhibition of antitumor action by cells of the immune system (Atefi et al., 2014; Dermani et al., 2018). As a result, checkpoint protein inhibition directed against the programmed death-1 axis (e.g., nivolumab, pembrolizumab) on tumor cells has emerged as an effective therapeutic option for melanoma, (Dermani et al., 2018; Rizvi et al., 2015; Robert et al., 2015; Weber et al., 2015; Wells et al., 2018), resulting in dramatic improvements in the prognosis of patients (Martins et al., 2019).

1.1.2. Cancer Immunotherapy and Cancer-associated fibroblasts (CAFs)

Although immunotherapy for solid tumors has shown promise in preclinical as well as early clinical studies, its efficacy remains limited. The hindrance to achieving objective, long-lasting therapeutic responses in solid tumors is in part mediated by the dynamic nature of the tumor and its complex microenvironment. Cancer-associated fibroblast (CAFs) form the most preponderant cell type in the solid tumor microenvironment and due to their pervasive role in facilitating tumor growth and metastatic dissemination, they have emerged as attractive therapeutic targets in the tumor microenvironment (TME) (Kakarla et al., 2012).

CAFs also contribute to the immunosuppressive tumor microenvironment and thus are excellent therapeutic targets to improve current immunotherapy approaches for cancer (Kakarla et al., 2012).

1.2. Cancer-associated fibroblasts (CAFs)

Cancer-associated fibroblasts (CAFs) are considered major players in tumor fibrotic microenvironment, which represent one of the most heterogeneous, yet abundant stromal cell types of several carcinomas (Prajapati et al., 2016; Ziani et al., 2018). They exhibit a classic spindle-shaped morphology, with a not well-defined origin; they are non epithelial (EpCAM), non endothelial ($CD31^-$) and non-immune ($CD45^-$) cells of a mesenchymal lineage origin (Kalluri 2016; Ziani et al., 2018), generated by differentiation of resident fibroblasts, or from progenitors, such as mesenchymal stem cells, smooth muscle cells, cells of epithelial origin, endothelial cells, perivascular cells, and adipose tissue-derived stem cells (**Figure 1.2.1.**). These various sources represent an important determinant that contributes to the heterogeneity of CAFs (Kalluri 2016; Tommelein et al., 2015; Ziani et al., 2018).

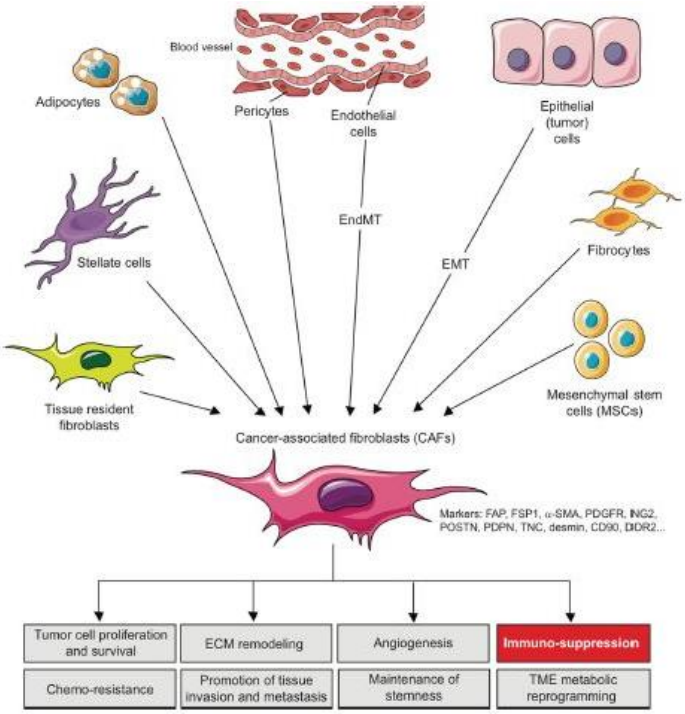


Figure 1.2.1.: The origins of cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME) and their role in cancer progression (Ziani et al., 2018).

In normal tissue, fibroblasts are usually considered quiescent cells, but with the ability to respond to growth factors to become activated. During this activation process, fibroblasts acquire proliferation and migration properties and become transcriptionally active, leading to

the secretion of several factors (cytokines, chemokines) and ECM components. The ability of resting fibroblasts to become activated is observed in the context of wound healing, as well as in pathologic conditions, such as acute or chronic inflammation or tissue fibrosis (Ziani et al., 2018).

In their activated state, they are characterized by the expression of α-smooth muscle actin (α-SMA), a cytoskeletal protein associated with smooth muscle cells (Kalluri 2016). In addition to α-SMA, CAFs express a variety of proteins that have an independent association with survival (Yan et al., 2019). This includes fibroblast-specific protein 1 (FSP1), platelet-derived growth factor (PDGF) receptor-α (PDGFRα), PDGFRβ, tenascin C, fibroblast activation protein (FAP), fibronectin, and podoplanin (Kalluri 2016; Sun et al., 2018). However, the expression levels of these proteins vary in different CAF populations, and none of them are unique to activated fibroblasts (Sun et al., 2018). Moreover, many activated fibroblasts may not express all of these markers at the same time, reflecting the high degree of heterogeneity of CAFs in the TME (Ziani et al., 2018).

Activated fibroblasts, although not by themselves malignant, play a critical role in the complex process of tumor cell-stroma interaction and have emerged as important regulators of the anti-tumor immune response (Austen et al., 2014; Capparelli et al., 2012; Kalluri 2016; Kalluri et al., 2006; Kumar et al., 2017;, Liang et al., 2018). Several evidence demonstrate that CAFs contribute actively to tumor growth, tumor invasiveness and tumor resistance to chemo/radiotherapy (Ngabire et al., 2017; Straussman et al., 2012; Paulsson et al., 2017), while they reduce anti-tumor immunity (Denton et al., 2014; Ruhland et al., 2016; Yang et al., 2016). Furthermore, studies in immune competent mice showed that FAP-positive CAFs drive immunosuppression and resistance to anti-PD-L1 immunotherapy (Costa et al., 2018). They are also involved in the evolution of a tumor by the production of cytokines. Cytokines play a major role in the development of chronic inflammation and in the anti-tumor response, but they also participate in all phases of cancer development through inflammation, which can also induce autophagy (Ngabire et al., 2017).

CAFs also possess antigen presenting functions, being potent stimulators of antigen-specific $CD4^+$ and $CD8^+$ T cells (Balmelli et al., 2008; Chesney et al., 1997; Costa et al., 2018; Zhang et al., 2013; Ziani et al., 2018). Lakins et al. have demonstrated a new biological function for CAFs in the tumor, showing that CAFs directly contribute to the suppression of anti-tumor T-cell responses by adopting similar characteristics of antigen presenting cells. This CAF-mediated mechanism reveals new insight into the cell biology, helping to explain why CAFs are associated with poor patient prognosis, and illustrating a novel mechanism of T cell depletion and dysfunction within tumors (Lakins et al., 2018).

Research has lately focused on the evaluation of drugs, which deplete CAFs (Duluk et al., 2015). However, therapeutic means for eliminating CAF-driven pro-tumor effects remain elusive (Chauhan et al., 2019). Subsequent clinical trials demonstrated that reducing fibrosis in addition to a first-line therapy was not beneficial for patients (Guet al., 2018), possibly due to the fact that all types of CAFs are eliminated, such as immunosupportive CAFs, CAF precursors and related cells like quiescent fibroblasts, stellate cells, and pericytes that have critical roles in tissue homeostasis (Chauhan et al., 2019). This suggests that reduction of CAFs is not a good option for cancer therapy. A better option may be to modify specific aspects of interactions between CAFs and carcinoma cells (Zhang et al., 2018).

1.3. Autophagy

Under nutrient starvation, cells initiate a lysosomal-dependent self-digestive catabolic pathway known as autophagy, whereby cytoplasmic contents, such as damaged proteins and organelles are captured and degrades to generate nutrients and energy, in order to maintain essential cellular activities (Alissafi et al., 2018; Alissafi et al., 2017; Amaravadi et al., 2016; Kim et al., 2011; New et al., 2017; Wang et al., 2016; Wang et al., 2017). Cells utilize autophagy to dispose of wastes and eliminate hazards, while recycling nutrients and tuning metabolism in the process. Through these functions, autophagy promotes cell fitness, genomeintegrity, tissue homeostasis, cell survival and growth under stress (Alissafi et al., 2018; Alissafi et al., 2017; Amaravadi et al., 2016; Cicchini et al., 2015).

To date, three forms of autophagy are known; chaperone-mediated autophagy, microautophagy and macroautophagy. Both differ from each other due to their initiation, the mechanisms involved and the mode of destruction during delivery to the lysosome. Of these types of autophagy, macroautophagy has the largest known impact on human health and disease and for this reason this type will be described analytically in the following paragraphs (Kuballa et al., 2012; Ngabire et al. 2017).

Autophagy is a survival process displayed when cells face stressful and abnormal conditions, like elevated temperature, lack of sufficient nutrients, or low level of oxygen and therefore plays a crucial role in the maintenance of the cellular homeostasis (Ngabire et al. 2017). Under normal nutrient conditions, autophagy functions at a constitutive, low basal level to maintain protein and organelle quality, quantity, and functionality (Amaravadi et al., 2016). More than being a guardian to cells, autophagy is also implicated in a significant number of human disorders like cancer, but it is also implicated in immune system defense, both innate and adaptive. Autophagy plays also an important role in the elimination of

invaders like parasites, viruses, and bacteria, and additionally participates in the presentation of antigens of the major histocompatibility complex (MHC) Class II molecules (Ngabire et al. 2017).

1.3.1. The process of autophagy

Initial steps in autophagy include the nucleation, elongation, and isolation of an isolated membrane usually called a phagophore. The ends of the formed phagophore are then united to form the autophagosome, which is a vesicle with double membranes that locks in specific targeted cytoplasmic components. After this step, the autophagosome fuses with the lysosome to form an autophagolysosome, where the captured materials, both together with the inside membrane, are destroyed (**Figure 1.3.1.1.**) (Jung et al., 2010; Ngabire et al., 2017).

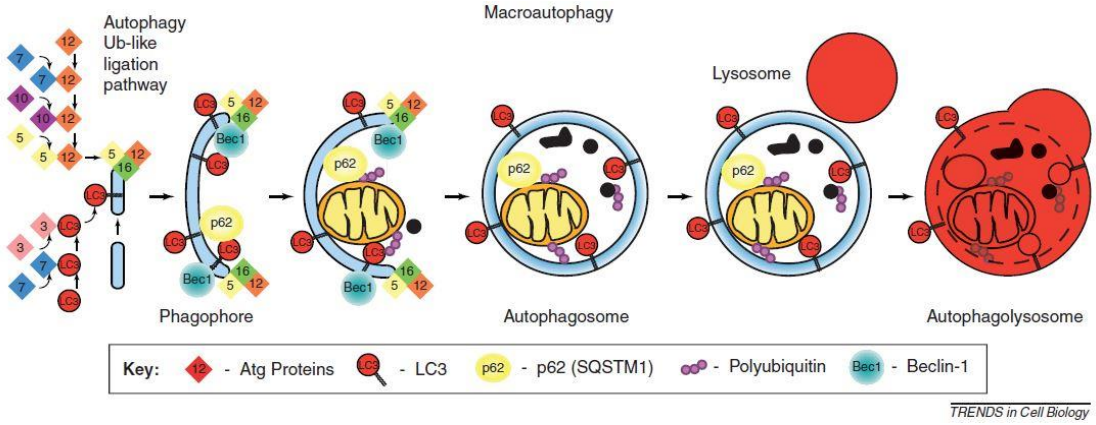


Figure 1.3.1.1.: The process of autophagy (Gump et al., 2011).

The formation and maturation of autophagosome in the cytoplasm are controlled and regulated by a set of multiple proteins both related to autophagy (ATG proteins) complex 1 (mTORC1). First, the pre-initiation complex is formed and is made of Unc-51-like kinase 1 (ULK1), FIP200 protein, and ATG13. Two major proteins regulate this complex: the mammalian target of rapamycin complex 1 (mTORC1) from the PI3k-Akt pathway, a key mediator of growth factor signaling to autophagy, which negative regulates autophagy, and adenosine monophosphate (AMP)-activated protein kinase (AMPK) that inhibits mammalian target of rapamycin complex 1 (mTORC1) (Jung et al., 2010; Ngabire et al., 2017).

The elongation phase and the late phase in the formation of autophagosomes are controlled by two separate but complementary ubiquitin like pathways: the ATG5-12 proteins and LC3-PE proteins. All these pathways start with a unique activating enzyme E1-ligase-like, ATG7, enrolled to the phagophore through PI3P from the initiation complex. At the beginning, ATG7, through its active cysteine residue, is strongly attached to a small protein ATG12 by a thioester bond. ATG12 is further transferred to another conjugating enzyme E2-like, ATG10,

which later sends away ATG12 to ATG5.At the end, the ATG5-ATG12 complex interacts with ATG16L to create a larger multimeric complexneeded to stabilize the forming phagophore (**Figure 1.3.1.2.**).

The LC3-PE complex connection pathway starts after cleavage of LC3 by ATG4. The cleaved LC3 then interacts with ATG7 and is instantly shifted away to the E2-like enzyme ATG3. The previously formed complex ATG5-ATG12-ATG16L works as an E3ligase, associating LC3 (Light chain 3) to phosphatidylethanolamine (PE) to form a LC3-PE complex, which is also called LC3-II. While LC3I remains in the cytoplasm, LC3II binds to both the outer and the inner membranes of the autophagosome; therefore, it is considered a marker of the autophagosome (**Figure 1.3.1.2.**) (Ndoye et al., 2016).

This newly formed complex, LC3-II, is very important for the fusion of autophagosomes with lysosomes. As soon as this step is terminated, the autophagosome fuses with lysosomes, and from the fusion, will result in one double membrane vesicle called autophagolysosome. The fusion procedure is terminated after membranes from both units are gathered together with the help of autophagosomal proteins syntaxin 17 (Stx17), SNAP29, and VAMP8. More interestingly, two known proteins that belong to lysosomes, LAMP1 and LAMP2, are needed for the fusion process, and when they are not available, the autophagosome fails to fuse to lysosomes. Inside the autophagolysosome, the trapped and engulfed cell components will be destroyed (**Figure 1.3.1.2.**) (Cicchini et al., 2015; Ngabire et al. 2017).

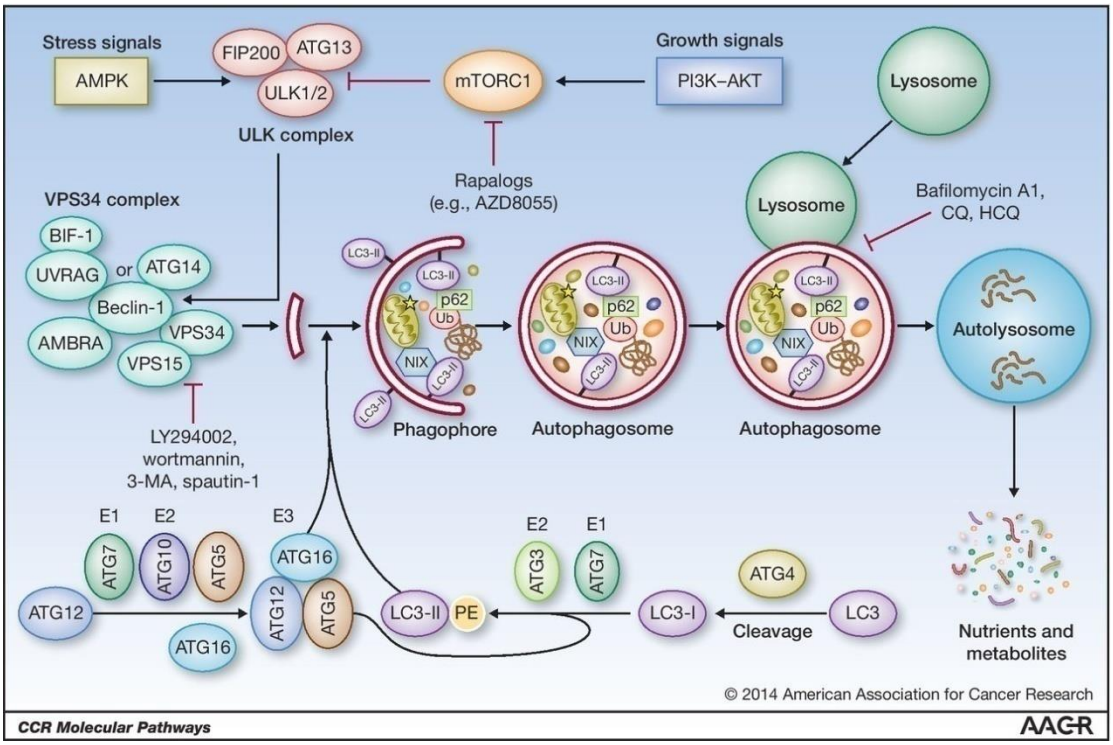


Figure 1.3.1.2.: The autophagic process (Cicchini et al., 2014)

Basal autophagy uses adaptor proteins, such as p62/SQSTM1 (sequestome) to identify and deliver misfolded or aggregated proteins and damaged organelles to the autophagosome for degradation, thereby preserving cellular fitness. P62 has emerged as a regulator of multiple types of autophagy (Kuballa et al., 2012). Key to this selective cargo delivery is the specific interactions between the adaptor proteins and the phagophore membrane bound LC3 (LC3-II), which serves as a cargo receptor (Cicchini et al., 2015).

