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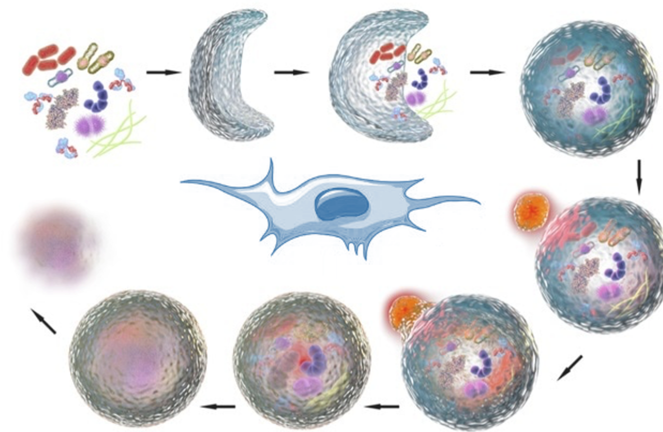
Delineation of the role of Cancer-associated fibroblast autophagy and phagocytic contexture in shaping tumor development and anti-tumor immunity



Master thesis

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A Thesis for the Degree of International Master of Science in Molecular Biomedicine

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The following Master thesis was carried out in the Laboratory of Immune Regulation and Tolerance, supervised by Panayotis Verginis, PhD. The laboratory of Dr. Verginis is located at the Center of Clinical, Experimental Surgery and Translational Research at the Biomedical Research Foundation of the Academy of Athens (BRFAA). This thesis was undertaken for fulfillment of the requirements for the degree of Master of Science in “Molecular Biomedicine: Mechanisms of Disease, Molecular and Cellular therapies and Bioinnovation”, National Kapodistrian University of Athens, Medical School.

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Abstract

Tumor microenvironment (TME) is considered crucial promoter of tumorigenesis, while it plays essential role in the regulation of immune response inside the tumors. The highly hypoxic, nutrient-deficient and rich in inflammatory factors TME activates several stress response pathways in cells, in order to enable their adaptation to these abnormal conditions. Autophagy is a fundamental catabolic pathway which is induced as a response to these conditions inside the cells while it is considered as a cardinal feature of many tumors. Autophagy can have either tumor promoting or tumor suppressing functions in cancer and this dual role has been studied extensively. Cancer-associated fibroblasts (CAFs) are the predominant non-hematopoietic cell population of TME, characterized by the expression of α-smooth muscle actin (αSMA) in their activated stage. CAFs are known for their tumor promoting functions and their critical implication in the regulation of anti-tumor immunity. However, less are known about the underlying mechanisms driving CAF-related functions. The process of autophagy in CAFs has been studied in a limited extent providing evidence for its tumor promoting function. Nevertheless, its role in shaping the anti-tumor immune response remains elusive.

Herein, we aimed to investigate the potential regulatory role of CAFs autophagy in tumor progression and anti-tumor immune response. We used αSMA-RFP transgenic mice that have a DsRed fluorescent reporter in αSMA-expressing cells, in order to identify the unique morphology and the spatial location of CAFs in αSMA-RFP mouse melanoma tumors. CAFs were found scattered among the cancer cells as well as in the periphery creating a “barrier”. Autophagy was activated in fibroblasts cultured under conditions mimicking the TME providing evidence for upregulation of autophagy in CAFs. The specific depletion of autophagy in CAFs resulted in reduced tumor growth as indicated by melanoma tumor inoculation experiments in αSMA-creAtg5$^{fl/fl}$ transgenic mice, highlighting the considerable role of CAFs autophagy in tumor progression. Despite this, the analysis of the infiltrating immune populations was characterized by great heterogeneity which may indicate that the immune alterations were a secondary phenomenon.

Collectively, our data bring into focus CAFs as an important cell population characterized by upregulated autophagy, with essential role in the regulation of tumor progression. Elucidation of this process would provide a great mechanistic insight of how CAFs influence tumor immune response and ultimately lead to development of more efficacious immunotherapeutic approaches.

Περίληψη

Το μικροπεριβάλλον του όγκου είναι κρίσιμο στην καρκινογένεση, ενώ παίζει σημαντικό ρόλο στην ρύθμιση της ανοσοαπόκρισης στους όγκους. Το άκρως υποξικό, ανεπαρκές σε θρεπτικά και πλούσιο σε φλεγμονώδης παράγοντες μικροπεριβάλλον του όγκου ενεργοποιεί μονοπάτια ανταποκρινόμενα σε στρες στα κύτταρα, προκειμένου να διευκολύνει την προσαρμογή τους σε αυτές τις συνθήκες. Η αυτοφαγία είναι ένα θεμελιώδες καταβολικό μονοπάτι που επάγεται ως απάντηση σε αυτές τις συνθήκες μέσα στα κύτταρα, ενώ θεωρείται και κύριο χαρακτηριστικό πολλών όγκων. Η αυτοφαγία μπορεί να έχει προωθητικές είτε κατασταλτικές λειτουργίες στον καρκίνο και αυτός ο διπλός ρόλος έχει μελετηθεί εκτεταμένα. Οι ινοβλάστες που σχετίζονται με τον καρκίνο (CAFs) είναι ο επικρατέστερος μη-αιμοποιητικός κυτταρικός πληθυσμός του μικροπεριβάλλοντος του όγκου, χαρακτηριζόμενοι από την έκφραση του αSMA στην ενεργοποιημένη τους μορφή. Τα CAFs είναι γνωστά για τις λειτουργίες τους στην προώθηση του καρκίνου και την κρίσιμη εμπλοκή τους στη ρύθμιση της αντι-καρκινικής ανοσίας. Παρόλα αυτά, λίγα είναι γνωστά σχετικά με τους υποκείμενους μηχανισμούς που κατευθύνουν τις λειτουργίες τους. Η διαδικασία της αυτοφαγίας στα CAFs έχει μελετηθεί σε περιορισμένο βαθμό παρέχοντας αποδείξεις για την προωθητική τους λειτουργία στον καρκίνο. Ωστόσο, ο ρόλος τους στην διαμόρφωση της καρκινικής ανοσοαπόκρισης παραμένει ασαφής.

Στην παρούσα μελέτη, στοχεύσαμε την διερεύνηση του πιθανού ρυθμιστικού ρόλου της αυτοφαγίας των CAFs στην εξέλιξη του όγκου και στην ανοσολογική απάντηση. Χρησιμοποιήσαμε αSMA-RFP διαγονιδιακά ποντίκια που εμφανίζουν κόκκινο φθορισμό στα κύτταρα που εκφράζουν αSMA, ώστε να ταυτοποιήσουμε την μοναδική μορφολογία και την χωρική διάταξη των CAFs σε μελανώματα ποντικών. Τα CAFs βρέθηκαν διάσπαρτα ανάμεσα στα καρκινικά κύτταρα καθώς και στην περιφέρεια τους δημιουργώντας ένα «φράγμα». Η αυτοφαγία ήταν ενεργοποιημένη στους ινοβλάστες που καλλιεργήθηκαν κάτω από συνθήκες που μιμούνται το μικροπεριβάλλον του όγκου υποδηλώνοντας αυξημένη ενεργοποίηση της αυτοφαγίας στα CAFs. Η ειδική απαλοιφή της αυτοφαγίας στα CAFs οδήγησε σε μειωμένη ανάπτυξη του όγκου, όπως υποδεικνύεται από πειράματα εμβολιασμού με καρκινικά κύτταρα μελανώματος στα διαγονιδιακά ποντίκια αSMA-creAtg5$^{fl/fl}$, τονίζοντας τον αξιοσημείωτο ρόλο της αυτοφαγίας των CAFs στην εξέλιξη του όγκου. Παρόλα αυτά, η ανάλυση των εισχωρούντων ανοσολογικών πληθυσμών χαρακτηρίστηκε από σημαντική ετερογένεια που μπορεί να υποδηλώνει ότι οι ανοσολογικές αλλαγές ήταν δευτερεύον φαινόμενο.

Συνολικά, τα δεδομένα παρουσιάζουν τα CAFs ως σημαντικό κυτταρικό πληθυσμό χαρακτηριζόμενο από αυξημένη αυτοφαγία, με καθοριστικό ρυθμιστικό ρόλο στην εξέλιξη του καρκίνου. Η διαλεύκανση αυτής της διαδικασίας θα παρέχει μια σημαντική μηχανιστική εικόνα για τον τρόπου που τα CAFs επηρεάζουν την ανοσοαπόκριση του όγκου καταλήγοντας έτσι στην ανάπτυξη πιο αποτελεσματικών ανοσοθεραπειών.