1.3.2. Autophagy and the PI3K/mTOR pathway

Eukaryotic cells have developed a mechanism through which autophagy induction is tightly coupled to the regulation of cell growth. Among the various components involved in the regulation of autophagy and growth, mammalian target of rapamycin (mTOR) is a key

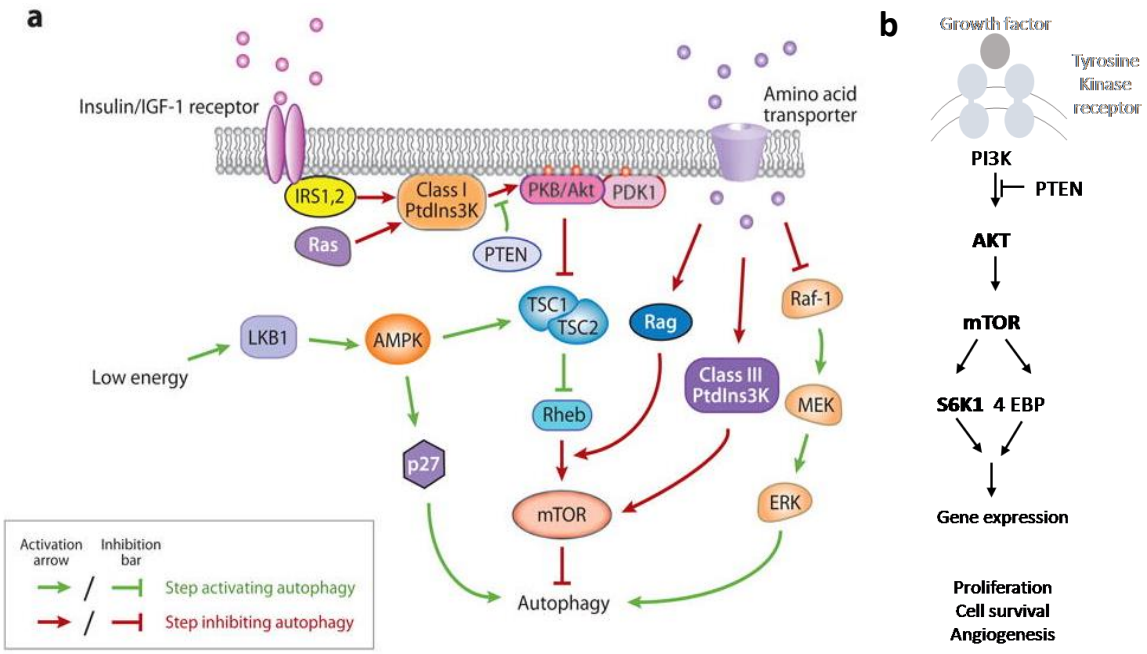


Figure 1.3.2.: **a**. Regulatory pathways of autophagy (Congcong He and Daniel J. Klionsky, 2009). **b**. Simplistic illustration of the PI3K-AKT-mTOR axis.

component that coordinately regulates the balance between growth and autophagy in response to cellular physiological conditions and environmental stress (Jung et al., 2010). The mTOR signaling pathway plays an essential role in maintaining protein synthesis and metabolic homeostasis in response to low energy production as well as hypoxia and nutrient deprivation (Daskalaki et al., 2018). Loss of energy as well as nutrient balance activate AMP-activated protein kinase (AMPK) and negatively regulate mTOR signaling, which in turn results in

autophagy induction through ULK1 phosphorylationat Ser317 and Ser777 (**Figure 1.3.2.**) (Jung et al., 2010; Kim et al., 2011).

1.3.3. Autophagy and immune response

Recent studies have demonstrated expanding roles for autophagy genes in both autophagy dependent and independent processes. Many of these processes have effects on the immune system, including immune cell differentiation, the coordination of metabolic signals, and both innate and adaptive immune defenses against pathogens (Kuballa et al., 2012).

Along with activation of other innate immune responses, autophagy induction represents the first line of defense during pathogen infections. It has been shown to function as a mechanism of intracellular pathogen sensing, while defects in autophagy can lead to increased susceptibility to infection. Further roles of autophagy in innate immunity include regulation of the inflammasome and cell-specific pattern-recognition receptor (PRR) signaling. Finally, it is another form of cell death, called the autophagic cell death, which is a potential mechanism to control inflammation (Deretic et al., 2013; Kuballa et al., 2012; Singh et al., 2017).

In the adaptive immune response, it has been shown that autophagy can deliver cytoplasmic material or pathogens to the lysosome and can contribute to antigen presentation by MHC class II molecules, as well as in the presentation of pathogen antigens by DCs, which are involved in T cell priming. Some other evidence also indicate a role for autophagy in MHC class I antigen presentation (Deretic et al, 2013; Kuballa et al. 2012).

1.3.4. Autophagy and Cancer

Hypoxia and nutrient deprivation are cardinal features of most tumors that posses a major role in tumor progression, metastasis and response to therapy (Daskalaki et al., 2018; Jiang et al., 2017). For tumor cells to adapt and survive in such hypoxic and hypo-nutrient conditions, the coordination of several stress response pathways is required, including HIF-1, mTOR, UPR, and autophagy (Daskalaki et al., 2018; Kalluri 2016). Low levels of oxygen and lack of nutrients, which are the most representative of metabolic stresses, can activate autophagy and therefore help to maintain cellular biosynthesis and survival (**Figure 1.3.4.1.**) (Ngabire et al., 2017).

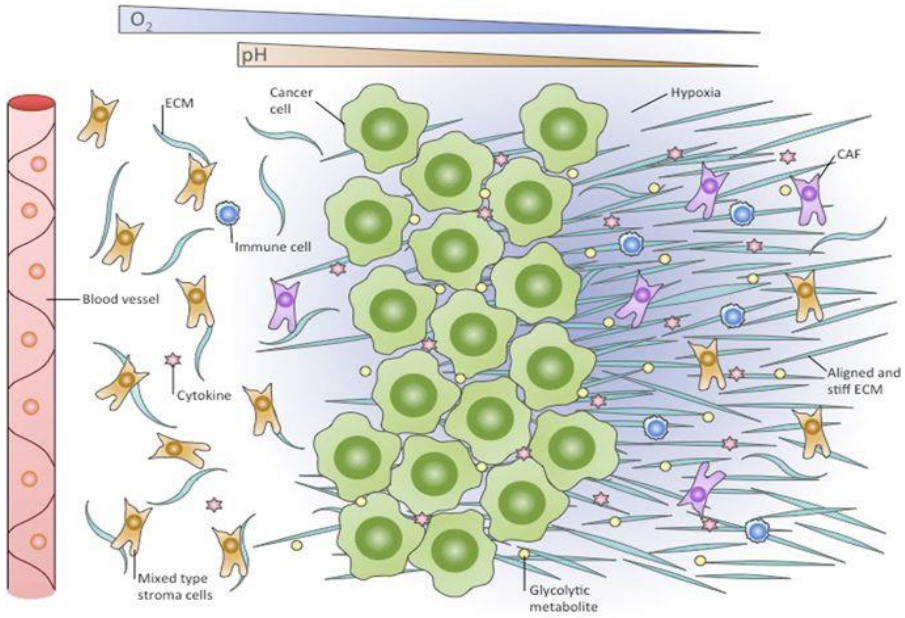


Figure 1.3.4.1.: Hypoxia is a cardinal feature of the TME and a major regulator of autophagy (Petrova et al., 2018).

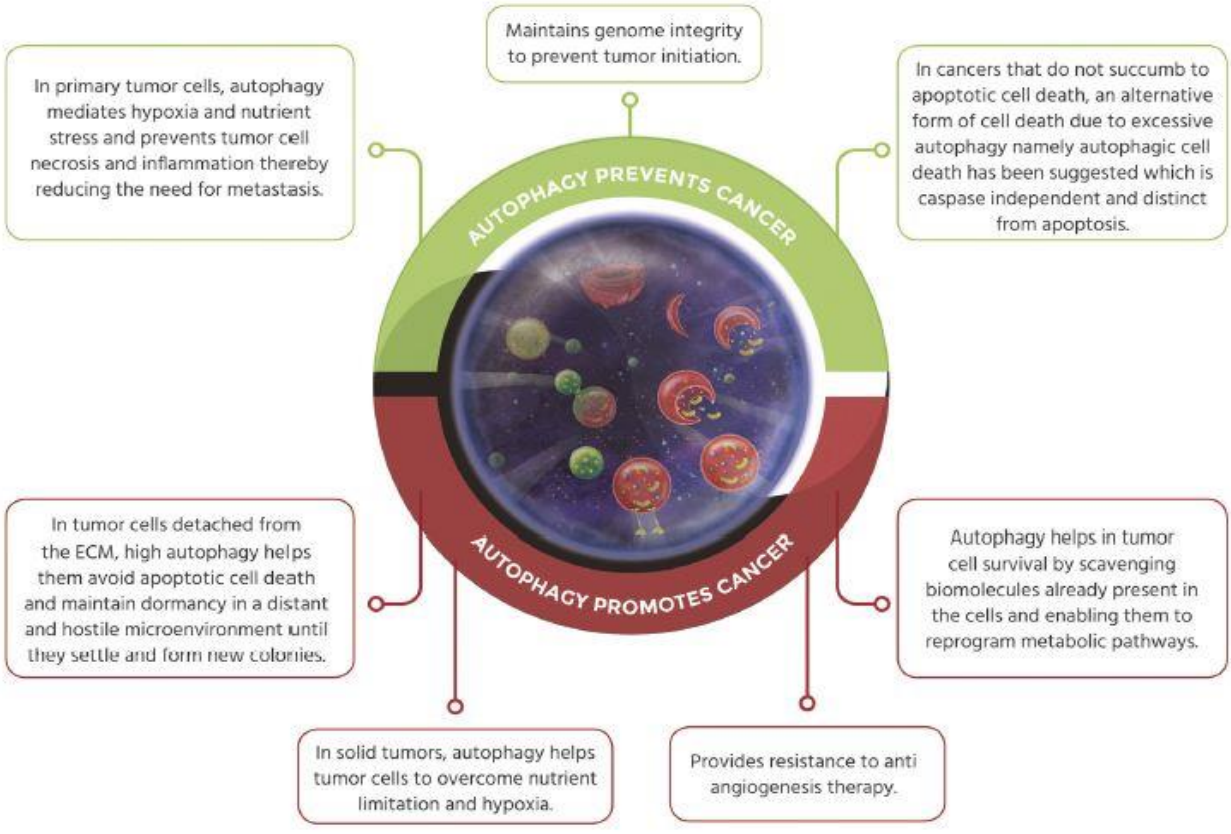


Figure 1.3.4.2.: The bipolar role of autophagy in cancer (Singh et al., 2018)**.**

Chronic hypoxia is characteristic of the tumor growth, which provokes the reduction of oxygen and its sources, and leads to the hypoxic conditions. These conditions cause the ATP levels to decrease, and subsequently the energy levels of the cells, as they inhibit the oxidative phosphorylation pathway. Therefore, the combination of hypoxia and low energy levels activates the autophagy pathway (Azad et al., 2008; Pouyssegur et al., 2006). The activation of autophagy facilitates the removal of damaged cellular compartments and recycles components (Daskalaki et al., 2018). As a highly conserved catabolic process, it is responsible for the maintenance of cellular homeostasis and cell survival, and is involved in multiple diseases, including cancer (Liu et al., 2018).

In cancer, autophagy has a bipolar role, either tumor-suppressive, or tumor-promoting, in different contexts (**Figure 1.3.4.2.**) (Sign et al., 2018). Autophagy impacts cellular metabolism, the proteome, and organelle numbers and quality, which alter cell functions in diverse ways. Moreover, autophagy influences the interaction between the tumor and the host by promoting stress adaptation and suppressing activation of innate and adaptive immune responses. Additionally, it can promote a cross-talk between the tumor and the stroma, supporting tumor growth, particularly in a nutrient-limited microenvironment. Thus, the role of autophagy in cancer is determined by nutrient availability, microenvironment stress, and the presence of an immune system (Amaravadi et al., 2016).The regulation of autophagy can either be elevated or suppressed by anticancer agents, and an increase of autophagy during cancer therapy can be in favor of survival, or induce cell death in tumor cells (Ngabire et al., 2017).

Autophagy may function as a tumor-suppressive mechanism during early tumorigenesis, but its role in advanced cancer remains unclear. Autophagy was first linked to cancer when 40%, 50%, and 70% of prostate, breast, and ovarian cancers, respectively, were reported to have allelic loss of *BECN1*, which encodes the essential VPS34 complex component Beclin-1, suggesting that Beclin-1 and autophagy in general may be a tumor suppressor (Cicchini et al, 2015). Inactivation of autophagy-related genes, such as Beclin-1, leads to increased tumorigenesis in mice while the over-expression of these genes (Beclin-1, Atg5) inhibit the formation of human breast tumors in mouse models. Also, mice with deficiency of Atg5 and Atg7develop liver tumors due to mitochondrial damage and oxidative stress (Ngabire et al., 2017; Singh et al., 2018) and concomitant deletion of p62 reduces tumor size, implying that accumulation of p62 due to deficient autophagy contributes to tumor progression (Ndoye et al., 2016).

Distinct to the role of autophagy in early tumorigenesis, it is also now widely accepted that autophagy is required for the survival of established cancers. Recent available data indicate that the leading role of autophagy in cancer cells is to provide them abilities to overcome induced stress, maintain homeostasis, survive in the poor physical environment and avoid apoptotic cell death and therefore helps to keep tumor cell alive. In established tumors, increased proliferation in cancer cells correlates with increased energetic and metabolic demands (Ngabire et al., 2017).

Thus, by enhancing stress tolerance and providing an alternative avenue through which cancer cells can power their massive nutrient and energy demands, autophagy is well-regarded as a mechanism for tumor cell survival (Singh et al., 2018). In this regard, autophagy inhibitors could be useful as anticancer therapeutic agents. However, the regression of tumor xenografts derived from a large number of human cancer cell lines is not detected upon inhibition of autophagy. However, the autophagy inhibitor, chloroquine (CQ), suppressed the growth of cancer cell lines, even if its effect is autophagy-dependent (Ngabire et al., 2017).

1.3.5. Autophagy and Melanoma

Melanoma accounts for only 5% of all cancers but is the leading cause of skin cancer death due to its high metastatic potential. Patients with metastatic melanoma have a 10-year survival rate of less than 10%. While the clinical landscape for melanoma is evolving rapidly, the lack of response to therapies, as well as resistance to therapy remain critical obstacles for treatment of this disease. In recent years, a myriad of therapy resistance mechanisms has been unraveled, one of which is autophagy (Ndoye et al., 2016). Melanomas have a high level of basal autophagosomes, suggestive of increased autophagy. Moreover, patients with melanoma with higher levels of autophagosomes had decreased survival (Amaravadi et al., 2016).

1.3.6. Autophagy and CAFs

Autophagy plays a major role in cancer, particularly in the TME, where it has a paradoxical function in acting as a tumor suppressor, but also as a tumor promoter. The induction of autophagy in stromal cells releases nutrients into the tumor microenvironment, supporting cancer cell growth, while the activation of autophagy in cancer cells drives the consumption of cellular components and effectively reduces tumor growth (Ngabire et al., 2018).

Proliferating tumor cells generate high consumption of oxygen and the growing tumor mass leads to progression of a hypoxic and acidic environment, with low nutrients, increased

reactive oxygen species (ROS), and hypoxia, towards which cancer cells must exhibit rapidly an adaptive response (Avagliano et al., 2018; Martinez-Outschoornetet al., 2010). The adaptation to hypoxia and hypo-nutrient conditions is sustained by the so-called "metabolic reprogramming", a reverse Warburg effect, in which changes of bioenergetics and biosynthesis occur both in cancer cells and CAFs. In this way, the alteration of cancer metabolism ensures sufficient building blocks for biosynthesis and facilitates cancer cells to survive a harsh hypoxic and nutrient-deprived microenvironment by promoting tumor growth, metastasis and bypassing cancer immunity (Avagliano et al., 2018; Goruppi et al., 2018; Kalluri 2016; Martinez-Outschoorn et al., 2011).

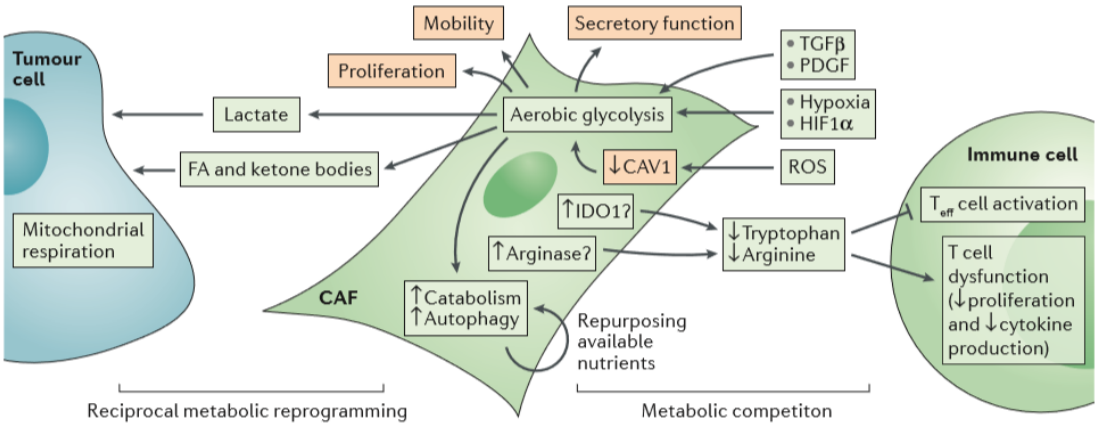


Figure 1.3.6.: CAFs and metabolic reprogramming of the tumor microenvironment (Kalluri, 2016).