Introduction

1.Cancer and Tumor microenvironment

Cancer is one of the leading causes of death worldwide according to the World Health Organization (WHO). Nevertheless, despite the constant research it remains in the center of scientific interest due to the complexity of the implicating mechanisms that lead to cancer initiation, progression and surveillance. Tumorigenesis is a dynamic procedure, consisting of tumor initiation, progression and metastasis (Maonan Wang et al., 2017). According to several evidence it seems that this multistep process reflects genetic alterations leading to progressive transformation of normal cells into highly malignant derivatives (Hanahan et al., 2000). The biological capabilities acquired during the multistep procedure of tumor development comprise the hallmarks of cancer (*Figure 1*) (Hanahan et al., 2011). Over the last decades, the role of immune system in response to tumor generation as well as its functional regulatory role has been studied extensively. The better understanding of the biological background implicated in tumorigenesis is essential in order to achieve the improved efficacy of anti-cancer therapy.

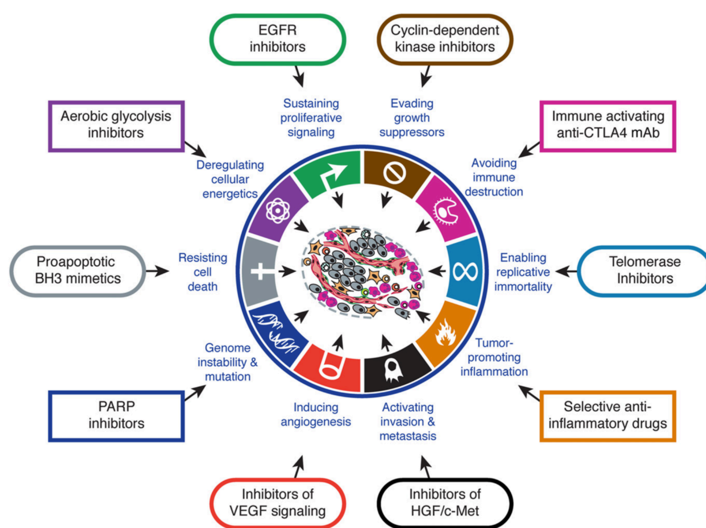


***Figure 1**. The hallmarks of cancer (Hanahan et al., 2011).*

During the last decades it has become clear that tumors are highly heterogeneous and should be conceived as organs composed by specialized cell types (Maes et al., 2013). The tumor masses are comprised not only by tumor cells, but also by non-cancerous compartments that surround the cancer cell niche (*Figure 2*). These non-cancerous components along with their interactions with the tumor cells constitute the tumor microenvironment (TME) or tumor stroma. TME consists of extracellular matrix (ECM), capillaries and a variety of cell populations, including innate and adaptive immune cells, endothelial cells, pericytes and fibroblasts. It is characterized by the presence of various stress factors, such as intratumoral hypoxia, lack of growth factors and tumor

acidosis (Maes et al., 2013; Monteran et al., 2019). The complexity of cancer biology is characterized by multicellular interactions between tumor cells and tumor microenvironment, demonstrating TME as a major regulator of tumorigenesis and therapy response. As a result, the components of TME emerge as attractive therapeutic targets (Kalluri et al., 2016). Cancer-associated fibroblasts or CAFs is the predominant population of TME in many types of cancer, with vital role in the regulation of the tumor progression and immunosuppression. Thence, CAFs are considered excellent therapeutic target that could improve the current approaches for cancer immunotherapy (Karkala et al., 2012).

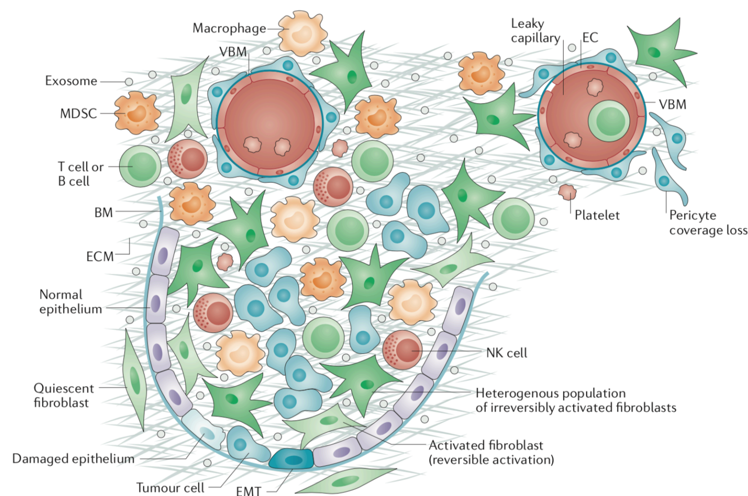


Figure 2. *The cellular components of tumor microenvironment (Kalluri et al., 2016).*

2. Immunotherapy

Immunotherapy is a rapidly advancing area of clinical oncology, which dramatically changed the clinical landscape of multiple malignancies (Fridman et al., 2017; Yu et al., 2018). Over the past years, the introduction of the immune checkpoints inhibitors for the treatment of different types of cancer has gained great interest (Fridman et al.,2017). The importance of immunotherapy has been acknowledged by the Nobel prize for physiology or medicine 2018 to James P.Alison and Tasuku Honjo for their discovery of cancer therapy by the inhibition of negative immune regulation. Tumors manage to evade the immune system through the inhibitory pathways of CTLA-4 cytotoxic-T-lymphocyte associated protein (CTLA-4)-CD28 and the programmed cell death protein 1 (PD-1) (Gajewski, Schreiber et al., 2013; Altmann et al., 2018; Kruger et al., 2019) .The specific inhibition of the immune checkpoints molecules CTLA4 and PD-1/PD-L1 with the usage of specific antibodies, allows T cells to more effectively eradicate cancer cells.

CTLA-4 modulates the CD28 co-stimulatory signaling as it is expressed on the surface of T cells and competes the activating ligands, which are expressed on the surface of antigen-presenting cells, while, it is also continuously expressed on T regulatory cells (Martins et al., 2019). Likewise,

PD-1 is also a surface receptor binds to the endogenous ligands PD-L1 and PD-L2, expressed on T cells, B cells and innate immune cells like NK and myeloid (Martins et al., 2019; Keir et al., 2008). In the absence of malignancy the activation of these signals inhibits T cell responses leading to immune tolerance and prevention of autoimmunity (Martins et al., 2019 ; Okazaki and Honjo 2006). Nevertheless, in the presence of malignancy this activation drives impaired anti-tumor immune responses, shaping an immunosuppressive environment tolerant for tumor development (Martins et al., 2019; Freeman et al., 2017). The disruption of the immune checkpoints axis with the usage of monoclonal antibodies reverses the escape of immune surveillance and promote tumor cell death in many types of cancer (*Figure 3*).

Ipilimunab (anti-CTLA-4), nivolumab (anti-PD-1) and perbrolizumab (anti-PD-L1) are the FDA-approved immune checkpoints inhibitors (ICIs), used for treatment of several malignancies including lung cancer, urothelial cancer, head and neck squamous cell carcinoma (HNSCC), renal cell cancer (RCC) and Hodgkin's disease (Kruger et al., 2019). Their usage has demonstrated significant efficacy and durable responses, whereas combination with the conventional therapeutic approaches, chemotherapy and radiotherapy seems propitious for the improved treatment of these malignancies. One more type of cancer that has shown effective response to immunotherapy is melanoma. Approximately 38% of melanoma tumors are positive for PD-L1 expression. In a recent study, increased expression of PD-L1 was detected in melanoma cancer cells compared with inflammatory signaling activators that upregulate the expression of PD-1 on T cells, driving impaired anti-tumor immune response(Atefi et al. 2014; Dermnai et al. 2018). The combination of immune checkpoints inhibitors has resulted in more effective responses for melanoma treatment, improving the patients prognosis (Dermnai et al. 2018).

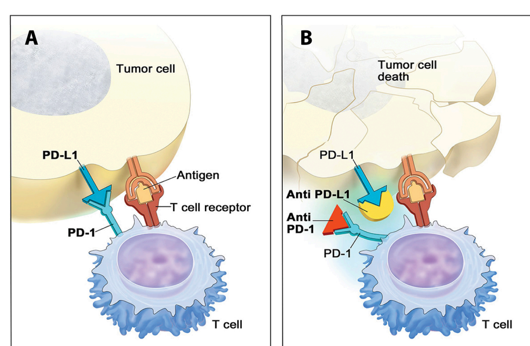


Figure 3. *Monoclonal antibodies for the blockade of immune checkpoints inhibitors PD-1 and PD-L1, promoting anti-tumor immune response and tumor cell death.*

Despite of the long-lasting responses of the therapeutic approaches in immunotherapy, its efficacy remains limited. There is still a remarkable portion of patients who do not respond (Borcoman et al. 2019; Hanahan et al. 2011; Yu et al. 2018) and a fraction of responders that become resistant to therapy (Yu et al., 2018). Tumors have the ability to form immunosuppressive networks that

strangle the possible anti-tumor immune responses evading the efficacy of immunotherapy. Therefore, a better understanding of the complexity and dynamic nature of the cell interactions that occur inside the tumor remains critical. Likewise, revealing mechanisms by which immune components can define the response to immunotherapy generates opportunities for improved patient prognosis and anti-cancer therapy.