Stromal autophagy releases nutrients that can be directly utilized by cancer cells to sustain growth and maintain cell viability. At the same time, HIF-1α activation and consequent mitophagy in CAFs induces mitochondrial dysfunction and enhances aerobic glycolysis, leading to the secretion of high energy nutrients that can directly feed oxidative mitochondrial metabolism in cancer cells. CAFs directly fuel tumor cells by producing and exporting high energy metabolites, especially lactate, pyruvate, and ketone bodies, which are used by adjacent cancer cells. Increased catabolic and autophagic pathways in CAFs may regulate the bioavailability of metabolites to immune cells, and such metabolic competition may impair tumor immunity (**Figure 1.3.6.**) (Chaudhri et al., 2013; Gorrupi et al., 2018; Kalluri, 2016; Martinez-outschoorn et al., 2010).

Despite many studies have investigated the role of autophagy in the tumor microenvironment, the mechanisms behind the interaction between autophagy and the components of the tumor environment still remains unclear and need to be explored (Ngabire et al. 2017).

1.3.6.1. The role of CAFs autophagy in cancer

The role of the autophagy in CAF biology is perplexing and plays crucial roles that differ depending on the chemical treatment and biological context (Li et al., 2016). The tumor mass reaction impairs the vasculature, leading to a highly hypoxic and hypo-nutrient environment (Feig et al., 2012). As a result, the oxidative stress induces the autophagic pathway in CAFs, which in turn positively influence the proliferation and metabolism of cancer cells (Kalluri, 2016; Yan et al., 2019; Zhou et al., 2017).

Secretory autophagy is involved in the export of a variety of cellular cargoes, including cytosolic proteins and inflammatory mediators, such as interleukin 1β (IL-1β), IL-6, IL-8 and IL-18 (Ponpuak et al., 2015). New et al. showed that mitigating autophagy greatly reduced CAF-induced progression through IL-6, IL-8 and bFGF in head and neck cell squamous carcinoma (HNCSC). Treatment with autophagy target Vps34 inhibitor, SAR405, attenuated xenograft growth and inhibited the effects of standard therapy (New et al., 2017). However, the inhibition of autophagy was not specific in CAFs, but in total organism. Similar results were found both *in vitro* and *in vivo* in various types of cancer, such as breast cancer, ovarian cancer, liver cancer, colorectal cancer and pancreatic adenocarcinoma (Ngabire et al., 2017; Wang et al., 2017; Yan et al., 2019).

Furthermore, Katheder et al. studied the role of autophagy in a *Drosophila melanogaster* malignant tumor model and showed that ablation of autophagy in the tumor had only a modest effect on tumor growth, but inhibition of autophagy in the non-tumor epithelia surrounding the tumor, had a marked effect on both tumor growth and invasiveness (Katheder et al., 2017). Moreover, Zhao et al. 2017 found increased CAFs autophagy in human luminal breast cancer tissues, which was correlated with poor prognosis. They also knocked down *ATG5* in CAFs and transplanted both CAFs and MCF-7, a breast cancer cell line, in NOD-SCID mice to study the effect of CAFs autophagy on breast cancer progression. Their research showed that CAFs promoted the tumorigenicity of MCF-7 cells in mice and *ATG5* depletion in CAFs significantly decreased tumor growth, demonstrating that CAFs autophagy increase the tumorigenicity of luminal breast cancer cells (Zhao et al., 2017). However, it remains elusive what impact has the specific ablation of autophagy in CAFs on tumor immune profile.

1.4. Aim

The immunosuppressive and hypoxic conditions of the tumor microenvironment (TME) impair the success of immunotherapy (Costa et al., 2018; Hanahan et al., 2011). A mechanism that is induced by hypoxia, a cardinal feature of most tumors (Daskalaki et al., 2018; Jiang et al., 2017), is autophagy (Azad et al., 2008; Pouyssegur et al., 2006); even though most studies have focused on the role of autophagy in tumor cells, the involvement of autophagy in the stromal compartment, and in particular in cancer-associated fibroblasts (CAFs), has been investigated to a limited extend (Capparelli et al., 2012; Chaudhri et al., 2013; Levy et al., 2017; Liang et al., 2018). CAFs represent a highly heterogeneous yet abundant cell population of the TME, quite resistant to chemo/radiotherapy (Augsten et al., 2014; Capparelli et al., 2012; Kalluri, 2016; Kalluri et al., 2006; Kumar et al., 2017; LeBleu et al., 2018; Ziani et al., 2018). Although their tumor-promoting function is acknowledged (Castells et al., 2012; Hammer et al., 2017; Tommelein et al., 2015; Trimboli et al., 2009; Sun, 2015; Ziani et al., 2018), their role in shaping the anti-tumor immune response remains elusive.

Recent evidence indicate that CAFs exhibit increased autophagic levels (Chaudhri et al., 2013; Martinez-outschoorn et al., 2010; New et al., 2017; Wang et al., 2017) and that autophagy contributes to their tumor promoting function (Sousa et al., 2016). Moreover, the inhibition of autophagy in a mouse xenograft model led to significant reduction in tumor volume (New et al., 2017), while the ablation of autophagy in the non-tumor epithelia of the TME on *a Drosophila melanogaster* malignant tumor model had a marked effect on both tumor growth and invasiveness compared to the inhibition of autophagy in only the tumor cells (Katheder et al., 2017). However, it remains unknown what impact has the specific ablation of autophagy in CAFs on tumor growth and on tumor immune response.

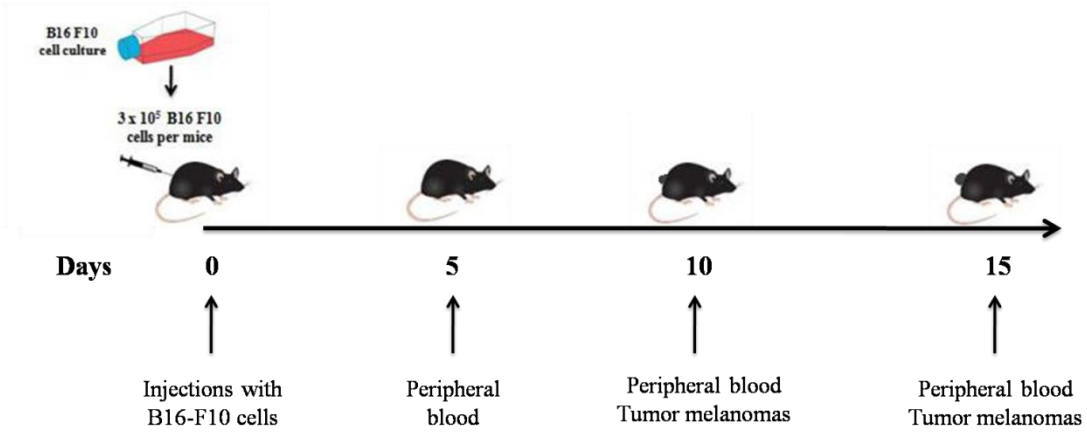
Taking the above into consideration, we formed the hypothesis that the upregulated autophagy in CAFs dictates their tumor promoting function. In this project, we aim to investigate how CAFs autophagy shapes tumor growth, anti-tumor immune response and response to immunotherapy.

In particular, by first establishing CAFs presence in the tumor microenvironmnent, we plan to examine CAFs autophagy levels during tumor development and in response to immunotherapy in B16-F10 inoculated C57BL/6 mice. Furthermore, in order to assess the functional role of CAFs autophagy in tumor growth, tumor immune response and tumor's response to immune checkpoint inhibitors, we will perform experiments on the first-time-generated transgenic mice that lack the autophagic process specifically from CAFs.

2. Methods

2.1. Mice: C57BL/6, Atg5fl/fl and αSMA-Cre mice (kindly provided by Dr.Raghu Kalluri, Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX) were used for the experiments; the mice were maintained in a pathogen-free environment in the animal facility of the Biomedical Research Foundation of Academy of Athens (BRFAA). For tumor experiments, gender-matched C57BL/6 mice were injected subcutaneously with 3×10^5 B16-F10 melanoma cells in the back. Tumors were measured regularly with digital calipers, and tumor volumes were calculated by the formula length × width × (length × width) $0.5 \times \pi/6$. At 10^{th}, 11^{th}, 15^{th}, 16^{th} and 18^{th} day of tumor growth, mice were killed and tumors and blood were collected for analysis (**Figure 2.1.a.**). For the PD-L1 blockade experiment, 200 μg anti-PDL1 (100μl per mouse; MIH5; bioceros) were administered intra-peritoneally (i.p.) every three days for a total of four times, until day 12 of tumor growth (**Figure 2.1.b.**), while the control mice received PBS 1x at the same quantity (100μl) and frequency of the anti-PD-L1 injections. Animals were excluded only if tumors failed to form or if health concerns were reported.

a.



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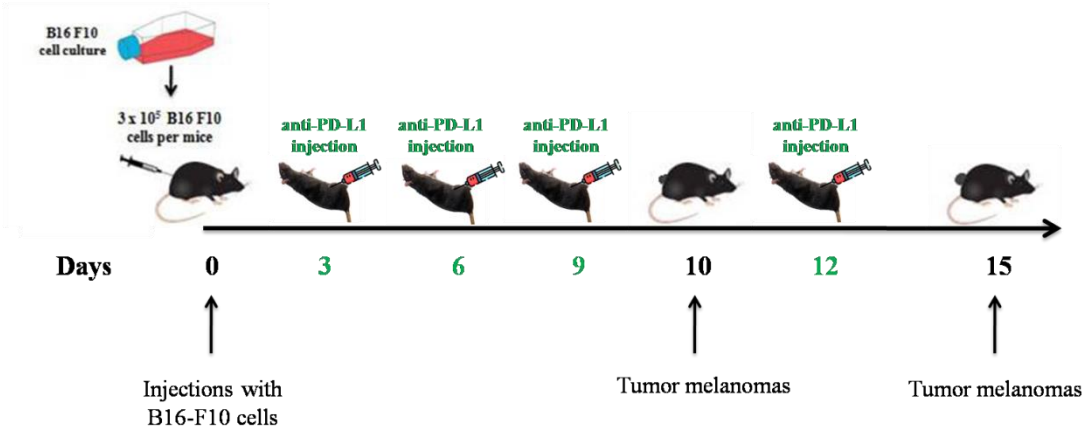


Figure 2.1.: Schematic illustration of tumor melanomas timepoint experiment; gender-matched C57BL/6 mice were injected subcutaneously with 3×10^5 B16-F10 melanoma cells in the back.(**a**) At 5th day of tumor growth, peripheral blood was obtained for analysis, while at 10th and 15th day of tumor growth, mice were killed and tumors and blood were collected for analysis. (**b**) For the PD-L1 blockade experiment, 200 μg anti-PD-L1 were administered per mouse intraperitoneally (i.p.) every three days for a total of four times, until day 12 of tumor growth. At 10th and 15th day of tumor growth, mice were killed and tumors and blood were collected for analysis. For control mice, PBS 1x was administered i.p. at the same frequence of anti-PD-L1 injections.

α-SMA-Cre Atg5$^{fl/fl}$ mice were generated by crossing Atg5$^{fl/fl}$ mice (Hara et al., 2006) (RIKEN BioResource Center) and α-SMA-Cre mice (kindly provided by Dr.Raghu Kalluri, Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX) (described in more details in Results Section 3.6). This mouse line was genotyped according to previously published protocols included the: floxed *Atg5* mice from RIKEN and α-SMA-Cre mice from Jackson Laboratories (Bar Harbor, ME, USA). The following primers were used: for Atg5$^{fl/fl}$ mice: A: (exon3-1) 5'-GAATATGAAGGCACACCCCTGAAATG-3', B: (short2) 5'-GTACTGCATAATGGTTTAACTCTTGC-3', C: (check2): 5'-ACAACGTCGAGCACAGCTGCGCAAGG-3'; for α-SMA-Cre mice: 1: 5'-ACATGTCCATCAGGTTCTTGC-3', 2: 5'-AGTGGCCTCTTCCAGAAATG-3', 3: 5'-TGCGACTGTGTCTGATTTCC-3', 4: 5'-GGTGTTAGTTGAGAACTGTGGAG-3'.

2.2. Cell culture: B16-F10 melanoma cells (kindly provided by Prof. Aristedes Eliopoulos, Medical School, University of Crete, Heraklion, Greece) were maintained in our laboratory and cultured in RPMI (Gibco) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (STEMCELL Technologies), 5% (vol/vol) penicillin-streptomycin (Gibco) and 0,05 mM β-mercaptoethanol (Gibco) (Complete RPMI Medium). NIH/3T3 fibroblast cells (kindly provides by Dr.Dimitris Thanos, Biomedical Research Foundation Academy of Athens) were cultured in DMEM with 1.5 g L−1 NaHCO3 (Gibco), 10% fetal bovine serum (STEMCELL Technologies) and 5% (vol/vol) penicillin-streptomycin (Gibco) (Complete DMEM Medium). NIH/3T3 cells were treated with 20% v/v Tumor Explant Supernatant (TES; described below), chloroquine diphosphate (CQ 50mM, Sigma Aldrich) overnight (O/N; 16-18 hours)

Morphological characteristics were assed and visualized, using an optical microscope where they displayed a typical CAF morphology.

2.3. Tumor-tissue processing for Fluorescence–Activated Cell Sorting (FACS): Cancer tissues were excised and cut into smaller possible fragments by using an ophthalmic scissor. The minced tissues were afterwards incubated for 45 minutes in RPMI medium (Gibco) containing 1 mg/mL collagenase D (Sigma-Aldrich) and 0.128 mg/mL DNAase I (Sigma-Aldrich) and then they were strained through a nylon filter with a pore size of 40μm (BD Falcon). Cells were pelleted through centrifugation (1650 rpm, 10 minutes,4°C) and re-suspended in PBS - 5% FBS buffer; staining with proper antibodies followed.

2.4. Tumor-tissue processing for Tumor Explant Supernatant (TES): We isolated B16/F10 melanomas of day 15th of tumor growth from C57BL/6 mice. Tumor homogenization was performed as described above. We plated 10^7 cells/ml on 6-well plates in complete RPMI medium (as described above) and the plates were cultured O/N in the incubator (37°C, 5% CO2). The next day, we collected the supernatant (S/N), centrifuged it (2000 rpm, 10 minutes, 25°C) and used it for our experiments.

2.5. Lung-tissue processing for Fluorescence–Activated Cell Sorting (FACS): In order to obtain normal fibroblasts (NFs), lung tissues were isolated from C57BL/6 mice and Atg5 fl/fl mice and cut into smaller possible fragments by using an ophthalmic scissor. The minced tissues were afterwards incubated for 45 minutes in RPMI medium (Gibco) containing 2 mg/mL collagenase D (Sigma- Aldrich), 0.128 mg/mL DNAase I (Sigma-Aldrich) and 2 mg/mL dispase (GIBCO). Then they were strained through a nylon filter with a pore size of 40 μm (BD Falcon). The cells were pelleted through centrifugation (1650 rpm, 10 minutes, 4°C) and re-suspended in PBS-5% FBS buffer; staining with proper antibodies followed.

2.6. Mouse Peripheral blood mononuclear cell isolation: Heparinized blood (500μl) was collected from mice. PBMCs were isolated on Histopaque-1077 (Sigma-Aldrich) density gradient. Briefly, blood was diluted 1:1 with PBS and carefully layered over Histopaque medium. Tubes were centrifuged at 400 g for 30 minutes with no break at room temperature. PBMC layer was collected, and cells were washed with PBS (**Figure 2.6**).

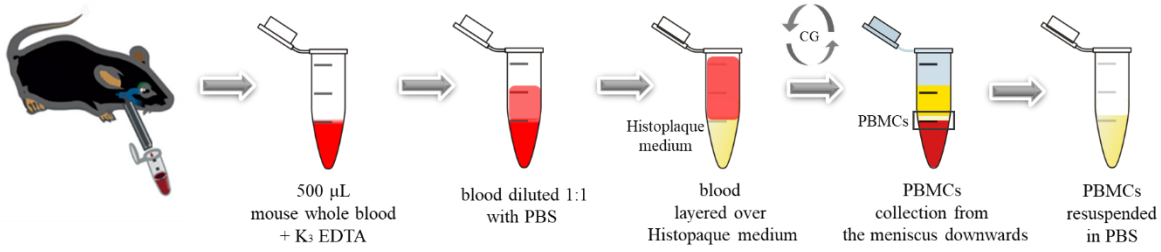


Figure 2.6.: Experimental protocol for isolating mouse peripheral blood mononuclear cells from whole blood (PBMCs: Peripheral blood mononuclear cells, CG: centrifugation).

2.7. Inguinal lymph node processing for Fluorescence–Activated Cell Sorting (FACS): The inguinal lymph nodes (**Figure 2.7.**) were isolated from Atg5$^{fl/fl}$ mice and α-SMA-Cre Atg5$^{fl/fl}$ mice. Afterwards, they were strained through a nylon filter with a pore size of 40 μm (BD Falcon). The cells were pelleted through centrifugation (1650 rpm, 10 minutes, 4°C) and re-suspended in PBS-5% FBS buffer; staining with proper antibodies followed.

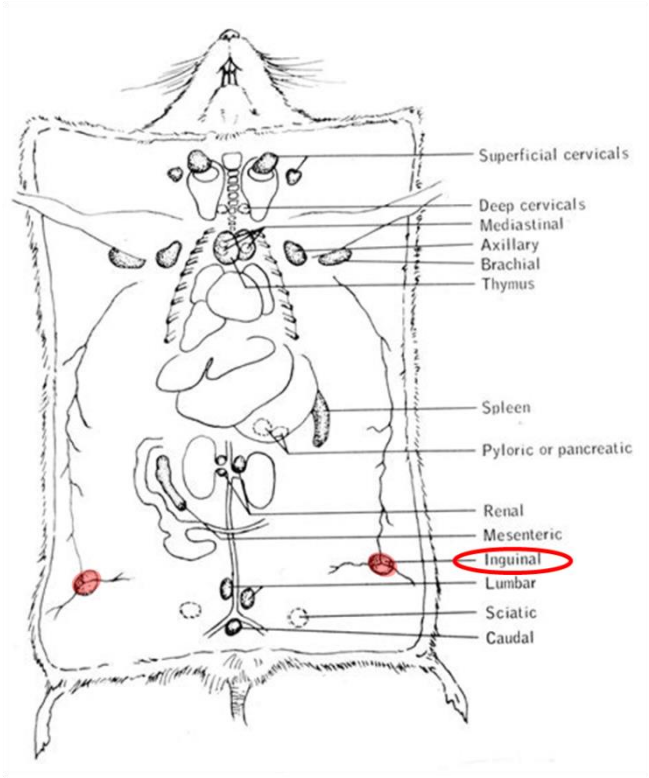


Figure 2.7.: Diagram to show the locations of the principal lymph nodes, the spleen, and the thymus; Inguinal lymph nodes are depicted in red (Dunn et al.,1954).

2.8. CAF characterization: FACs analysis of fibroblasts was performed using the following antibodies:

- For **extracellular staining**, cell suspensions were incubated at 4°C for 20 minutes with markers to exclude leucocytes (CD45; PerCp-Cy5.5; 1:200; clone 30-F11; BioLegend), epithelial cells (EpCAM, 1:200; clone G8.8; Biolegend) and endothelial cells (CD31, 1:200; clone 390; BioLegend) and also with a panel of typical CAFs markers; PDGFRα (APC; 1:100; clone APA5; BioLegeend), PD-L1 (BV421; 1:200; 10F.9G2; BioLegend). All antibodies were diluted in PBS-5% FBS.
- For **intracellular staining**, the Intracellular Fixation & Permeabilization Buffer Set (eBioscience) was used, according to the manufacturer's instructions. After 45 minutes (RT in the dark) cell permeabilization with the Fixation Buffer, cell suspensions were

washed with Permeabilization Buffer (centrifuged at 1800 rpm, 10 minutes, 4°C) and then incubated for 45 minutes (RT in the dark) with α-SMA antibody (FITC; 1:50; 1A4; ab8211 abcam), diluted in Permeabilization Buffer. After incubation, cell suspensions were washed with Permeabilization Buffer (centrifuged at 1800 rpm, 10 minutes, 4°C) and re-suspended in PBS-5% FBS.

The flow cytometry data were analyzed with Flowjo Software (Tree Star).

2.9. Immune cell populations characterization: FACs analysis of different immune cell populations was performed using the following antibodies:

- **$CD45^+$ cells**: CD45 (PerCp-Cy5.5; 1:200; clone 30-F11; BioLegend; extracellular staining)
- **$CD4^+$ T cells**: CD4 (PE; 1:200; GK1.5; BioLegend; extracellular staining)
- **$CD8^+$ T cells**: CD8 (PE-Cy7; 1:200; 53-6.7; BioLegend; extracellular staining)
- **Natural killer cells (NK)**: NK1.1 (APC; 1:200; PK136; BioLegend; extracellular staining) ($CD4^-$ $NK1.1.^+$ cells)
- **Dendritic cells (DCs)**: CD11c (APC; 1:200; N418, BioLegend; extracellular staining) ($CD45^+$ $CD11c^+$ cells)
- **Myeloid Derived Suppressor Cells (MDSCs)**: CD11b (PE-Cy7; 1:200; M1/70; BioLegend; extracellular staining), GR1 (PE; 1:200; RB6-8C5; BioLegend; extracellular staining) ($CD45^+$ $CD11b^+$ $GR1^+$ cells)
- **Regulatory T cells (Tregs)**: Foxp3 (Alexa488; 1:50; 150D; BioLegend; intracellular staining*****) ($CD4^+$ $Foxp3^+$ cells)

*****For the intracellular staining of Foxp3, the Foxp3 staining Buffer Set (eBioscience) was used, following the same procedure as mentioned above, according to manufacturer's instructions.

The flow cytometry data were analyzed with Flowjo Software (Tree Star).

2.10. Confocal Microscopy: Cultured NIH/3T3 cells were plated on poly-Lysine coated glass coverslips. Afterwards, they were fixed with 4% paraformaldehyde (PFA) in PBS. The confocal staining protocol that was implemented included permeabilization 0.1% saponin 2% BSA in PBS 1x for 15 minutes in room temperature followed by 10 minutes of fixation with ice-cold methanol (Sigma). Cells were afterwards incubated with primary antibodies for autophagy staining. Cells were stained with Lamp-1 (rat; 1:400; sc-19992/1D4B; Santa Cruz Biotechnology), p62 MBL (rabbit; 1:500; PM045/SQSTM1; MBL) and LC3 (mouse; 1:20; catalog 0231/clone 5F10; nanoTools) for 1 hour and then stained with Alexa Fluor 555 anti-mouse IgG (1:500; A28180; Invitrogen), Alexa Fluor 647 anti-rabbit IgG (1:200; A21245;

Invitrogen), and Alexa Fluor 488 anti-rat IgG (1:250; A11006; Invitrogen) for one hour and DAPI (1:100; Sigma-Aldrich), for visualization of the nuclei for 3 minutes. Images were obtained using an inverted confocal live cell imaging system Leica SP5. The numbers of LC3 spots/cell, p62 spots/cell, Lamp-1 spots/cell were calculated using a macro developed in Fiji software (SciJava).

2.11. Quantitative PCR (qPCR) analysis: Total RNA from FACS-sorted cell populations was isolated with NucleoSpin® RNA kit (Macherey-Nagel), followed by reverse transcription with ThermoScript Reverse Transcriptase Kit (Invitrogen), according to manufacturers' instructions. Transcripts were quantified by incorporation of Platinum SYBR Green (Bio-Rad Laboratories Inc.) with a Step One Plus Real-Time PCR System (Applied Biosystems), and expression was calculated by the change-in-threshold method (ΔΔCT) with Hprt mRNA (encoding hypoxanthine phosphoribosyltransferase 1).The following primers were used, all purchased by Invitrogen: mouse *TYRP1* forward 5'- CCGCCATTATCCCCACGATG-3', *TYRP1* reverse 5'- GCCCTGACAAAGTGGCTCT-3', mouse SQSTM1 forward 5'- AGGATGGGGACTTGGTTGC- 3', mouse SQSTM1 reverse 5'- TCACAGATCACATTGGGGTGC- 3', mouse *Hprt* forward, 5'- GTGAAACTGGAAAAGCCAAA-3', *Hprt* reverse, 5'- GGACGCAGCAACTGACAT-3'.

2.12. Statistical analysis: Statistical analyses were performed using Student's t test. Two-way ANOVA statistical tests were applied in experiments with multiple comparisons. Data are presented as means ± S.E.M. Differences were considered statistically significant at $P < 0.05$. All data were analyzed using GraphPad Prism v5 software.

3. Results

3.1 Isolation and characterization of fibroblasts in lung and melanoma tissue

Fibroblasts were isolated from lung tissues (NFs) and B16-F10 tumor melanomas (CAFs), to analyze their profile with Fluorescence–Activated Cell Sorting (FACS) and Confocal Immunofluorescence microscopy. At first, we properly selected markers that would efficiently identify CAFs, differentiating them from other cell populations (**Table 3.1.1.**). The lack of a reliable and specific molecular fibroblast marker is a limiting factor in studying fibroblasts *in vivo*. There are several well-established indicators of fibroblast phenotype, but none of them are both exclusive to fibroblasts and present in all fibroblasts (Kalluri, 2016).

Table 3.1.1. Antibodies used for positive and negative selection of CAFs

Marker	Selection	Description
CD45	Negative	Leukocyte common antigen, marker of all differentiated hematopoietic cells expects for red blood cells (Costa et al., 2018; Zhang et al., 2013; Kraman et al., 2010; Takahashi et al., 2017)
CD31	Negative	130 kDa platelet–endothelial cell (EC) adhesion molecule (Liu et al.., 2012)
PDGFRa	Positive	Important mesenchymal stem cell marker (Farahani et al., 2015)
PD-L1	Positive	Transmembrane protein that inhibits TCR-mediated immune responses. (Dezutter-Dambuyant et al, 2016; Dunne et al., 2016)
α-SMA	Positive	Cytoskeletal protein associated with smooth muscle cells that is expressed on activated fibroblasts (Costa et al., 2018; Kalluri, 2016; Kalluri et al, 2006; Shiga et al., 2018)

As fibroblasts are the non-vascular, non-epithelial and non-inflammatory cells of the connective tissue (Kalluri, 2016), we used markers expressing those characteristics to exclude those cells. The best characterized marker of CAFs is α-smooth muscle actin (α-SMA), a cytoskeletal protein associated with smooth muscle cells that is expressed on activated fibroblasts (Costa et al., 2018; Kalluri, 2016; Kalluri et al, 2006; Shiga et al., 2015). However, we only used this marker to perform analysis of their frequencies; due to the fact that α-SMA is an intracellular marker.

After thorough research, for isolation of CAFs through Fluorescence-Activated Cell Sorting (FACS), we chose the following markers: as positive selection of we utilized Programmed Death Ligand-1 (PD-L1), since within the tumor microenvironment, PD-L1 is constitutively expressed on several non-hematopoietic cell types, including tumor-associated fibroblasts (Dezutter-Dambuyant et al, 2016; Dunne et al., 2016). Furthermore, previous experiments on our laboratory have shown that cells stained with α-SMA displayed high

expression of PD-L1 at their majority (>80% in all cases, results not shown), as was expected concerning their role as suppressors of the anti-tumor immune response, proving that PD-L1 is an adequate marker in successfully isolating CAFs. Moreover, another marker we used for positive selection of CAFs was Platelet-Derived Growth Factor Receptor Alpha (PDGFRa), a marker of mesenchymal stem cells (Farahani et al., 2015), as from the existing literature it is expressed also in the surface of activated fibroblasts (Kalluri, 2016; Kalluri et al., 2006; Shiga et al., 2015). Using the combination of the above markers (positive and negative selection), we isolated CAFs through FACs in order to further examine them.

We used tumor melanomas of B16-F10-bearing C57BL/6 mice at their 15th day of tumor growth to isolate CAFs, while as control we used lung tissues of Atg5$^{fl/fl}$ mice, to obtain normal fibroblasts (NFs). Cells suspensions that were prepared were incubated with the extracellular antibodies CD45-PerCP/Cy5.5, CD31-PerCP/Cy5.5, PD-L1/BV421 in 1/200 and PDGFRa (CD140a)/APC in 1/100 (**Table 1 for description**), according to well-established protocols (described in more details in the Materials and Methods section).

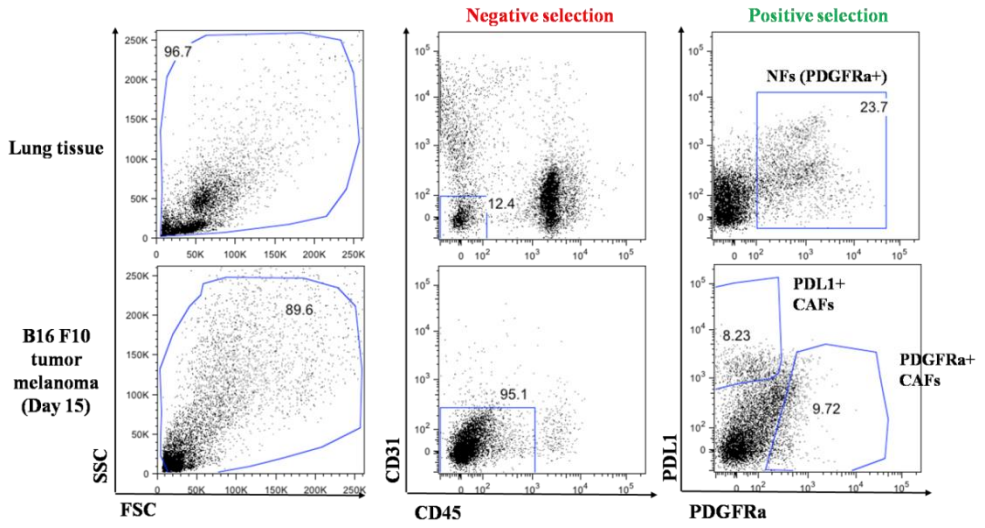


Figure 3.1.1.: Representative plots depicting the gating strategy we used to isolate the population of CAFs inside the melanomas of B16/F10 injected mice and the population of NFs from lung tissues of Atg5$^{fl/fl}$ mice. Numbers of FACS plot denote frequency of gated populations.

We sorted cells that were negatively stained for all markers we used except for PDGRa^{+} and PD-L1^{+}. Out of CD45- cells, a clear population of PDGFRa^{+} NFs was detected in lung tissues, but on tumor melanomas we distinguished two possible populations as CAFs; a population that was positive for PDL1^{+} expression and another population that was positive for PDGFRa^{+} expression (**Figure 3.1.1.**). Many activated fibroblasts may not express all their

markers at the same time (Ziani et al., 2018) and for that reason we proceeded in examining both those CAFs populations.

We then cultured FACS sorted CAFs (PDL1$^+$ CAFs and PDGFRa$^+$ CAFs) and sorted NFs in DMEM high glucose, with 10% FBS and Pen/Strep (37^{o}C, 5% $C0_2$), in order to observe if their morphological characteristics *in vitro* exhibit a large spindle-shape mesenchymal morphology, with a potential for planar polarity, as mentioned in the literature (Kalluri et al., 2016; Xing et al., 2015).

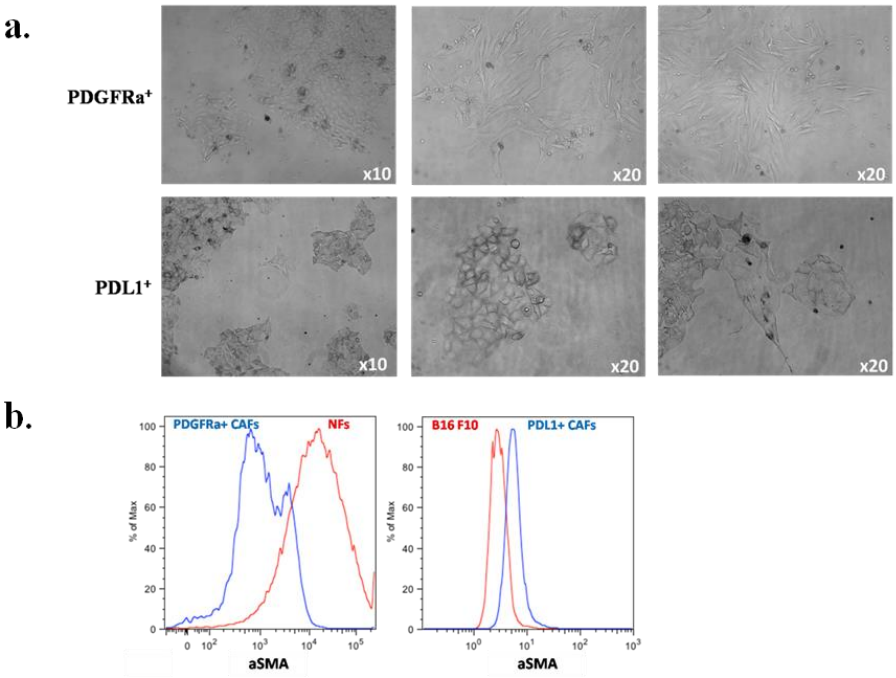


Figure 3.1.2.: (**a**) Morphological characteristics of FACS sorted CAFs (PDGFRa$^+$ and PDL1$^+$); PDGFRa$^+$ CAFs were cultured for 13 days in DMEM high glucose, 10% FBS and Pen/Strep (37^{o}C, 5% $C0_2$), while PDL1$^+$ CAFs for 5 days accordingly. Images were obtained with an Optical Microscope, zoom x10, x20. (**b**) Representative histograms for the expression of α-SMA in cultured PDGFRa$^+$ CAFs, PDL1$^+$ CAFs, B16-F10 melanocytes and NFs.