3. Fibroblasts

3.1. Normal fibroblasts (NFs)

Fibroblasts are non-epithelial (EpCAM$^-$), non-immune (CD45$^-$) cells with mesenchymal origin and characteristics of connective tissue. They have large, spindle-shape morphology and a potential for planar polarity (Kalluri et al., 2006; Kalluri et al. 2016). In normal tissues fibroblasts are inactivated with negligible metabolism and transcriptome, sharing many characteristics with mesenchymal stem cell (MSC) precursors. In their inactivated state fibroblasts are defined as quiescent or resting fibroblasts (Kalluri et al., 2016). The quiescent fibroblasts become activated upon tissue damage as they participate in the process of wound healing, or under pathological conditions such as acute or chronic inflammation and tissue fibrosis. In this stage they are termed as "myofibroblasts" and are characterized by the expression of α-smooth muscle actin or α-SMA, a cytoskeletal protein related with smooth muscle cells (Kalluri et al. 2016; Sahai et al. 2020). Under normal circumstances they are responsible for the modulation of extracellular matrix (ECM), the production of collagens and fibronectin as well as the secretion of cytokines and chemokines (Kalluri et al., 2016).

3.2. Cancer-associated fibroblasts (CAFs)

The activation of fibroblasts inside the TME can occur through several stimuli that among others include cellular stress, DNA damage, contact signals and inflammatory factors (*Figure 4*) (Kalluri et al., 2006; Öhlund et al., 2014; Sahai *et al.*, 2020). The activated fibroblasts that are found in cancer are defined as Cancer-associated fibroblasts or CAFs. CAFs constitute the most abundant, non-hematopoietic cell population of the TME and therefore, they play a major role in its regulation (Prajapati et al., 2016; Kalluri et al. 2016; Ziani et al. 2018). The recruitment of CAFs around the tumor niche depends on the secreted factors and cytokines, derived from the tumor cells or the immune cells of the TME. The cellular metabolism of CAFs relays on aerobic glycolysis and mimics the metabolism of highly proliferating cells.

The presence of hypoxia, hypoxia inducible factor 1α (HIF1α), reactive oxygen species (ROS), the absence of nutrient and an amount of soluble factors such as, transforming growth factor-β (TGF-β), interleukin-6 (IL-6), fibroblasts growth factor-2 (FGF-2), and platelet-derived growth factor (PDGF), inside the TME can drive the metabolic shift of fibroblasts leading to their activation

(Kalluri et al., 2016). In many types of cancer high levels of TGF-β expression have been detected (Xing et al., 2010). TGF-β is considered as the major regulator of stromal fibroblasts activation and proliferation as it is known for its capability to transdifferentiate normal fibroblast to CAFs (Löhr *et al.*, 2001; Aoyagi *et al.*, 2004; Kalluri et al., 2016).

On the other hand, CAFs act via a paracrine or autocrine way, by the secretion of cytokines and inflammatory mediators. For example, CAFs themselves are considered as a major source of TGF-β inside the TME (Xing et al., 2010). Through their secretome CAFs can regulate their main functions, including ECM remodeling, angiogenesis (Vong et al., 2011), as well as the crosstalk with the tumor cells and the recruitment of the immune cells inside the TME (Tomasek *et al.*, 2002; Kalluri, 2016; Sahai *et al.*, 2020).

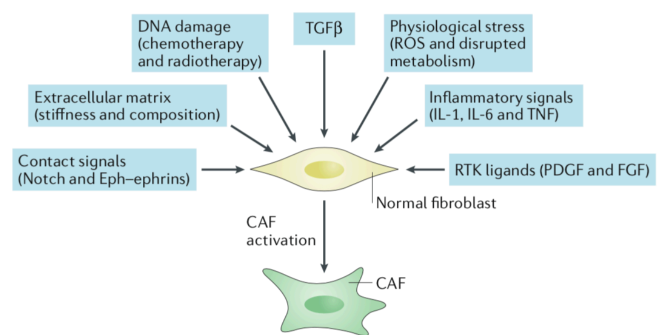


***Figure 4**. Factors of the tumor microenvironment mediate the activation of cancer-associated fibroblasts (Sahai et al., 2020).*

3.3. Origin and characterization of CAFs

The origin of CAFs is not well defined as they may come from different sources. They are derived from reprogrammed resident tissue fibroblasts, bone marrow-derived mesenchymal stem cells (BM-MSCs), adipocytes, liver and pancreas stellate cells and epithelial or endothelial cells through the processes of epithelial-mesenchymal transition (EMT) or endothelial-mesenchymal transition (EndMT), respectivelly (Xing et al., 2010; Kalluri et al., 2016; Monteran et al., 2019). This variety of origins indicates that there must be different subpopulations of CAFs in the TME, which can be characterized by a variety of markers. CAFs express fibroblast-specific protein 1 (FSP-1), vimentin, platelet-derived growth factor receptor-α or β (PDGFR-α/β), tenasin C, fibroblast activation protein (FAP), fibronectin, podoplanin and CD-90.2 and α-smooth muscle actin (α-SMA) (Kalluri et al., 2016; Monteran et al., 2019). However, none of these markers is unique for CAF population, as they may be expressed in various cell types. For example, vimentin is expressed in epithelial cells and α-SMA is expressed in smooth-muscle cells. Moreover, activated fibroblasts may express only some of these markers, depending on the biological conditions or the phase of their cell cycle. Taking together all these reflect the high heterogeneity of CAFs and the difficulty to

identify this population only by its expression markers. The characterization of CAFs must come from the combination of morphological features, spatial location and expression of proteins (Kalluri et al., 2016; Sun et al., 2018).

3.4. Function and role in cancer

In many types of cancer, the presence of CAFs is associated with more aggressive disease phenotype, poorer prognosis, increased relapse time and resistance to therapy.
Inside the tumor microenvironment CAFs accelerate a pivotal regulatory role in the cancer biology. They are implicated in the modulation of tumor-promoting inflammation in various types of cancers, either by the paracrine secretion of cytokines or by the direct interactions with the cancer cells and the immune compartments (Monteran et al. 2019). CAFs support tumor growth, protect tumor cells from the anti-tumor immune response and facilitate drug resistance, reflecting their tumor-promoting functions (Calon *et al.*, 2015; Fiori *et al.*, 2019; Monteran et al., 2019). They participate in the generation and maintenance of cancer stem cell niche through their ability to remodel ECM, enhancing the stemness and tumorigenic capacity of tumor cells (Kalluri et al. 2016; Xing et al. 2010). In addition, CAFs can influence ECM stiffness in primary tumors supporting the invasion of tumor cells (Levental *et al.*, 2009; Kalluri et al., 2016; Monteran et al., 2019). The induction of angiogenesis, the secretion of pro-tumorigenic factors and the recruitment of immune cells in the TME make CAFs important mediators of tumor progression, invasion and metastasis in distant sites (Kalluri et al. 2016; Xing et al. 2010). Furthermore, CAFs promote the resistance to therapy as they can alter the implicating signaling pathways in cancer cells, inhibit the uptake of anticancer drugs and participate in the metabolic reprogramming of cancerous and non-cancerous components of TME (Meads et al., 2009; Kalluri, et al., 2016). According to recent studies, it is getting clear that the plethora of tumor-promoting functions of CAFs is mediated by functionally distinct CAFs subpopulations (Monteran et al., 2019).