We observed that PDGFRa$^+$ CAFs exhibited the classic spindle-shape morphology in contrast with PDL1$^+$ CAFs, which resembled more with B16-F10 melanoma cell morphology (**Figure 3.1.2.a**). It is worth mentioning that PDGFRa$^+$ CAFs started proliferating and expanding in their petri-dish only after their 10th day of culture, while PDL1$^+$ CAFs at their 3rd day of culture had already shown signs of proliferation and expansion.

We proceeded by performing intracellular staining of α-SMA on those cultured PDGFRa$^+$ CAFs and PDL1$^+$ CAFs, along with B16-F10 melanocytes (cultured for 2 days in RPMI with

10% FBS, Pen/Strep (37°C, 5% $C0_2$)) and NFs (cultured for 4 days in DMEM high glucose, 10% FBS, Pen/Strep (37°C, 5% $C0_2$)) (**Figure 3.1.2.b**). The results showed that normal fibroblasts (NFs) exhibited the highest expression of α-SMA, which was not expected, as α-SMA is a marker of activated fibroblasts and not quiescent fibroblasts (Kalluri et al., 2016; Kalluri, 2006). Interestingly, $PDGFRa^+$ CAFs had higher α-SMA expression than $PD\text{-}L1^+$ CAFs, while B16-F10 cells also expressed α-SMA, which came by surprise, as there is no evidence in the existing literature that cancer cells express α-smooth muscle actin.

The findings above troubled us, so we proceeded with further experiments in order to delineate whether the cells that we isolate are indeed fibroblasts or a mixture of fibroblasts and B16-F10 melanoma cells. We performed quantitative PCR analysis (qPCR) on different sorted cell populations (**Figure 3.1.3.a**), measuring the relative expression of *TYRP1*, which is among critical enzymes that are expressed on melanocytes (Mello et al., 2016) versus *HPRT*, a house keeping gene (described in more details in Materials and Methods section).

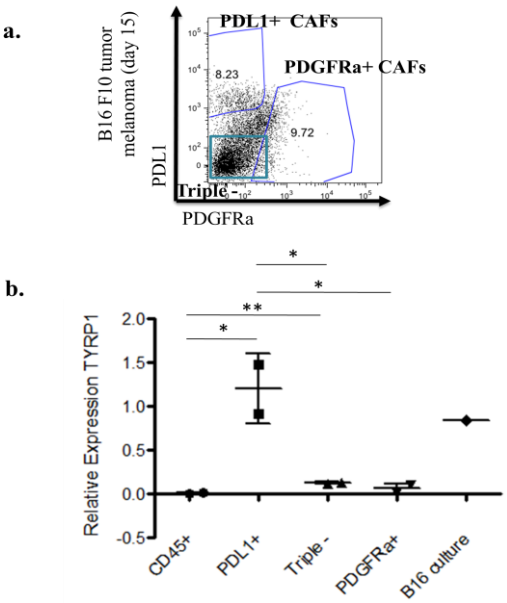


Figure 3.1.3.: (**a**) Representative plot depicting the gating strategy we used to isolate $PDL1^+$ CAFs, $PDGFRa^+$ CAFs, and triple negative cell population (CD45- , PDL1- , PDGFRa-). (**b**) Results from relative expression of *TYRP1* / *HPRT* at different sorted populations and at B16 F10 cultured cells (***P<0.01 / *P<0,05, N=2 mice per group*).

The results indicated that $PDL1^+$ CAFs exhibit higher expression of *TYRP1* even from B16-F10 melanocytes, where we expected to find the highest expression. The expression of *TYRP1* was also present in $PDGFRa^+$ cells, but at lower levels from B16-F10 melanocytes (**Figure 3.1.3.b**).

The positive selection of CAFs through PDL1 and PDGFRa accordingly could not be further utilized in our experiments, as it wasn't an efficient way to isolate fibroblasts *in vivo*. There is no specific extracellular marker for CAFs, a major barrier for isolating and studying them *in vivo*, except from the intracellular marker α-SMA, as already mentioned above. For future experiments, we decided to isolate CAFs from α-SMA-RFP mice, a transgenic strain that has a DsRed fluorescent reporter for *α-SMA* expressing cells, and has applications in studies related to the role of *Acta2* expressing myofibroblasts in cancer (Magness et al., 2004). Through this transgenic mouse, we will be able to identify the fluorescent α-SMA positive cells, isolating efficiently activated fibroblasts, in order to perform further analysis in *ex vivo* studies.

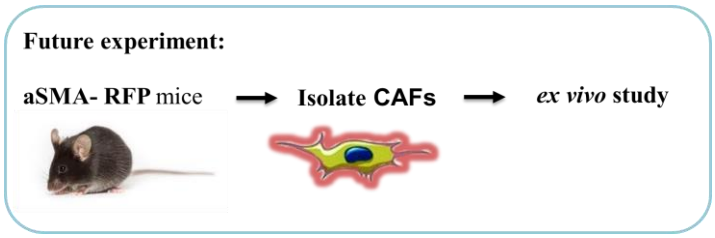


Figure 3.1.4.: For future experiments, we will use transgenic α-SMA-RFP mice in order to successfully isolate CAFs and investigate them *ex vivo*.

However, in the meantime, as an alternative we chose to identify CAF population in tumor melanomas, by assessing the levels of α-SMA expression intracellularly (**Figure 3.1.5.**); in that way, we could only evaluate their frequencies in tumors and not isolate them to perform further experiments, such as confocal microscopy to monitor their levels of autophagy. For Confocal Immunofluorescence microscopy, we used a fibroblastic cell line, NIH/3T3 cells that will be described further below.

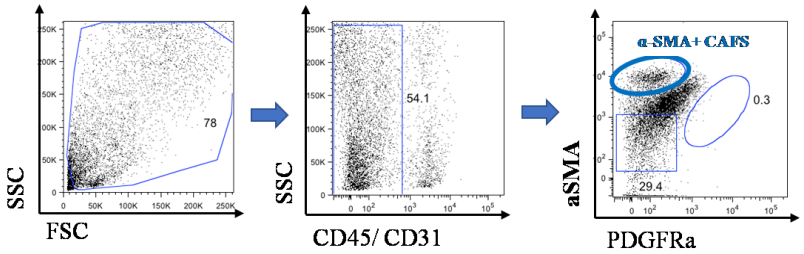


Figure 3.1.5.: Representative plot depicting the gating strategy we concluded in studying α-SMA$^+$ CAFs. Numbers of FACS plot denote frequency of gated populations.

3.2. α-SMA$^+$ CAFs enriched in the TME and the circulation of melanoma model

First, we wanted to evaluate whether CAFs accumulate during tumor progression. We performed a timepoint experiment, where we analyzed α-SMA$^+$CAFs frequencies in the TME

and in the circulation of melanoma-bearing mice during tumor development. C57BL/6 mice were inoculated with B16-F10 cells (described in more details in Materials and Methods section, **Figure 2.1.a**.); tumors were isolated and day 11th, 15th and 18th day of tumor growth and peripheral blood was obtained from naïve mice, and from day 6, 11, 15 and 18 of tumor development (**Figure 2.1.a.**, **Figure 2.6.**).

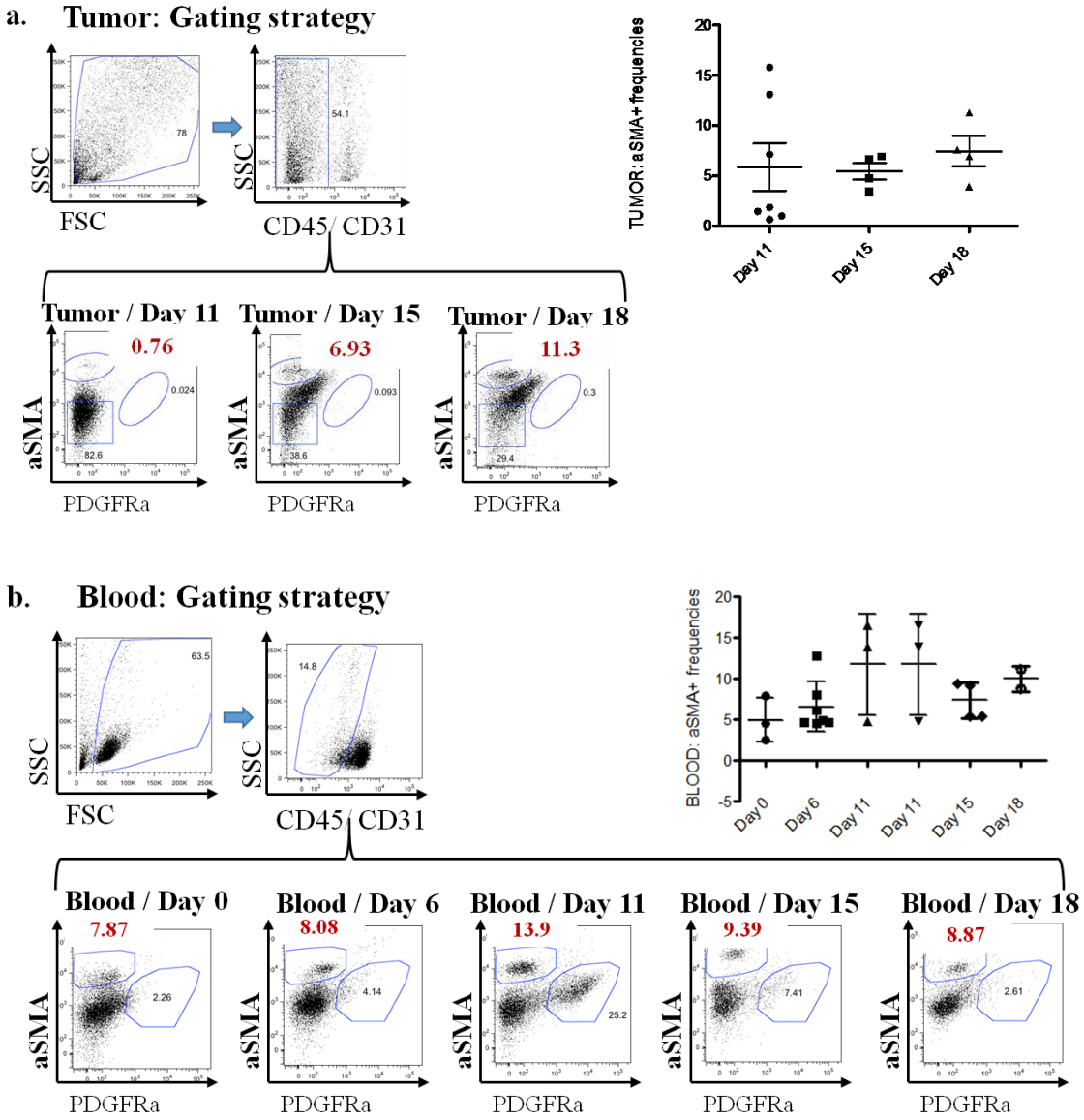


Figure 3.2.1.: Gating strategy; frequencies of α-SMA^{+} CAFs (CD45- CD31-) in (**a**) B16-F10 tumor melanomas of Day 11, 15 and 18 of C57BL/6 mice and in (**b**) peripheral blood of naïve (Day 0), Day 6, 11, 15 and 18 B16-F10 melanoma-bearing C57BL/6 mice.

From these results, we observed a significant enrichment of α-SMA^{+} CAFs in the TME of melanoma-bearing mice, with an increased frequency of α-SMA^{+} CAFs in blood as tumor progresses. The identification of circulating CAFs (cCAFs), which form heterotypic clusters

with circulating tumor cells (CTC), is of quite interest, as they are considered to be pre-cursors of metastatic colonies (McCarthy et al., 2018). Further research on targeting cCAF/CTC co-clusters may provide novel avenues to abrogate melanoma metastasis.

3.3. Downregulation of PI3K/AKT/mTOR pathway in α-SMA^{+} CAFs upon tumor progression

Once we found that CAFs do accumulate in the TME of melanoma mouse model, we next sought to investigate whether autophagy is upregulated in CAFs upon tumor progression, in order to support our hypothesis. We chose to study the kinase mTOR-dependent pathway, which is the best-characterized regulator of autophagy, and activation of the PI3K/AKT axis is an upstream modulator of mTOR activity (Alissafi et al., 2018). By assessing the levels of phosphorylated AKT (pAKT), phosphorylated mTOR (pmTOR) and phosphorylated S6 (pS6) in α-SMA^{+} CAFs (CD45- CD31-; described in more details in Results section 3.1., **Figure 3.1.4**) of day 11th and day 15th of B16-F10 tumor melanomas, we detected that the PI3K/AKT/mTOR axis was downregulated upon tumor progression.

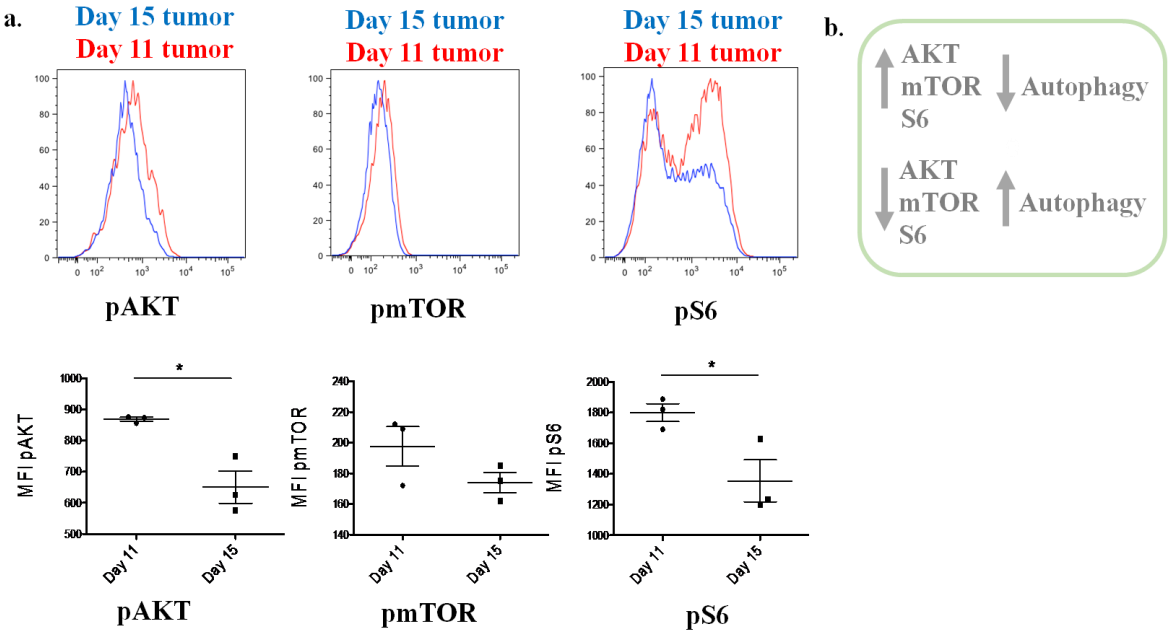
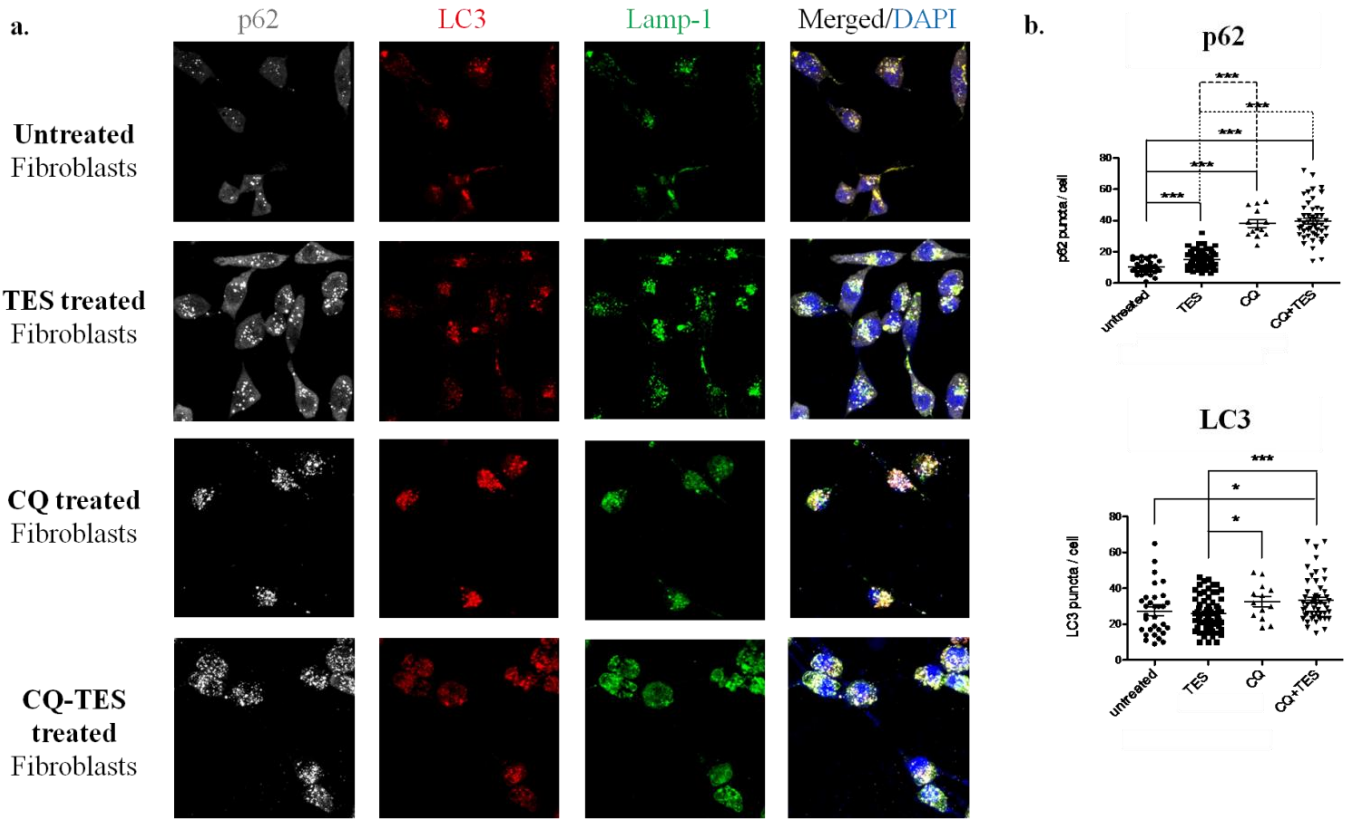


Figure 3.3.: (a) Representative histograms and MFI for the expression of pAKT (***P*= *0.0138*), pmTOR and pS6 (***P = 0.0378)* in α-SMA^{+} CAFs of tumor melanomas (Day 11 vs 15) of C57BL/6 mice, n=3 mice per group. **(b)** Schematic illustration of PI3K/AKT/mTOR axis in relation with autophagy.

We know that the initiation of autophagy takes place when PI3K/AKT/mTOR pathway is downregulated. Indeed, our results demonstrate that pAKT and pS6 levels are downregulated upon tumor progression; therefore, autophagy is initiated properly as tumor develops.

3.4. Deregulated autophagy in TES-treated 3T3 fibroblasts versus untreated

In order to assess whether autophagy is being completed, we proceeded by monitoring autophagy in 3T3 fibroblastic cells, treated with tumor explants supernatant (TES), chloroquine (CQ), which inhibits autophagy by impairing autophagosome fusion with lysosomes, TES-CQ and untreated cells as controls (**Figure 3.4.1.**), following the updated guidelines for autophagy (Klionsky et al., 2016). Using confocal immunofluorescence microscopy, we determined formation of functional autophagolysosomes (a hybrid organelle formed by fusion of the autophagosome and lysosome) based on the expression of LC3 that denotes formation of autophagosomes (Alissafi et al., 2017; Alissafi et al., 2018), the lysosomal-associated membrane protein 1 (LAMP-1), and the adaptor protein SQSTM1/p62 that targets ubiquitinated proteins for lysosomal degradation (Pankiv et al., 2007).



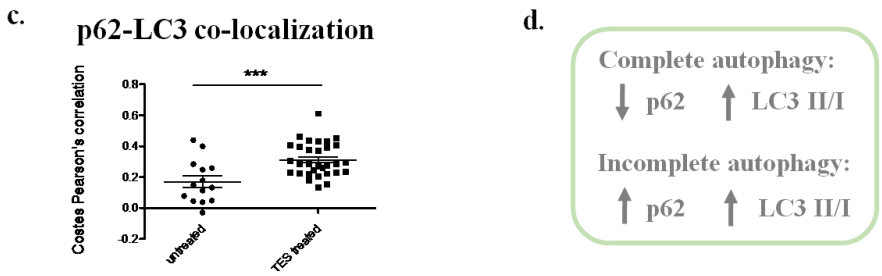


Figure 3.4.1.: (**a**) Representative Confocal Immunofluorescence Microscopy images for p62 (silver white), LC3 (red), LAMP-1 (green) and DAPI (blue) in 3T3 fibroblastic cells (untreated, TES-treated, CQ-treated, CQ/TES-treated). (**b**) p62 puncta/cell and LC3 puncta/cell are depicted (****P < 0.0001; *P <0.05*). Results are expressed as mean ± SEM. (Images analyzed with the image processing software Fiji (SciJava), zoom 2x). (**c**) Pearson's correlation of LC3 versus p62 (****P < 0.0001*) in 3T3 fibroblastic cells (TES-treated vs untreated). (**d**) Schematic illustration of p62 and LC3 protein levels in completed or incompleted autophagy. TES: tumor explant supernatant; CQ: chloroquine.

We detected that the expression levels of LC3-II remained unchanged between untreated and TES treated cells (**Figure 3.4.1**); this could indicate that autophagosome accumulation occurred due to inhibition of autophagic degradation, such as blockage of autophagosome-lysosome fusion (Mizushima et al., 2007). We also observed that p62, which is a downstream protein regulated by LC3, was considerably upregulated in TES-treated cells, in comparison with untreated cells (*P****<0.0001*) (**Figure 3.4.1**.). Those finding indicate that TES-treated fibroblasts exhibit deregulated autophagy; unchanged LC3 II/I levels in combination with high cytoplasmic p62 indicates impairment of autophagy flux (Liang et al., 2018).

Inhibition of autophagy correlates with increased levels of p62 in mammals and in *Drosophila* (Valencia et al., 2014). *SQSTM1* (p62) changes can be cell type and context specific. In some cell types, there is no change in the overall amount of p62 despite strong levels of autophagy induction, while in other contexts, a robust loss of p62 does not correlate with increased autophagic flux. Moreover, p62 may be transcriptionally upregulated under certain conditions, further complicating the interpretation of results (Klionsky et al., 2016).

In order to address whether the upregulated levels of p62 were correlated with deregulated autophagic flux or with transcriptional upregulation of *SQSTM1* gene, we performed an quantitative PCR analysis (qPCR) of *SQSTM1* gene (p62) versus HPRT gene (**Figure 3.4.2**), a house keeping gene as mentioned above (described in more details in Materials and Methods section, 2.9).

Those results indicated that there is no difference between the expression levels of *SQSTM1* (p62) gene of untreated fibroblasts versus the TES-treated fibroblasts (**Figure 3.4.2.**), demonstrating that the highly enriched p62 levels in TES-treated cells do not derive from upregulated *SQSTM1* transcription, but possibly due to increased autophagic flux; yet deregulated.

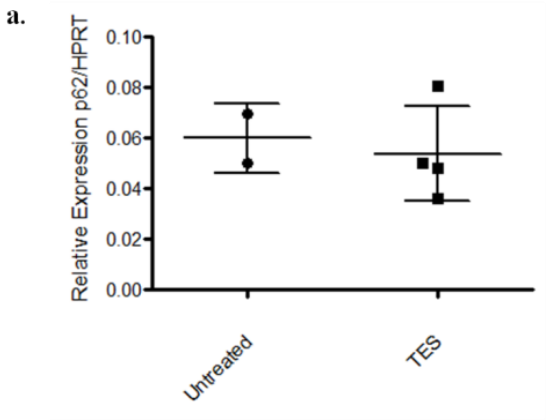


Figure 3.4.2.: Results from relative expression of *SQSTM1(p62)* / *HPRT* at 3T3 untreated fibroblasts vs TES-treated (Results from 2 experiments combined).

3.5. Downregulation of PI3K/AKT/mTOR pathway in α-SMA$^+$ CAFs upon tumor progression in C57BL/6 mice that have received anti-PD-L1

Within the tumor microenvironment, Programmed Death Ligand-1 (PD-L1) is constitutively expressed on several non-hematopoietic cell types, including tumor-associated fibroblasts (Dezutter-Dambuyant et al, 2016; Dunne et al., 2016). We also found that CAFs exhibit increased expression of PD-L1 ligand (**Figure 3.5.1**), a fact that makes them potential therapeutic targets for immunotherapy.

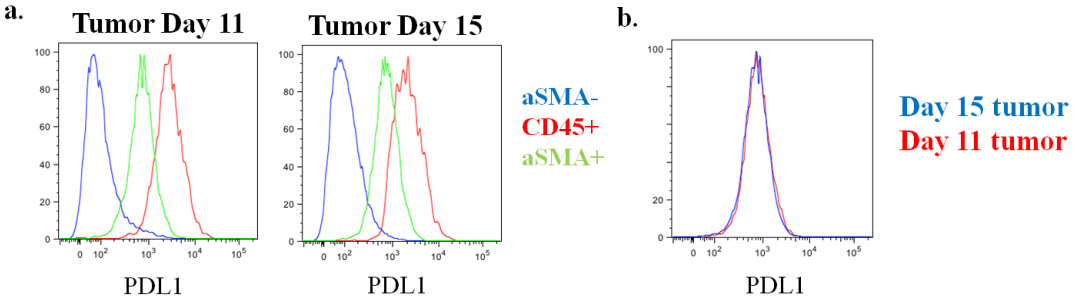
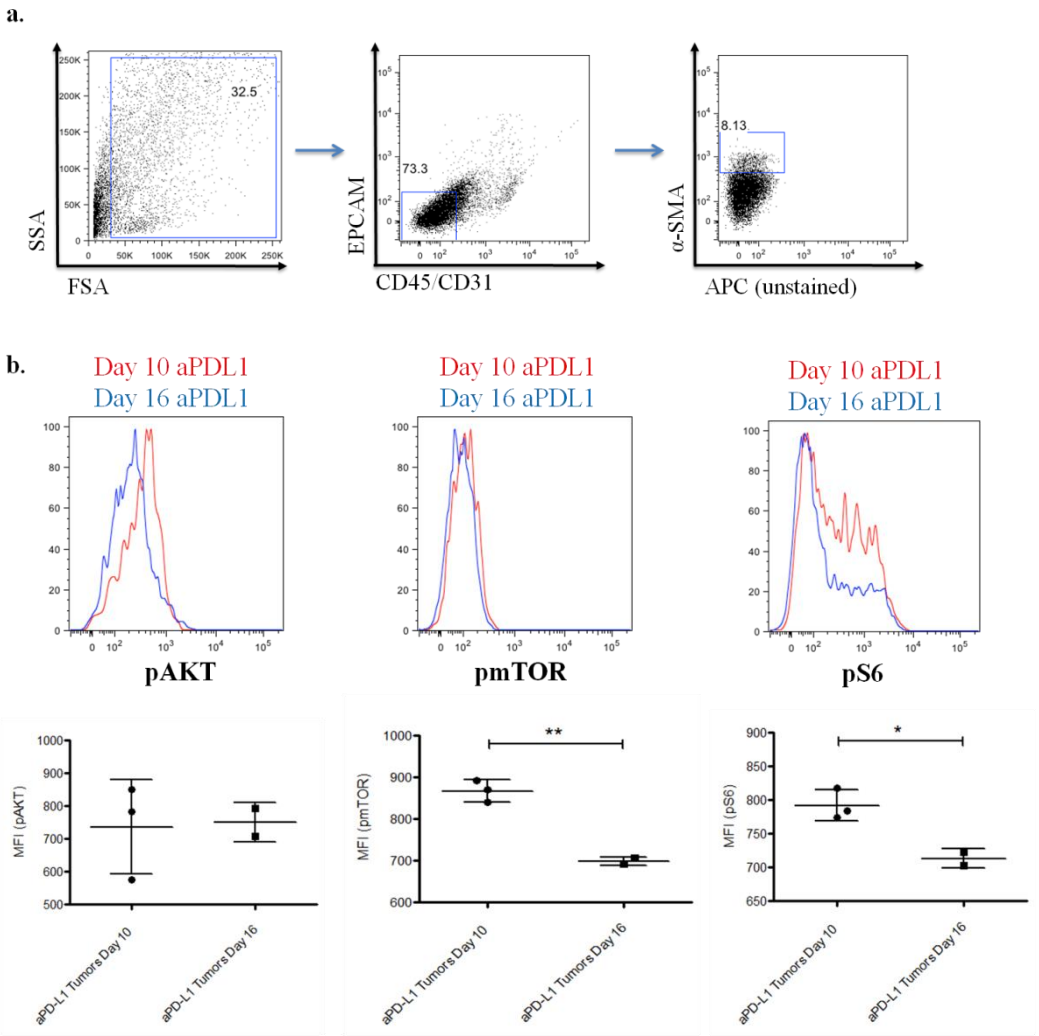


Figure 3.5.1.: (**a**) (**b**) α-SMA$^+$ CAFs express high expression of PD-L1 as tumor develops.

Next, we wanted to investigate the autophagic levels of CAFs in melanoma-bearing mice that receive immune checkpoint inhibitors and in particular, anti-PD-L1. We performed a timepoint experiment on C57BL/6-melanoma bearing mice, where we administered anti-PD-L1 inhibitor intraperitoneally (i.p.) every three days for a total of four times, until day 12 of tumor growth (**Figure 2.1.b.**), while the control mice received PBS 1x at the same quantity and frequency. Tumor melanomas of day 10 and 16 of tumor growth were analyzed, from mice receiving a-PD-L1 (aPDL1) and from control mice (control) accordingly (**Figure 2.1.b**., described in more details in the Materials and Methods section).

We assessed the levels of phosphorylated AKT (pAKT), phosphorylated mTOR (pmTOR) and phosphorylated S6 (pS6) in α-SMA^{+} CAFs (CD45- CD31-, EPCAM-; **Figure 3.5.a.**) of day 10th and day 16th of B16-F10 tumor melanomas of aPDL1 mice and control mice. We detected that the PI3K/AKT/mTOR axis was downregulated upon tumor progression in aPDL1 mice (**Figure 3.5.b.**), indicating that autophagy is initiated properly as tumor develops in mice that receive anti-PD-L1 checkpoint inhibitor.



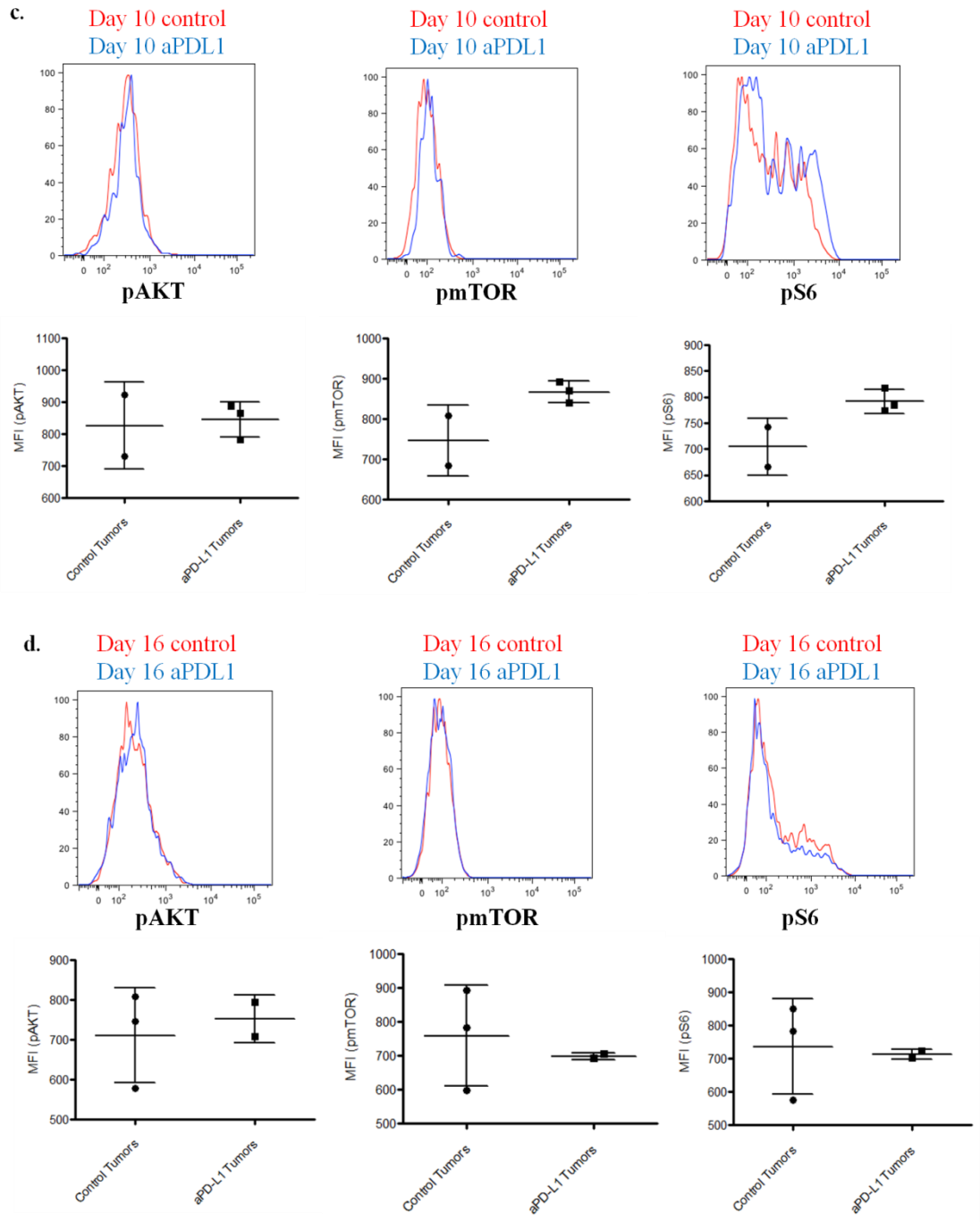
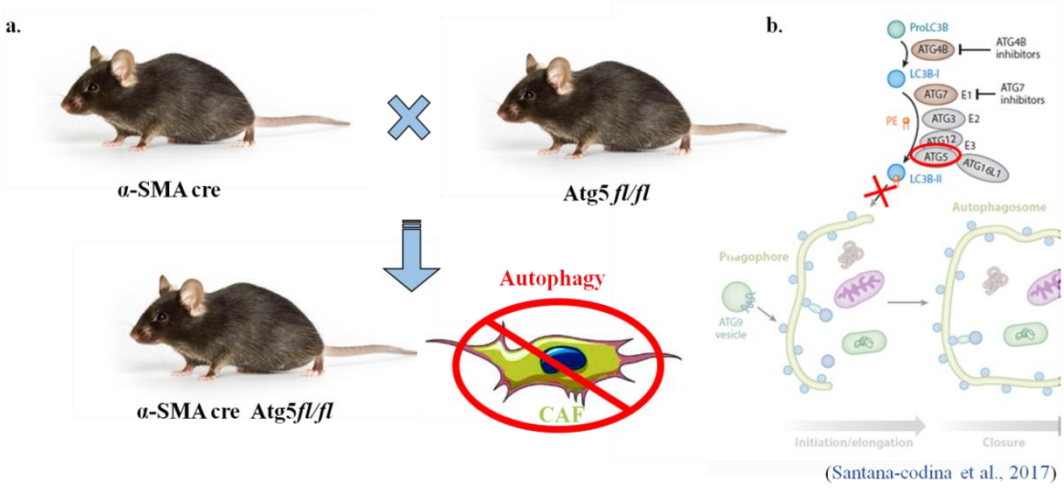


Figure 3.5.: anti-PD-L1 timepoint expetriment (**a**) Representative plot depicting the gating strategy we used in studying α-SMA$^+$ CAFs. Numbers of FACS plot denote frequency of gated populations. (**b**) Representative histograms and MFI for the expression of pAKT, pmTOR (***P< 0.01*) and pS6 (**P<0.05)* in α-SMA$^+$ CAFs of tumor melanomas (Day 10 vs16) of C57BL/6 mice that received anti-PD-L1 (aPDL1). (**c**) Representative histograms and MFI for the expression of pAKT, pmTOR and pS6 in α-SMA$^+$ CAFs of tumor melanomas (Day 10) of C57BL/6 mice that received PBS 1x (control) and of aPDL1 mice. (**d**) Representative histograms and MFI for the expression of pAKT, pmTOR and pS6 in α-SMA$^+$ CAFs of tumor melanomas (Day 16) of control and of aPDL1 mice , n=3 mice per group.