3.5. Modulation of the immune system

Among the tumor-promoting functions of CAFs is their ability to regulate immune response by shaping an immunosuppressive microenvironment (*Figure 5)*. CAFs protect tumor cells from the anti-tumor immunity contributing to the escape of immunosurveillance. The pleiotropic immunomodulatory functions of CAFs may be indirect, via the paracrine secretion of inflammatory cytokines, or direct via interactions with immune cells (Kalluri et al., 2016; Monteran et al., 2019). CAFs can recruit tumor-infiltrating immune cells in the TME, including myeloid cells and regulatory T cells (Tregs) (Augsten *et al.*, 2014; Cohen *et al.*, 2017; Monteran et al., 2019). Besides to the recruitment of immune cells, CAFs also have the ability to drive their functional differentiation into more immunosuppressive phenotypes. They can promote the activation of mast cells, the

differentiation of monocytes into macrophages and the M2 polarization of macrophages as well as the differentiation and survival of Tregs (Kalluri et al, 2016; Liu *et al.*, 2019; Monteran et al., 2019; Newton *et al.*, 2019). Furthermore, CAFs have the ability to affect the behavior of immune cells. TGF-β secretion by CAFs suppresses the activation and cytotoxic functions of Natural Killer (NK) cells and inhibits the stimulation of CD4+ Th1 cell-mediated anti-tumor immune response (Flavell *et al.*, 2010; Batlle et al., 2019; Liu *et al.*, 2019). Moreover, CAFs can restrain the antigen-presenting function of dendritic cells (DCs), by expressing TGF-β and IL-6, and disable the activation of T cells, inducing T cell anergy inside the tumor (Kitamura *et al.*, 2017; Nagarsheth et al., 2017). In addition, CAFs have the ability to behave as antigen-presenting cells, regulating the specific activation of CD4+ and CD8+ cells (Costa *et al.*, 2018; Ziani et al., 2018). A recent study indicated the antigen-presenting function of CAFs through which they suppress the anti-tumor immune response by the antigen-specific deletion of CD8+ cytotoxic T cells (Lakins et al., 2019). The shaping of an immunosuppressive TME by CAFs has also a crucial impact in resistance to immunotherapy. In this notion, a recent study showed that FAP^{high} CAFs can recruit myeloid cells via the secretion of CCL2 leading to resistance in immune checkpoint inhibitor therapy, anti-PD-L1 (Chen et al., 2017).

Therefore, due to the fundamental regulatory role of CAFs in the immune system it is essential to reveal the underlying mechanisms that drive the interactions of CAFs with the immune compartments.

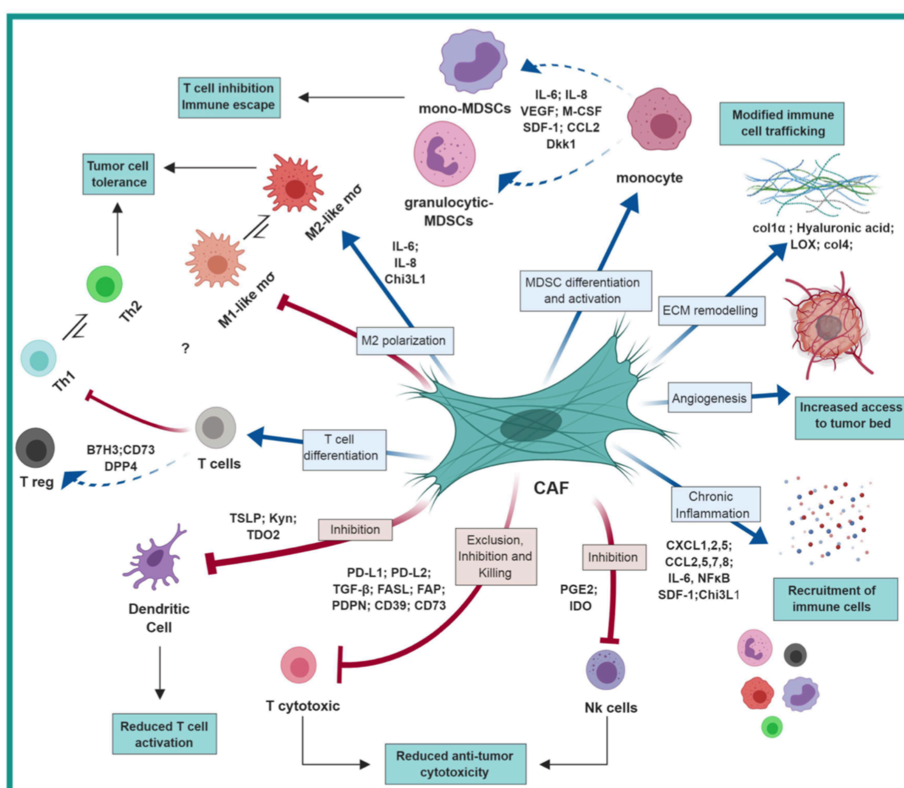


***Figure 5**. The immunosuppressive functions of CAFs (Monteran et al., 2019).*

3.6. Therapeutic approaches

CAFs have been considered as promising therapeutic targets due to their tumor-promoting functions and their ability to suppress the anti-tumor immune responses. Over the past few years several therapeutic approaches targeting CAFs have been attempted. CAF-directed therapy has designed to eliminate CAFs by depleting the expression of α-SMA or FAP or by the usage of antibodies that inactivate FAP (Özdemir et al., 2014; Takeda et al., 2014; Kalluri et al., 2016). Despite of the direct depletion of CAFs, the indirect approaches involve the targeting of the transcriptional reprogramming of CAFs that contribute to their activation or the targeting of CAF-derived cytokines in combination with immunotherapy (Sherman et al., 2014; Kalluri et al., 2016; Ziani et al., 2018; Liu et al., 2019). Nevertheless, the therapeutic targeting of CAFs remains elusive (Chauhan et al., 2019). Even though targeting CAFs seems to provide a powerful tool for anti-cancer treatment, the functional complexity and heterogeneity of CAFs, as well as the lack of unique markers require a more precise targeting and a better understanding of the underlying mechanisms driving CAFs responses.

4. Autophagy

Autophagy is a fundamental, highly conserved catabolic procedure essential for the maintenance of cell homeostasis and survival. Autophagy is known as a self-eating process through which cells manage to degrade and recycle intracellular materials and damaged proteins or organelles (Amaravadi et al., 2016; Alissafi et al., 2017, 2018; Ngabire et al., 2017). This lysosomal-dependent process occurs in every cell in basal levels, called basic autophagy, and it has an important cytoprotective role, participating in the elimination of pathogens and the engulfment of apoptotic cells (Ngabire et al., 2017). Due to the vital role of autophagy for cell survival the regulatory mechanisms that delineate its induction, have been studied extensively. Besides to its normal activation, the stimulation of autophagy can occur under conditions of cellular stress, a process called induced autophagy (Maes et al., 2013; Ngabire et al., 2017). Among others, the activating stimuli of autophagy include hypoxia, deficiency of nutrients and growth factors, DNA damage, viral infections and unfolded proteins (Pierzynowska et al., 2018). The deregulated autophagy activation is associated with a variety of disorders, including cancer. Induction of autophagy in cancer cells as well as in the other compartments of TME occurs as a response to the stressful conditions that define tumors (Ngabire et al., 2017), facilitating the adaptation of the cells in this abnormal environment.

4.1. The forms of autophagy

There are three forms of autophagy in the cells: chaperone-mediated autophagy, microautophagy and macroautophagy, which are different regarding to the implicating mechanisms and the

initiating stages (Cuervo 2004; Ngabire et al., 2017). Chaperone-mediated autophagy involves the binding of proteins or peptides by the Hsp70 chaperones, and the generation of chaperone-substrate complexes. Microautophagy is responsible for the degradation of small cytoplasmic molecules. In both processes, lysosome is the only necessary organelle, unlike with the process of macroautophagy that requires the formation of the unique double-membrane organelle called autophagosome and its fusion to the lysosome, for the effective degradation of selective cellular compartments (Maes et al., 2013). The best-characterized and most common form is macroautophagy, that's why it is usually referred as autophagy.

4.2. The process of autophagy

Autophagy is a highly dynamic, multistep procedure that involves the interaction of complex molecular mechanisms as well as the involvement of many regulatory signaling pathways. The best-characterized signaling pathway implicating in the regulation of autophagy is the PI3K/ AKT/mTOR pathway (Daskalaki et al., 2018). AKT is a serine-threonine kinase, found downstream of class I PI3K, activating mTOR, also a serine-threonine kinase that controls the transcription of many genes. Activation of this axis leads to the suppression of autophagy (Rybstein et al., 2018). The mTOR-signaling pathway maintains the balance of protein biosynthesis and cellular homeostasis under harsh metabolic alterations (Jung et al., 2010; Daskalaki et al. 2018).

Briefly, the process of autophagy requires the engulfment of a cytoplasm fragment along with the cargo proteins or organelles that aimed to be degraded, by a double membrane structure, which consists the phagophore. The capturing of the phagophore results in the formation of autophagosome, which further fuses with lysosome, generating an organelle called autophagolysosome. The internal membrane of the autophagosome is then degraded together with the enclosed molecules or organelles. The degradation products are released back to cell to the cytoplasm and can be used in a variety of cellular procedures (*Figure 6*) (Ngabire et al., 2017;Pierzynowska et al., 2018). The genes that regulate the autophagosome formation and maturation are termed as autophagy related genes (ATG). Autophagy occurs through the following distinct phases: 1) the initiation, 2) the phagophore nucleation, 2) the autophagosome formation or elongation, 4) the lysosome fusion and 5) the cargo degradation.