Although we didn't find any significant difference between the phosphorylated levels of AKT (pAKT), mTOR (pmTOR) and S6 (pS6) in α-SMA^{+} CAFs of Day 10 aPDL1 mice (**Figure 3.5.c.**) and control mice, it seems that in aPDL1 mice, they are slightly increased versus control mice; this could indicate that anti-PD-L1 treatment tries to block the autophagic pathway in CAFs. However, on Day 16, the phosphorylated levels of the AKT/mTOR pathway in aPDL1 mice are lower in comparison with control mice (**Figure 3.5.d.**), possibly illustrating that in the end, anti-PD-L1 treatment couldn't manage to inhibit autophagy in CAFs.

3.6. Generation of transgenic mice α-SMA-Cre Atg5$^{fl/fl}$

In order to conclusively test the functional role of autophagy in CAFs, we generated the transgenic mice α-SMA-Cre Atg5 $^{fl/fl}$, in which autophagy is depleted from α-SMA^{+} cells. By crossing Atg5fl/fl and α-SMA-Cre mice using the Cre-LoxP system, *Atg5*, an essential gene for the formation of the autophagosome (Santana-codina et al., 2017), is deleted in α-SMA^{+} cells and as a result, the autophagic pathway cannot be initiated in CAFs (**Figure 3.6.**).



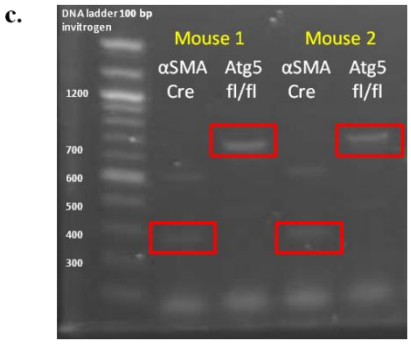


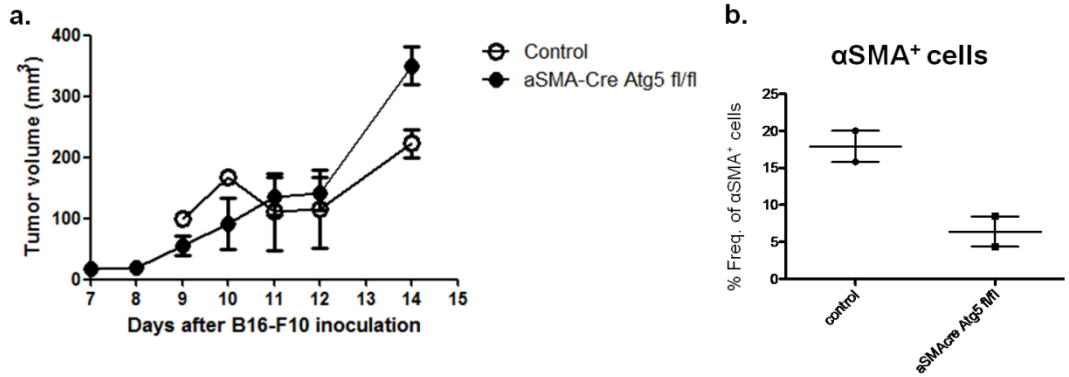
Figure 3.6.: (**a**) Generation of transgenic mice α-SMA-Cre Atg5$^{fl/fl}$ mice, which lack autophagy in α-SMA^{+} cells. (**b**) *Atg5* gene plays a significant role in the pre-initiation complex of autophagy and by depleting it, the autophagy pathway cannot occur. (**c**) Electrophoresis agarose gel (1%) depicting PCR products of α-SMA-Cre Atg5$^{fl/fl}$ mice (αSMA cre: transgene band: 300 bp, internal positive control: 521 bp (previously published protocols from Jackson Laboratories / Atg5: fl/fl band: 700 bp, wild type: 350 bp (previously published protocols from Hara et al., 2006, RIKEN BioResource Center).

In more details, α-SMA-Cre transgenic mice express Cre recombinase under the control of *Acta2* gene promoter that is expressed on CAFs. *Atg5* floxed mice have the exon 3 of *Atg5* gene flanked by *loxP* sites, containing neomycin resistant cassette. By crossing α-SMA-Cre with Atg5$^{fl/fl}$ mice, the Cre recombinase catalyses the recombination of DNA between *loxP* sequences; in our case *Atg5* and as a result the expression of this gene is depleted from activated fibroblasts.

Those transgenic mice have never been generated before according to existing literature; they mice were born at the expected Mendelian ratios with no obvious developmental defects. They were viable and fertile in adulthood and presented no obvious phenotype or altered life span. In future experiments, when we will have the adequate numbers of mice in order to perform experiments, we will specifically examine the role of autophagy in CAFs in tumor development, tumor immune response and in response to immunotherapy (aPD-L1).

3.7. Tumor growth and immune response assessments of α-SMA-Cre Atg5$^{fl/fl}$ mice

To dissect their potential role of CAFS autophagy as regulator of the anti-tumor immunity, we monitored the presence of immune cell populations infiltrating the tumor site. Specifically, we isolated and studied with flow cytometry the CD45^{+} cells, the myeloid derived suppressor cells (MDSCs), the dendritic cells (DCs), the CD4^{+} T cells, the CD8^{+} T cells, the natural killer (NK) cells and the regulatory T cells (Tregs) (**Figure 3.7.1.c.**).



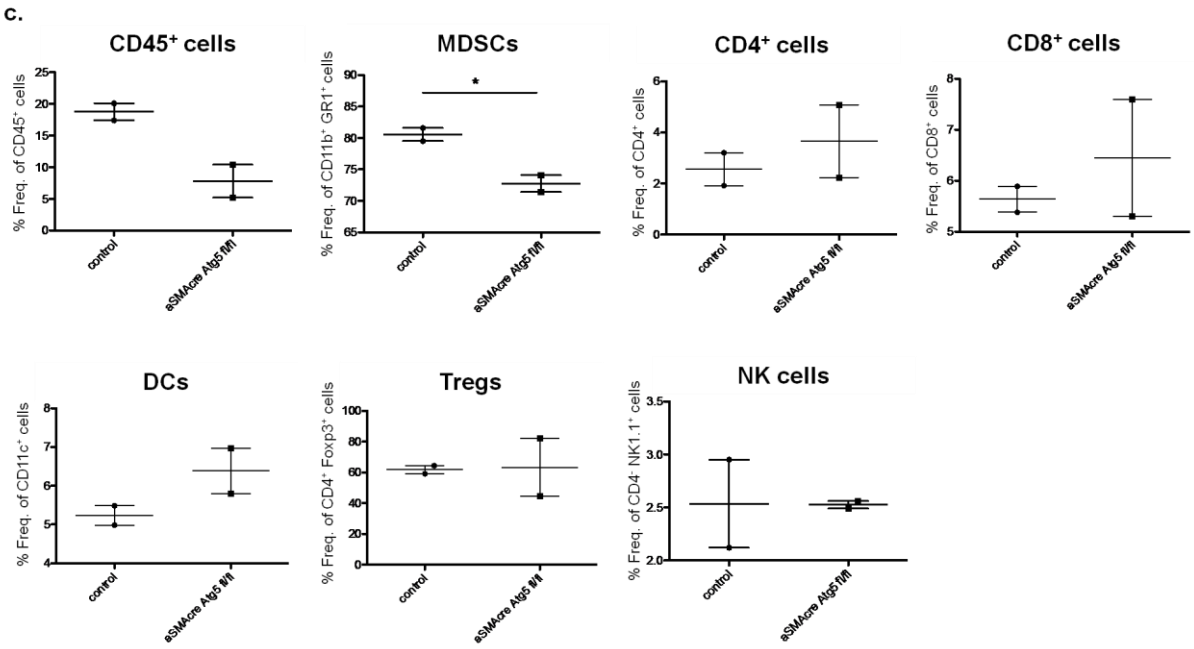


Figure 3.7.1.: (**a**) Measurement of control and α-SMA-Cre Atg5$^{fl/fl}$ tumor volume (mm^3) from Day 7 until Day 14 of tumor progression, upon B16/F10 inoculation, N=2 for control tumors (from Day 11 to 15); N=2 for α-SMA-Cre Atg $^{fl/fl}$ tumors. (**b**) Frequencies of αSMA$^+$ CAFs of tumor melanomas of control and α-SMA-Cre Atg5$^{fl/fl}$ mice on their 14th Day of tumor growth. (**c**) Frequencies of CD45$^+$ cells, CD11b$^+$GR1$^+$ MDSCs, CD4$^+$ T, CD8$^+$ T cells, CD11c$^+$ DCs, CD4$^+$ Foxp3$^+$ Tregs and CD4- NK1.1.$^+$ NK cells of tumor melanomas of control and α-SMA-Cre Atg5$^{fl/fl}$ mice on their 14th Day of tumor growth (***P <0.05*). Representative results from one experiment are shown. Results are expressed as mean ± SEM. N=2 mice per group.

Analysis from tumor melanomas revealed that upon ablation of CAFs autophagy, the frequencies of the hematopoietic CD45$^+$ cells were decreased (**Figure 3.7.1.c**), yet not significantly (*P=0.064*), indicating that α-SMA-Cre Atg $^{fl/fl}$ tumors are characterized by a lower immune infiltration in comparison with control mice (Atg5$^{fl/fl}$). Myeloid-derived suppressor cells (MDSCs) were significantly downregulated in tumor melanomas lacking autophagy from CAFs (***P<0.05*), while CD4$^+$ T cells (*P=0.55*), CD8$^+$ T cells (*P=0.56*) and dendritic cells (DCs) (*P=0.21*) showed a slight increased trend in α-SMA-Cre Atg $^{fl/fl}$ tumors in comparison to control tumors, with no statistical significance.

The frequencies of α-SMA$^+$ CAFs in Day 14th tumor melanomas appeared to be decreased in that α-SMA-Cre Atg $^{fl/fl}$ mice in comparison to control mice (**Figure 3.7.1.b.**), but with no statistical difference (*P=0.059*).

We observed that the B16-F10 tumors of α-SMA-Cre Atg5$^{fl/fl}$ were formed earlier than the tumors of control mice, and their volumes appeared to be higher in later tumor stages (Day 14th of tumor growth: *P=0.08*), yet we did not observe any significant difference (**Figure 3.7.1.a.**). These findings do not align with the existing literature, where the inhibition of autophagy in a mouse xenograft model led to significant reduction in tumor volume (New et al., 2017); nonetheless, it is worth mentioning that the inhibition of autophagy was not specific in the study of New et al. At the same direction, Zhao et al. showed that *ATG5* depletion in CAFs significantly decreased tumor growth (Zhao et al., 2017). However, in their studies, they indirectly address autophagy in CAFs, while our transgenic mouse model is suitable for directly examining the role of the autophagic pathway in CAFs, by analyzing the malignant growth and tumor immune responses of those mice.

The evaluation of the draining lymph nodes of control and α-SMA-Cre Atg5$^{fl/fl}$ mice on their 14th of tumor growth did not demonstrate statistical differences on the immune populations (CD4^{+} T cells, CD8^{+} T cells, Foxp3^{+} Tregs, NK cells) (**Figure 3.7.2.**). Further experiments are needed, with the adequate number of mice, in order to investigate whether there are differences regarding the immune profiles of α-SMA-Cre Atg5$^{fl/fl}$ mice versus the control mice.

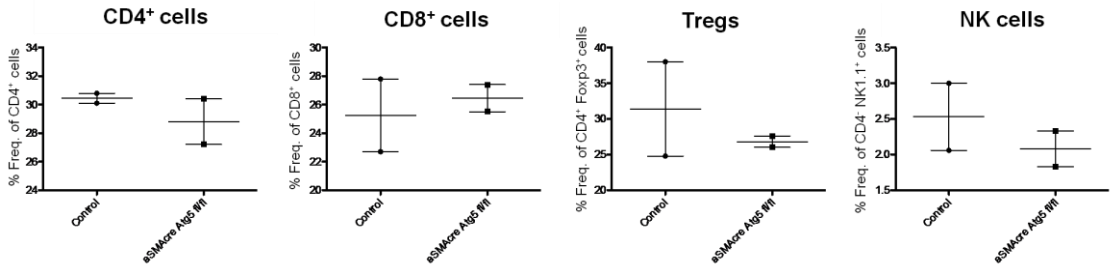


Figure 3.7.2.: Frequencies of CD4^{+} cells, CD8^{+} T cells, CD4^{+} Foxp3^{+} Tregs and CD4-NK1.1.$^{+}$ NK cells in draining lymph nodes of control and α-SMA-Cre Atg5$^{fl/fl}$ mice on their 14th Day of tumor growth. Representative results from one experiment are shown. Results are expressed as mean ± SEM. N=2 mice per group.

However, these data need to be re-evaluated, not only due to the modest number of mice used, but also because tumors were disrupted at later stages of their development and this unfortunate incident could interfere with the frequencies of those immune cell populations of the TME.

4. Discussion and Future Directions

The advents of immunotherapy have revolutionized cancer therapeutics; yet, a sizable portion of patients do not respond to these agents (Borcoman et al., 2019; Hanahan et al., 2011). Despite the operation of immune surveillance mechanisms, tumors form endowed immunosuppressive networks that impede the elicitation of potent antitumor immune responses, impairing the success of immunotherapy (Chen et al., 2014; Costa et al., 2018; Ziani et al., 2018). For that reason, a better understanding of the biology of cancer is necessary for improving these dismal outcomes.

To date, several evidence support critical roles of the tumor microenvironment (TME) in providing cancer cells with proliferative, migratory, survival and invasive propensities, favoring the processes of tumorigenesis. The cancerous reactive stroma coevolves alongside tumor progression, directly influencing the anti-tumor immune response, providing resistance to anticancer immunotherapies (Oliver et al., 2018; Polanska et al., 2013). The TME is populated by a large number of cancer-associated fibroblasts (CAFs), one of the major stromal cell types recognized in various human carcinomas (Kalluri, 2016; Kalluri et al., 2006; LeBleu et al., 2018; Polanska et al., 2013).

CAFs, although not by themselves malignant, play a critical role in the complex process of tumor cell-stroma interaction and have emerged as important regulators of the anti-tumor immune response (Augsten et al., 2014; Capparelli et al., 2012; Kalluri, 2016; Kalluri et al., 2006; Kumar et al., 2017; LeBleu et al., 2018; Ziani et al., 2018). They represent a highly heterogeneous yet abundant population of the TME, which they alter through secretion of several pro-tumorigenic molecules, while they support cancer progression (Oliver et al., 2018; Polanska et al., 2013), angiogenesis, cancer stemness, tissue invasion, metastasis, and even chemo-resistance (Augsten et al., 2014; Capparelli et al., 2012; Castells et al., 2012; Hammer et al., 2017; Kalluri, 2016; Kalluri et al., 2006; Kumar et al., 2017; LeBleu et al., 2018; Tommelein et al., 2015; Trimboli et al., 2009; Sun, 2015; Ziani et al., 2018). Although their tumor-promoting function is acknowledged (Castells et al., 2012; Hammer et al., 2017; Tommelein et al., 2015; Trimboli et al., 2009; Sun, 2015; Ziani et al., 2018), their role in shaping the anti-tumor immune response remains elusive.

Research has lately focused on the evaluation of drugs, which deplete CAFs (Duluk et al., 2015). However, subsequent clinical trials demonstrated that reducing fibrosis in addition to a first-line therapy was not beneficial for patients (Guet al., 2018). This suggests that reduction of CAFs is not a good option for cancer therapy. A better option may be to modify specific aspects of interactions between CAFs and carcinoma cells (Zhang et al., 2018). A mechanism that is involved in the interaction between tumor and CAFs and has a crucial role in the tumor

microenvironment, is autophagy, which is induced by the hypoxic conditions of the tumor stroma (Azad et al., 2008; Daskalaki et al., 2018; Jiang et al., 2017; Pouyssegur et al., 2006).

Autophagy has recently been shown to be important for multiple aspects of cancer biology (Kimmelman, 2011; Santana-codina et al., 2017; Zhang et al., 2018); its role in cancer is complex, as demonstrated by studies, describing situations in which autophagy can either promote or inhibit tumorigenesis (Kimmelman 2011; Levy et al., 2017; Santana-codina et al., 2017; Wang et al., 2016; Wang et al., 2017). Even though most studies have focused on the role of autophagy in tumor cells, the involvement of autophagy in the stromal compartment, and in particular in CAFs, has been investigated to a limited extend (Capparelli et al., 2012; Chaudhri et al., 2013; Levy et al., 2017; Liang et al., 2018).

Several studies indicate that autophagy is upregulated in various types of cancer in humans, such as primary human breast, melanoma, and esophageal cancer upon progression to advanced metastatic disease, while expression of autophagy markers in these cancers is correlated with poor prognosis (Espina et al., 2010; Galavotti et al., 2013; Whelan et al., 2017) shedding into light novel and valuable roles for autophagy at different points in the metastatic cascade (Mowers et al., 2018).

Regarding CAFs autophagy, recent evidence indicate that CAFs exhibit increased autophagic levels (Chaudhri et al., 2013; Martinez-outschoorn et al., 2010; New et al., 2017; Wang et al., 2017) and that autophagy contributes to their tumor promoting function (Sousa et al., 2016). Moreover, the inhibition of autophagy in a mouse xenograft model led to significant reduction in tumor volume (New et al., 2017), while the ablation of autohagy in the non-tumor epithelia of the TME on *a Drosophila melanogaster* malignant tumor model had a marked effect on both tumor growth and invasiveness compared to the inhibition of autophagy in only the tumor cells (Katheder et al., 2017). Zhao et al. found increased CAFs autophagy in human luminal breast cancer tissues, correlated with poor prognosis. They also showed that *ATG5* depletion in CAFs significantly decreased tumor growth, demonstrating that CAFs autophagy increase the tumorigenicity of luminal breast cancer cells (Zhao et al., 2017). However, it remains unknown what impact has the specific ablation of autophagy in CAFs on tumor growth and on tumor immune response.

Taking the above into consideration, we formed the hypothesis that the upregulated autophagy in CAFs dictates their tumor promoting function and in this project, we assesed how CAFs autophagy shapes tumor growth, anti-tumor immune response and response to immunotherapy.

Our first step was to isolate and examine CAFs *in vivo*; CAFs represent one of the most heterogeneous stromal cell populations of several carcinomas, arising from multiple origins and in that way they express various surface markers. However, the expression levels of these proteins vary in different CAF populations, where none of them are unique to activated fibroblasts (Sun et al., 2018), while many activated fibroblasts may not express all of these markers at the same time (Ziani et al., 2018). This lack of reliable and specific molecular fibroblast markers is a limiting factor in studying fibroblasts *in vivo*; in that way, as described thoroughly in the Results section 3.1., we concluded in performing analysis of CAFs by their expression of α-smooth muscle actin (α-SMA), a cytoskeletal protein associated with smooth muscle cells, which is best characterized marker of activated fibroblasts (Costa et al., 2018; Kalluri, 2016; Kalluri et al, 2006; Shiga et al., 2015). In that way, we could only evaluate their frequencies in tumors and not isolate them to perform further experiments, such as confocal microscopy to monitor their levels of autophagy. For Confocal Immunofluorescence microscopy, we used, as previously described, a fibroblastic cell line, NIH/3T3 cells.

For future experiments, we decided to isolate CAFs from α-SMA-RFP mice, a transgenic strain that has a DsRed fluorescent reporter for *α-SMA* expressing cells, and has applications in studies related to the role of *Acta2* expressing myofibroblasts in cancer (Magness et al., 2004). Through this transgenic mouse, we will be able to identify the fluorescent α-SMA positive cells, isolating efficiently activated fibroblasts, in order to perform further analysis in *ex vivo* studies.

Further on, we observed that CAFs accumulate in high frequencies in the TME of B16-F10 inoculated C57BL/6 mice during tumor progression, as expected, with a quite interesting finding that CAFs also have a strong presence in the circulation of those mice as well, as tumor develops. The identification of circulating CAFs (cCAFs), which form heterotypic clusters with circulating tumor cells (CTC), is of quite interest, as they are considered to be pre-cursors of metastatic colonies (McCarthy et al., 2018) and this population has been totally ignored from the existing literature. Further research on targeting cCAF/CTC co-clusters should provide novel avenues to abrogate melanoma metastasis.

Once we have established CAFs presence in the TME of melanoma mouse model, we next sought to investigate whether autophagy is upregulated in CAFs upon tumor progression, in order to support our hypothesis. By evaluating the kinase mTOR-dependent pathway, the best-characterized regulator of autophagy (Alissafi et al., 2018), we detected that the PI3K/AKT/mTOR axis was downregulated upon tumor progression, indicating that autophagy is properly initiated as tumor develops.

Then, in order to assess whether CAFs autophagy was being completed, we determined formation of functional autophagolysosomes *in vitro* in 3T3 fibroblastic cells (described in more details in the Materials and Methods section), based on the expression of LC3 (Alissafi et al., 2017; Alissafi et al., 2018), LAMP-1, and the adaptor protein SQSTM1/p62 (Pankiv et al., 2007). Our results also showed that p62 expression was highly enriched in TES-treated fibroblasts compared with untreated fibroblasts, while the levels of LC3 remained unchanged between the two conditions. Those finding indicate that TES-treated fibroblasts exhibit deregulated autophagy; consistent LC3 II/I levels in combination with high cytoplasmic p62 indicates impairment of autophagy flux (Liang et al., 2018). Additionally, p62 and LC3 puncta were highly co-localized, indicating aggregation of autophagolysosomes (Klionsky et al., 2016).

Accumulation of p62 could be a good indicator of autophagy suppression (Nakai et al. 2007; Wang et al., 2006). Inhibition of autophagy correlates with increased levels of p62 in mammals and in *Drosophila*, suggesting that that steady state levels of this protein reflect the autophagic status (Cui et al., 2011; Lee et al., 2010; Masiero et al., 2009; Nezis et al., 2008; Wang et al, 2006) and that p62 could exert its effect as a tumor suppressor in the tumor microenvironment (Valencia et al., 2014). *SQSTM1* (p62) changes can be cell type and context specific; in some cell types, there is no change in the overall amount of p62 despite strong levels of autophagy induction, while in other contexts, a robust loss of p62 does not correlate with increased autophagic flux. Moreover, p62 protein levels are affected by both increased transcription/translation and decreased degradation under certain conditions (Yoshii et al., 2017); for instance, p62 mRNA levels are upregulated by increased autophagic flux or activated signaling pathway, like RAF1/Raf-MAP2K/MEK-MAPK/ERK (Klionsky et al., 2016) and in muscles upon exercise, especially in combination with starvation, which can mask its degradation by autophagy even though autophagic flux is increased (Jamart et al., 2013; Sanchez et al., 2014; Yoshii et al., 2017). However, we found no difference between the expression levels of *SQSTM1* of TES-treated fibroblasts versus untreated fibroblasts, demonstrating that the highly enriched p62 levels in TES-treated cells derive from increased autophagic flux; yet deregulated.

LC3 is the only known protein that is specifically associated with all types of autophagic membranes, including phagophore, autophagosome, and autophagolysosome. Therefore, the number of LC3-II correlates well with the number of autophagosomes, which provides a good index of autophagy induction (Zhang et al., 2016). The fact that the LC3 II/I levels between TES-treated fibroblasts and untreated remained unchanged indicates autophagosome accumulation, possibly occurred due to degradation of autophagy; for example, blockage of autophagosome-lysosome fusion (Mizushima et al., 2007). The presence of LC3 II does not

always exclude the possibility that the cells have abnormal autophagy, and thus, we examined the autophagic flux using chloroquine, a lysosomal protease inhibitor that impairs autophagosome fusion with lysosomes (Mauthe et al., 2018), to confirm whether the cells are autophagy defective or competent (Mizushima et al., 2007). In our case, the amount of LC3 II/I further accumulated in the presence of chloroquine, which is in accordance with the guidelines of autophagy, demonstrating that the cells are not damaged (Klionsky et al, 2016; Mizushima et al., 2007).

The fact that TES-treated fibroblasts exhibit deregulated autophagy resulted from one experiment; further replicates of the same experiment did not demonstrate that p62 levels were elevated. Those results are inconclusive; there might be other factors that influence CAFs autophagy levels, such as cell – cell interactions, the hypoxic and hypo-nutrient conditions, oxidative stress and various extracellular and intracellular stresses, which are important for cells to adapt to or overcome unfavorable conditions. Maybe normal fibroblasts and CAFs differ in the activation of autophagy in response to nutrient limitations or CAFs further induce autophagy in response to nutrient stress. There is not always a clear correlation between changes in the levels of LC3-II/I and p62.Thus, although analysis of p62 can assist in assessing the impairment of autophagy or autophagic flux, in future experiments we would use p62, LC3 and Lamp-1 fluorescence microscopy in combination with other methods, such as Western Blotting, flow or multispectral imaging cytometry (Klionsky et al., 2016). Furthermore, in order to assess multiple possible implicating factors, we should include on our future experiments an autophagy inhibitor, such as VPS34 inhibitor, along with the already reviewed conditions.

Within the tumor microenvironment, Programmed Death Ligand-1 (PD-L1) is constitutively expressed on several non-hematopoietic cell types, including tumor-associated fibroblasts (Dezutter-Dambuyant et al, 2016; Dunne et al., 2016). We also found that CAFs exhibit increased expression of PD-L1 ligand, a fact that makes them potential therapeutic targets for immunotherapy.

We next investigated the autophagic levels of CAFs in melanoma-bearing mice that receive the immune checkpoint inhibitor, anti-PD-L1; our findings indicated that tumor-associated fibroblasts constitutively express Programmed Death Ligand-1 (PD-L1) within the tumor microenvironment, which came in accordance in the literature (Dezutter-Dambuyant et al, 2016; Dunne et al., 2016). We detected that the PI3K/AKT/mTOR pathway was downregulated upon tumor progression in anti-PD-L1treated mice, indicating that autophagy is enhanced as tumor develops, following the same trend as mice that did not receive immune checkpoint blockade. We also noticed a slight increase of the AKT/mTOR axis in α-SMA^{+}

CAFs at Day 10 of tumor growth in mice receiving anti-PD-L1 versus control mice, possibly indicating that anti-PD-L1 treatment tries to block the autophagic pathway in CAFs. However, on later tumor stages, those levels were lower in comparison with control mice, perhaps illustrating that in the end, anti-PD-L1 treatment fail to inhibit autophagy in CAFs. However, we need to re-evaluate this finding, using Confocal Immunofluorescence Microscopy etc.

In order to conclusively test the functional role of autophagy in CAFs, we generated for the first time the transgenic mice α-SMA-Cre Atg5 $^{fl/fl}$, in which autophagy is depleted from α-SMA^{+} cells. To dissect their potential role of CAFs autophagy as regulator of the anti-tumor immunity, we monitored the presence of immune cell populations infiltrating the tumor and the tumor-draining lymph nodes. We found that upon ablation of CAFs autophagy, α-SMA-Cre Atg5 $^{fl/fl}$ mice had decreased immune infiltration in comparison with control mice (Atg5$^{fl/fl}$), yet not significant (*P=0.064*). This result could be associated with growth and aggressiveness of tumors of α-SMA-Cre Atg5 $^{fl/fl}$ mice (Dunn et al., 2004), and could possibly explain the enhanced tumor volumes on later tumor stages on those mice.

The fact that B16-F10 tumor volumes appeared to be higher in α-SMA-Cre Atg5$^{fl/fl}$ mice in late tumor progression (Day 14th of tumor growth: *P=0.08*) do not align with the existing literature, where the inhibition of autophagy in a mouse xenograft model led to significant reduction in tumor volume (New et al., 2017); nonetheless, it is worth mentioning that the inhibition of autophagy was not specific in CAFs in the study of New et al. At the same direction, Zhao et al. showed that *ATG5* depletion in CAFs significantly decreased tumor growth (Zhao et al., 2017). However, in their studies, they indirectly address autophagy in CAFs, while our transgenic mouse model is suitable for directly examining the role of the autophagic pathway in CAFs, by analyzing the malignant growth and tumor immune responses of those mice.

The frequencies of myeloid-derived suppressor cells (MDSCs) that were greatly diminished (*P=0.044*) in tumor melanomas that lacked autophagy from CAFs. This result, according to the literature, could be associated with increased survival (Sevko et al., 2013) and a better clinical outcome, with restored anti-tumor immunity (Kanterman et al., 2014). Furthermore, the frequencies of T cells (CD4^{+}, CD8^{+}) and dendritic cells (DCs) had an increased trend in α-SMA-Cre Atg5 $^{fl/fl}$ tumor melanomas versus control tumors but not statistical significant, which could be an indication of good prognosis, improved survival and anti-tumor immunity (Clemente et al., 1996; Zhang et al., 2003). However, those results are do not agree with the finding above, while the analysis from the draining lymph nodes from

both of those two mice groups did not did not demonstrate statistical differences on the immune populations.

Last but not least, the α-SMA^{+} CAFs frequencies in the TME of Day 14th tumor melanomas appeared to be decreased in that α-SMA-Cre Atg $^{fl/fl}$ mice in comparison with control mice, but with no statistical difference (*P=0.059*). This result could possibly indicate that autophagy is needed for the survival of CAFs in the hypoxic and hypo-nutrient conditions of the TME. Moreover, in association with the marginally higher tumor volumes and the decreased immune cell infiltration of the tumor melanomas of α-SMA-Cre Atg5$^{fl/fl}$ mice (yet not significant), the decreased frequencies of CAFs in those transgenic mice could illustrate that autophagy in CAFs has a protective role towards malignant growth.

Overall, our results regarding the role of CAFs auutophagy in the tumor growth and immune response are ambiguous, probably resulting from the modest number of mice used for our experiments, and due to the disruption of tumor melanomas of α-SMA-Cre Atg5$^{fl/fl}$ mice. On future experiments, when we will have the adequate number of α-SMA-Cre Atg5 $^{fl/fl}$ transgenic mice, we will repeat the analysis of their tumor growth and the profile of tumor infiltrating immune cells, in order to have stronger results to support or reject our hypothesis. Moreover, we will investigate what effect has the ablation of autophagy from CAFs to tumor growth and tumor immune infiltration in response to immunotherapy, specifically to immune checkpoint inhibitor anti-PD-L1.

In addition, we plan to perform a transcriptomic study in CAFs from α-SMA-Cre Atg5 $^{fl/fl}$ mice versus control mice (Atg5 $^{fl/fl}$ mice) utilizing RNA-Seq, to identify transcripts that are responsible for possible differences in functional properties of CAFs that lack the process of autophagy. Utilizing this very sensitive technique, we would be able to investigate which cellular processes are active and which are dormant on those cells, in order to interpret the molecular mechanisms, signaling pathways and biological processes of those cells. To better understand cellular processes, a more precise comprehension of the transcriptome in individual cells will be essential for elucidating the role of CAFs autophagy in cellular functions and understanding how gene expression can promote beneficial or harmful states (Hwang et al., 2018). Furthermore, we could determine what types of molecules might be inhibited by the administration of immune checkpoint blockade (anti-PD-L1) and whether gene expression changes induced by anti-PD-L1 are likely to change the level of signaling through a given signal transduction cascade in our target cell type. Ultimately, we could potentially identify whether CAFs autophagy contributes to response or to resistance to anti-PD-L1 immunotherapy (Gide et al., 2019).

Cancer cells co-evolve with their tumor microenvironment and the role of autophagy in modulating how the cancer cell interacts with other cell types in the surrounding milieu is emerging as a key topic in determining whether autophagy inhibition is likely to be effective in cancer treatment (Mowers et al., 2018). The mechanisms underlying the effects of CAFs on cancer progression, along with the mechanisms behind the interaction between autophagy and the components of the tumor environment remain unclear (Ngabire et al. 2017).

This project addresses an unexplored hypothesis that brings into focus the functional role of autophagy of activated fibroblasts in the tumor microenvironment. Understanding the mechanism of autophagy, a major resistance mechanism to cancer therapy, at the level of CAFs in the tumor stroma, should produce novel mechanistic insights and prognostic or therapeutic procedures for the pathogenesis of a variety of solid tumors and cancer. This new knowledge can ultimately be exploited for establishing new approaches for cancer immunotherapy.

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