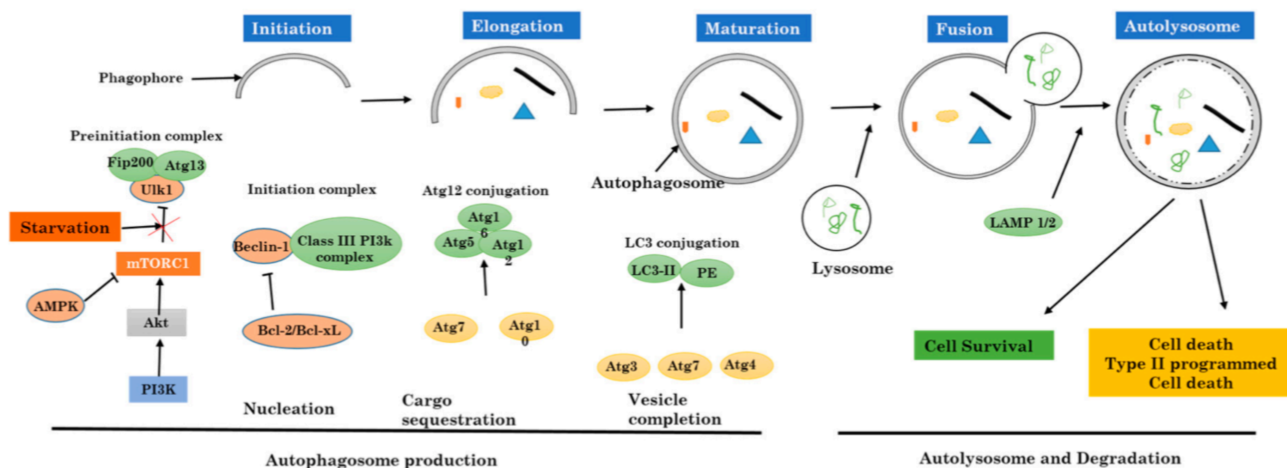


Figure 6*. The molecular process of autophagy (Ngabire and Kim).*

4.3. The molecular mechanism of autophagy

Mechanistically, the initiation step of autophagy begins with the formation of a preinitiation complex, constituted by Unc-51 like kinase (ULK-1), FIP200 protein and ATG13 (Jung et al., 2010; Ngabire et al., 2017). The mammalian target of rapamycin complex 1 (mTORC1 protein) derived from the PI3K/AKT pathway can inhibit the induction of autophagy as under normal conditions, it phosphorylates ULK-1 and ATG13 inhibiting the formation of the preinitiation complex. By contrast, under metabolic stress this effect is reversed. Adenosine monophosphatase (AMP) activated protein kinase (AMPK) can inhibit mTORC1, activating the formation of the preinitiation complex and accordingly the induction of autophagy. As a result, the ULK-1/AMPK axis is also considered one of the major regulators of autophagic pathway.

In the next step of autophagic process, during nucleation of the isolating membrane, the presence of another complex is required (Ngabire et al., 2017; Pierzynowska et al., 2018). This complex consists of class III phospatidilinositol kinase PI3K, Beclin-1 and the serine kinase p150. The core of this complex is located in the phagophore and its enzymatic activity leads the production of phosphatidylinositol-3-phosphate (PI3P). Beclin-1, is phosphorylated by ULK-1, facilitating the localization of autophagic proteins in the phagophore. The growing double membrane undergoes vehicle elongation to form an autophagosome (Levy et al., 2017). There are several regulatory elements implicating in this stage. For example, UVRAG binds to Beclin-1 and stimulates the kinase activity of PI3K facilitating the autophagosome maturation. On the other hand, proteins of Bcl-2 family are negative regulators that block these interactions inhibiting the formation of the complex (Ngabire et al., 2017; Pierzynowska et al., 2018).

Subsequently, the phase of autophagosome formation is regulated by two ubiquitin-like conjugation systems that involve ATG12-ATG5-ATG16L and LC3-PE complexes, mediated by the

ATG7, an E1-ligase like enzyme (Levy et al., 2017). At the first system ATG7 remains strongly attached to the small protein ATG12, which is further transferred to ATG10, E2-like conjugating enzyme. ATG10 sends ATG12 to ATG5, and then the ATG12-ATG5 complex interacts with ATG16L, creating a larger multimeric complex responsible for the stabilization of phagophore formation. At the second system, LC3 (Light chain 3) protein is cleaved by ATG4 protease and is converted to the cytoplasmic LC3-I form. This form is conjugated to phosphatidylethanolamine (PE) and is further modulated by ATG7 to eventually generate the lapidated LC3-II form. The large ATG12-ATG5-ATG16L complex acts like E3-ligase to the formation of LC3-PE and LC3-II complex. LC3-II is incorporated into the growing membrane of the autophagosome, while LC3-I remains in the cytoplasm (Levy et al., 2017; Ngabire et al., 2017; Pierzynowska et al., 2018). LC3-II is considered a marker for autophagosome formation (Ndoye et al., 2016). The adaptor protein sequestome 1 (p62 or SQSTM1), targets specific substrates, like misfolded or aggregated proteins, and deliver them to the autophagosomes for degradation. P62 interacts with LC3 II, and along with the other cargo proteins, they are degraded inside the autophagosomes (Levy et al., 2017). The lipid-conjugated form of LC3 and the adaptor protein p62 can be used as markers for autophagic flux.

Afterward, autophagosome fuses with early or late endosomes that provide them with factors required for the following fusion with the lysosome. This fusion results in the generation of autophagolysosome, a double membrane vehicle arising from the gathering of both compartments membranes (Levy et al., 2017; Pierzynowska et al., 2018). LC3-II, the SNARE protein STX17, the lysosomal proteins, LAMP-1 and LAMP-2, as well as the monomeric GTPases, Rab-7, Rab-22 and Rab-24, have pivotal regulatory roles in the efficient formation of a functional autophagolysosome (Levy et al., 2017; Ngabire et al., 2017; Pierzynowska et al., 2018).

The last step of autophagic procedure is the degradation of the captured compounds, which takes place inside the autophagolysosome (Ngabire et al., 2017). The low pH of this structure creates an acidic environment that activates the lysosomal hydrolases, facilitating the digestion of autophagosome contents. As a result, the trapped and engulfed compartments are degraded inside autophagolysosome and then released back to the cytoplasm, in order to meet the increased nutrient and energy demands of the cell.

4.4. Autophagy in cancer

The process of autophagy can be induced as response to various stress conditions, such as those that characterize tumors. In the central areas of tumors, the lack of vascularization leads to the deficiency of oxygen and nutrients, creating conditions of chronic hypoxia inside the TME. This can drive the inhibition of oxidative phosphorylation pathway and consequently decrease the energy supplies for the cells (Rybstein et al., 2018). Moreover, TME is characterized by increased

metabolic stress and production of inflammatory mediators (Pierzynowska et al., 2018). The combination of these events triggers the activation of autophagy both in the cancerous and in non-cancerous counterparts, maintaining their homeostasis and promoting their survival.

The role of autophagy in cancer has been studied extensively. Autophagy in cancer has a paradoxical dual role as it can act as tumor promoter and/or tumor suppressor, depending on the biological context (Singh et al., 2018).

More specifically, autophagy has a tumor suppressive function during the early stages of tumorigenesis. According to supporting evidence this function is related to the anti-oncogenic role of ATG genes. The inactivation of these genes in mouse models lead to increased tumorigenic capacity, whereas their overexpression inhibits the formation of human breast tumor in mice (Wilkinson et al., 2010; Ngabire et al., 2017).

Despite of its impact in early tumorigenesis, autophagy seems to have a positive effect in the survival of the already established tumors (Amaravadi et al., 2011; Gewirtz et al., 2016; Ngabire et al., 2017). Many evidence support the idea that activated autophagy at the later stages of tumorigenesis helps the cells to enhance stress tolerance, supporting their survival (White et al., 2009; Ngabire et al., 2017; Yun et al., 2018). Increased autophagy in cancer cells fulfills the elevated metabolic demands of these highly proliferating cells and in this way it supports the progression of tumor growth (Yun et al., 2018). Furthermore, increased autophagy is related to RAS-mutated cancer, including lung, colon and pancreatic cancer, enhancing oncogenesis and tumor aggressiveness (Masliah-Planchon, et al., 2016; Yun et al., 2018). The inhibition of autophagy or the knockdown of autophagy related genes results in enhanced tumor cell death (White et al., 2009; Yun et al., 2018).

Autophagy has been considered as a potential therapeutic target of anti-cancer therapy. On the one hand, due to the tumor suppressive function of autophagy, its induction in the early stages of tumorigenesis could lead to the prevention of tumor growth. For example, the usage of rapamycin, which inhibits mTOR pathway and induces autophagy, resulted in 90% reduction of tumor growth in murine experimental models (Ngabire et al., 2017; Yun et al., 2018).

On the other hand, as autophagy has a tumor-promoting function in the later stages of cancer progression, autophagy inhibitors, such as Chloroquine (CQ), have been used as potential therapeutic agents (Nyfeler et al., 2016) for the manipulation of tumor progression. Additionally, several studies demonstrate that induction of autophagy could increase the resistance to chemotherapy and promote cancer cell survival (Ngabire et al., 2017). As a result, the depletion of autophagy in combination with conventional anti-cancer therapies could provide powerful insights to anti-cancer therapy.

5. CAFs autophagy

Tumor microenvironment is known for the pivotal regulatory role in tumor initiation, progression, metastasis and therapy resistance. Inside the TME, cells undergo severe metabolic stress due to the hypoxic and starved conditions and the presence of inflammatory mediators. The adaptation of the cells in these harsh conditions occurs through the activation of stress response pathways, including the pathway of autophagy, which can be induced both in tumor and stromal cells (Kalluri et al., 2016; Goruppi et al., 2019; Yan et al., 2019). Recently the role of autophagy in the non-cancerous counterparts of tumors has gained attention. CAFs are the most prominent stromal population with regulatory role in tumorigenesis, tumor metabolism and anti-tumor immunity, so the better understanding of the implicating pathways that drive their activation and regulatory roles, remains critical. In this notion, many studies have focused their interest in the role of autophagy in CAFs. The role of autophagy in CAF biology is complex and may serves different functions depending on the biological context.

Activation of autophagy in CAFs is mediated by several factors, including hypoxia, glycolysis, senescence, antitumor chemicals, miRNAs and lnRNAs (*Figure 7*), which can further regulate cancer cell stemness and survival leading to increased tumor progression and recurrence (Yan et al., 2019).

The reverse Warburg effect drives the interactions of CAFs with neighboring tumor cells, resulting in the “metabolic reprogramming” of CAFs and the induction of autophagy. Lisanti and colleagues revealed the paracrine cross-talk of epithelial cancer cells and CAFs, through which increased autophagy in CAFs supported cancer metabolism and growth. Cancer cells released hydrogen peroxide and induced oxidative stress and senescence in adjacent CAFs. CAFs induced mitophagy forcing shift towards aerobic glycolysis leading to the release of metabolic products such as L-lactate, glutamine, ketone bodies, free fatty acids that can fuel the phosphorylation in cancer cell, creating a loop that supplied cancer cells with sufficient energy and nutrients to support their growth (Maes et al., 2013; Capparelli et al., 2012). Besides to the secretion of cellular cargoes in the TME, secretory CAFs autophagy can release a variety of inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-18 (Ponpuak et al., 2015), with a crucial influence on cancer cell behavior. In this way CAFs autophagy has a positive effect in the survival of tumor cells, supporting their metabolism and enhancing their proliferation.

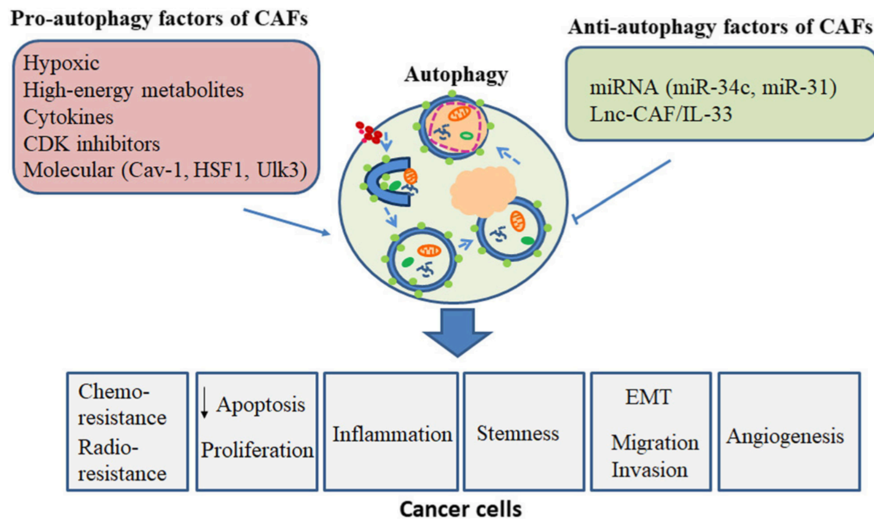


Figure 7. *Activation of autophagy in CAFs (Yan et al., 2019).*

5.1. The role of CAF autophagy

Lately, many studies highlighted the crucial role of CAFs autophagy in tumor progression. Some of them indicated metabolic alterations both in CAFs and cancer cells, due to upregulated autophagy in CAFs. In more detail, a recent study illustrated that elevated levels of autophagy in CAFs could result in metabolic changes that distinguish them from normal fibroblast or from CAFs derived from different types of cancer (Chaundri et al., 2014). According to another study, increased oxidative stress and autophagy in CAFs could positively influence the metabolism and proliferation of colorectal cancer cells (Zhou et al., 2017). In this context, Sousa et al, supported the idea that CAFs autophagy can regulate the metabolism of cancer cells, promoting the progression of pancreatic ductal adenocarcinoma (PDAC). More specifically, PDAC cells stimulated CAFs to upregulate autophagy in order to secrete alanine that fueled TCA cycle in cancer cells. According to their observations, alanine secretion via the increased CAFs autophagy promoted the proliferation of cancer cells in vitro, while, impaired CAFs autophagy (knock down of ATG7 or ATG5 with shRNA), led to decreased development of tumor growth in mice models, indicating its tumor-promoting function (Sousa et al., 2016).

Regarding to the tumor-promoting function of upregulated CAFs autophagy, a recent study, showed that upregulation of CAFs autophagy in head and neck squamous cell carcinoma (HNSCC) via paracrine and autocrine secretion of IL-6, IL-8 and bFGF, had a positive impact on tumor progression. In vitro inhibition of CAFs autophagy reduced the migration, invasion and proliferation of HNSCC cells, indicating its oncogenic function. Moreover, the combination of cisplatin, a conventional chemotherapy, with the autophagy inhibitors, SAR405 and CQ, enhanced the efficacy of treatment, resulting both from in vitro and in vivo experimental settings. Nevertheless, the inhibition of autophagy wasn't CAFs-specific, as these drugs blocked the process of autophagy generally in the cells (New et al., 2017). Furthermore, another study in a

setting of in vitro experiments showed that increased CAFs autophagy could increase the migration, invasion and proliferation of triple negative breast cancer cells (TBNC). In addition, the upregulated CAFs autophagy altered the expression of vimentin, N-cadherin and E-cadherin by cancer cells, promoting the EMT process, indicating its tumor-promoting function (Wang et al., 2017). Even though, these observations were not confirmed by in vivo experiments.

In this context, Zhao et al, found an association of increased autophagy in CAFs with more aggressive disease phenotype and poorer prognosis in luminal breast cancer. The increased autophagic activity in CAFs enhanced the stemness and tumorigenic capacity of breast cancer cells, though the HGMB1/TLR4 axis, promoting tumor progression. Further, decreased tumor growth was observed in vivo, after co-injections of CAFs with impaired autophagy (knock down of ATG5) with breast cancer cells in NOD-SCID mice, indicating that CAFs autophagy increased the tumorigenicity of luminal breast cancer cells (Zhao et al., 2017).

Despite of the extensive studies about the interactions of CAFs with the tumor cells and about the critical impact of CAFs autophagy on these cells, the role of CAFs autophagy on the immune compartments of the TME remains elusive.

Rationale and objective

The non-cancerous components that surround the tumor niche have the ability to create an immunosuppressive tumor microenvironment, promoting the progression of the tumor and preventing the efficacy of immunotherapy. The tumor microenvironment is characterized by hypoxic and low-nutrient conditions that influence the behavior and metabolism of the tumor cells as well as of the non-cancerous components of TME. The adaptation of the cells in these abnormal conditions is managed by the induction of several stress response pathways, including the pathway of autophagy. Autophagy is a fundamental self-digestive catabolic procedure, during which the cytoplasmic contents, damaged proteins and organelles are used for the recycling of nutrients and energy inside the cell, maintaining the cellular homeostasis and cell. Autophagy is considered a cardinal feature of many tumors and it is known for its dual role to act either as tumor promoter or/and as tumor suppressor, depending on the biological context. The process of autophagy has been studied widely regarding its functional role in tumor cells. However, less is known about the role of autophagy in the non-cancerous compartments of the TME. CAFs constitute the most dominant population of TME, known as a highly heterogeneous population with a pivotal role in the regulation of tumorigenesis and anti-tumor immunity. CAFs shape an immunosuppressive environment through which they promote tumor progression, invasion and metastatic dissemination. They affect the recruitment and functional differentiation of immune cells, modulating the anti-tumor immune responses. CAFs manage to suppress immune responses through functions that involve their secretome, through directly interactions with other cells or through their ability to adopt antigen-presenting functions. Furthermore, CAFs have the ability to promote resistance to conventional therapy and impair the efficacy of anti-PD-L1 immunotherapy. Targeting CAFs is an attractive therapeutic approach, even though its efficacy remains elusive. As a result, it is important to reveal the underlying mechanisms behind their tumor-promoting and immunosuppressive functions. A better understanding of the CAFs interactions with the immune compartments could lead to great insights for the improvement of anti-cancer therapy.

Recently several studies have focused on the functional role of autophagy in the non-cancerous compartments, especially in CAFs. The highly hypoxic and low-nutrient tumor conditions lead to the induction of autophagy in CAFs, which seems to have an important impact in the survival and proliferation of the tumor cells. CAFs autophagy is responsible for several metabolic alterations in tumor cells, supporting tumor growth. Yet increased autophagy in CAFs promotes the tumorigenic and migratory capacity of cancer cells in many types of cancer and has been linked to more aggressive progression and poorer prognosis of the disease. The inhibition of autophagy in CAFs reverses its tumor-promoting function, both at in vitro and vivo sets of experiments, however the autophagy depletion was not CAFs-specific.

Even though these studies provide great insights regarding to the role of CAFs autophagy, a more precise and in-depth investigation remains essential for the better understanding of CAFs interference in tumor biology. Moreover, despite of the essential regulatory role of CAFs, less is known about the implicating mechanisms that modulate the interactions between CAFs and immune cells. The role of CAF autophagy in the immune contexture of the tumor has not been studied yet. Therefore, pending questions like which is the role of CAF autophagy in the immune response, how the depletion of CAFs autophagy shapes the tumor progression and anti-tumor immunity or which is the impact of immunotherapy in CAF autophagy are generated. Further, the depletion of CAFs autophagy in combination with the immune checkpoint inhibitor, anti-PD-L1, could have a positive effect on tumor progression and increase the efficacy of immunotherapy.

Materials and Methods

Mice: αSMA-RFP, $Atg5^{fl/fl}$ 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). αSMA-cre;$Atg5^{fl/fl}$ mice were generated by crossing $Atg5^{fl/fl}$ mice (Hara et al., 2006) (RIKEN BioResource Center) and αSMA-cre mice (kindly provided by Dr.Raghu Kalluri, Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX). 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 (Invitrogen):

For $Atg5^{fl/fl}$ mice:

- exon 3-1: 5'-GAATATGAAGGCACACCCCTGAAATG-3',
- short2: 5'-GTACTGCATAATGGTTTAACTCTTGC-3',
- check2: 5'-ACAACGTCGAGCACAGCTGCGCAAGG3'

For αSMA-cre mice:

- 5'-ACATGTCCATCAGGTTCTTGC-3',
- 5'-AGTGGCCTCTTCCAGAAATG-3',
- 5'-TGCGACTGTGTCTGATTTCC-3',
- 5'-GGTGTTAGTTGAGAACTGTGGAG-3'.

Cell lines: B16-F10 mouse melanoma tumor cells (kindly provided by Prof. Aristedes Eliopoulos, Medical School, University of Crete, Heraklion, Greece) were 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 mouse fibroblastic cell line (kindly provided by Dr.Dimitris Thanos, Biomedical Research Foundation Academy of Athens) was cultured in DMEM with 1.5 g L^{-1} $NaHCO_3$ (Gibco), 10% fetal bovine serum (STEMCELL Technologies) and 5% (vol/vol) penicillin-streptomycin (Gibco) (Complete DMEM Medium). NIH/3T3 were treated with a variant of different stimuli that could induce autophagy: LPS (1μg/ml), IFNα (100ng/ml), TES (30 % v/v), NH_4Cl (15mM) overnight (O/N;16-18hr) and TGFβ (1ng/ml) for 12hr and 24 hr. Similarly, NIH/3T3 cells

were cultured under different conditions that could induce autophagy including serum starvation and hypoxia.

For the induction of serum starvation cells were cultured in DMEM with 1% FBS for 40hr. For induction of hypoxia, at first, cells were cultured in starvation (DMEM 1% FBS) for 2hr and then incubated with 75% mineral oil and 25% complete medium for 1hr. Then washed very well with DMEM and PBS and used for experiment. As control groups we used untreated, cultured cells for the corresponding period and untreated, uncultured cells.

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

Tumor induction experiments: For tumor inoculation experiments, gender-matched mice were subcutaneously injected in the flank with 3×10^5 B16-F10 mouse melanoma cells resuspended in 100μl of PBS. Tumors were measured regularly with digital calipers, and tumor volumes were calculated by the formula length × width × (length × width) 0.5 × π/6, during the days 10-15 of tumor growth. At day 15 mice were sacrificed for isolation of melanoma tumors and inguinal lymph nodes and analyzed with flow cytometry. Animals were excluded only if tumors failed to form or if health concerns were reported.

Tail and Lung tissue processing for Fluorescence–Activated Cell Sorting (FACS): In order to obtain normal fibroblasts (NFs), tail and lung tissues were isolated from naïve αSMA-RFP 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 DNase 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^oC), resuspended in PBS-5% FBS buffer and stained with the proper antibodies.

Lymph node processing for Fluorescence–Activated Cell Sorting (FACS): Mouse lymph nodes (total or draining inguinal lymph nodes) 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^oC), resuspended in PBS-5% FBS buffer and stained with the proper antibodies.

Spleen and Bone Marrow tissue processing for Fluorescence–Activated Cell Sorting (FACS): Bone marrow and spleen were isolated from $Atg5^{fl/fl}$ and αSMA-cre; $Atg5^{fl/fl}$ mice. For bone marrow isolation, cells were flushed out with ice cold PBS, the femurs, tibiae and humeri. The single-cell suspension of spleen and bone marrow was pelleted by centrifugation (1650 rmp,

4°C), incubated with erythrolysis medium (2 min, RT). The cells were pelleted through centrifugation (1650 rpm, 10 minutes, 4°C), resuspended in PBS-5% FBS buffer and stained with the proper antibodies.

Tumor-tissue processing for Fluorescence–Activated Cell Sorting (FACS): Melanoma tumors from αSMA-RFP, αSMA-cre;Atg5$^{fl/fl}$ and control mice 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 DNase 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 min,4°C) and resuspended in PBS-5% FBS buffer; staining with the proper antibodies as described below.

Flow Cytometry and Cell Sorting: The characterization and isolation of different immune cell populations of the tumor was performed using the following antibodies:

Immune population	Markers
Leukocytes	**$CD45^+$**
Endothelial	**$CD31^+$**
CD4 T cells	**$CD45^+CD4^+$**
CD8 T cells	**$CD45^+CD8^+$**
Regulatory Tcells (T-regs)	**$CD45^+CD4^+Foxp3^+$**
Dendritic cells (DCs)	**$CD45^+CD11c^+$**
Macrophages (MΦs)	**$CD45^+CD11c^-CD11b^+F4/80^+$**
Myeloid-derived suppressor cells (MDSCs)	**$CD45^+CD11c^-CD11b^+GR1^+$**
Cancer-associated fibroblasts (CAFs)	**$CD45^-CD31^-\alpha SMA^+CD90.2^+$**

For extracellular staining single-cell suspensions were incubated with the proper amounts of extracellular antibodies diluted 1:200 in PBS-5% FBS, at 4°C for 20 min. The following antibodies (Biolegend) were used: CD45 (clone 30-F11), CD31(clone 390), CD4 (clone GK1.5), Cd8 (clone 53-6.7), CD11c (clone N418), CD11b (clone M1/70), GR-1 (clone RB6-8C5), PD-L1 (clone

10F.9G2), CD90.2(clone Thy-1.2), PDGR-α (clone APA5). For Foxp3 intracellular staining with anti-Foxp3 (1:50; clone 150D; Biolegend), the cells were fixed, permeabilized and stained with eBioscience Foxp3 Staining Buffer Set (ThermoFisher), following the procedure according to manufacturer's instructions. For intracellular staining with anti-αSMA (FITC; 1:100;1A4;ab8211; Abcam) and phosphorylated proteins: pmTOR (PE; clone MRRBY ;Biologend;1:100;), pAKT (PE-CF594;clone M89-61;BD Bioscences;1:50;), pS6 (efluor 450;clone cupk43k;ThermoFisher;1:50) and p4EBP1 (efluor 660; clone V3NTY24;ThermoFisher;1:50), cells were fixed and permeabilized using the eBioscience Intracellular IC Fixation Buffer and Permeabilization Buffer (ThermoFisher) according to manufacturer's instructions. Following the staining, cells were washed with PBS and resuspended in PBS-5%FBS.

Tumor Explant Supernatant (TES): B16-F10 mouse melanoma tumors were isolated at day 15th of tumor growth and prepared as described above. Then the single cell suspension was plated on 6-well plate (10^7 cells/ml) in complete medium (RPMI 10% FBS) and cultured O/N (37°C, 5%CO_2). The next day, the supernatant (S/N) was collected, centrifuged (2000 rpm, 10min, 25°C) and used for experiment.

Immunofluorescence experiments: For immunofluorescence experiments in melanoma tumors derived of αSMA-RFP mice, tissues were fixed with 4%PFA (paraformaldehyde) in PBS (2hr, 4°C), washed three times with PBS and placed in 30% sucrose solution (overnight, 4°C). Next day, they frozen in optimal cutting temperature (OCT) compound (Tissue Tek, Sakura). Frozen 10 μm sections were obtained using a Leica (CM1950) cryostat. Sectioned tissues were washed in PBS (5 min, RT) and blocked in Blocking solution (1% bovine serum albumin, containing 0.1% Triton X-100 in PBS) for 30 min. For visualization of the nuclei, tissues were stained with DAPI (1:100, 3min; Sigma-Aldrich). Images were obtained using an inverted confocal live cell imaging system Leica SP5. Image processing was performed using Fiji software.

Immunohistochemistry (IHC) experiments: Lung, intestine and melanoma tumors from αSMA-cre;Atg5$^{fl/fl}$ and ATG5$^{fl/fl}$ mice, were fixed in formalin solution overnight, then transferred to 70% EtOH and embedded in paraffin. Paraffin sections were cut and stained with Hematoxylin and Eosin (H&E), which performed using standard histology procedures.

Immunocytochemistry (ICC) experiments: For immunofluorescence experiments NIH/3T3 cells were plated and treated on poly-Lysine coated glass coverslips. Afterwards, they were fixed with 4% PFA (paraformaldehyde) in PBS (15 min, RT) and followed by post fixation with ice-cold methanol (10 min, RT; Sigma). The confocal staining protocol that was implemented included blocking with Permeabilization (PS) Buffer (0.1% saponin, 2% BSA in PBS; 15 min, RT). Cells

were afterwards incubated with primary antibodies in PS Buffer for autophagy staining 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. Then stained with the secondary antibodies in PS Buffer: 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 45 min. For visualization of the nuclei, cells were stained with DAPI (1:100, 3min; Sigma-Aldrich). 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).

Western blot analysis: Whole cell lysates from NIH/3T3 cells, were subjected to 12% SDS-Polyacrilamide gel electrophoresis and then transferred to Nitrocellulose membrane (Millipore). Membranes were blocked with 5% blocking solution (5% non-fat, dry milk/1% BSA), overnight at 4°C. The next day, membranes were incubated with purified rabbit anti-p62, (1:1000; PM045/SQSTM1; MBL), mouse anti-LC3I/II (1:1000; Novus) and monoclonal mouse anti-β-actin, (1:5000, Millipore) as a loading control (1:30hr, RT). Afterwards, they incubated with the secondary detection antibodies rabbit, anti-mouse IgG, HRP conjugate (1:5000, MIllipore) and anti-rabbit IgG HRP conjugated (1:2000) in 2% blocking solution (2% non-fat, dry milk, 1% BSA) for 1 hour at RT. Detection was performed using Pierce ECL Western Blotting Substrate kit (Thermo Scientific).

Quantitative PRC (qPCR) analysis: Total RNA from NIH/3T3 cells treated with TGFβ and control untreated cells 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 (Ivitrogen):

For mouse SQSTM1:

- Fwd: 5'- AGGATGGGGACTTGGTTGC- 3',
- Rev: 5'- TCACAGATCACATTGGGGTGC- 3',

For mouse Hprt:

- Fwd: 5'- GTGAAACTGGAAAAGCCAAA-3',

- Rev: 5’- GGACGCAGCAACTGACAT-3’.

Phagosome purification:

NIH/3T3 cells were incubated with magnetic beads (2,8μm, Dynabeads, Invitrogen) at a ratio 3 beads/cell, for 1hr in pulse at 37°C (CO2-independent conditions) and then treated with LPS (1μg/ml) for 4hr and 16hr or treated with TGFβ (1ng/ml) for 8hr and 16hr. At the end of the chase cells washed with Homogenization Buffer (8% sucrose, 3mM Imidazole, 2mM DTT, a complete 1x ULTRA protease inhibitor cocktail solution) and pelleted by centrifugation (1800rpm,10min, 4°C). Pelleted cells containing the magnetic beads, were resuspended in 1ml of HB and an 1ml syringe with a 22g needle was used in order to break them. Then, the eppendorf was placed on a magnetic stand and washed three times with ice-cold 0.1%PBS-BSA. The remaining beads were resuspended with the proper amount of RIPA buffer, incubated on ice for 30min and centrifuged (full speed, 10min, 4°C) in order to collect the supernatant which contained the phagosomal content. A panel of antibodies was used in order to check the purity of the purified phagosomes with western blot analysis, including mouse anti-Rab7 (1:1000; Abcam), rat anti-LAMP-1 (1:500; Santa Cruz), mouse anti-LC3I/II (1:1000; Novus) and monoclonal mouse anti-β-actin, (1:5000, Millipore) as a loading control.

CD90.2 tumor-associated fibroblasts isolation kit

We isolated CD90.2 CAFs from tumors of αSMA-cre;Atg5$^{fl/fl}$ and αSMA-cre female mice at day 20 upon the tumor inoculation according to the manufacturer’s instructions. Further, we isolated total RNA with NucleoSpin RNA XS kit (Macherey-Nagel) in order to perform RNA-sequencing analysis in these cells.

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 ± SD. Differences were considered statistically significant at $P < 0.05$. Data were analyzed using GraphPad Prism v8 software.

Results

1. Isolation and characterization of fibroblasts from naïve and melanoma bearing αSMA-RFP mice

The lack of reliable and specific molecular markers for fibroblast characterization remains a major limiting factor in studying CAFs in vivo. We used the transgenic mouse model αSMA-RFP in order to identify fibroblasts either in normal tissues and or inside the melanoma tumors. These transgenic mice have a DsRed fluorescent reporter in cells that express α-smooth muscle actin (αSMA), a well-established marker for the characterization of activated fibroblast. The red fluorescent αSMA$^+$ cells exhibited the classical large, spindle-shape mesenchymal morphology of CAFs (Xing et., 2015; Kalluri et al., 2016) in cryosections derived from melanoma bearing αSMA-RFP male mice, sacrificed at day 15 after the tumor inoculation (*Figure 1.1*).

CAFs were found both scattered among the tumor cells and arranged in the periphery, creating a “barrier” around the tumor cells niche. CAFs have a promoting role in tumor progression by interacting with other cell populations, including tumor and immune cells, according to the literature (Xing et., 2015; Kalluri et al., 2016). The “CAF barrier” could indicate that CAFs prevent the infiltration of immune cells, shaping an immunosuppressive TME.

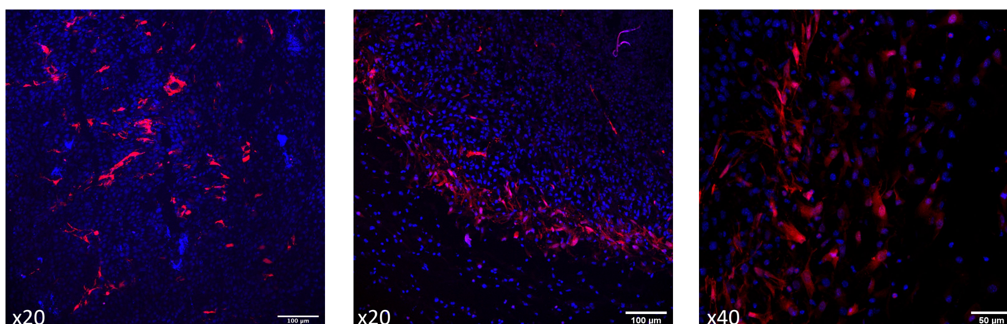


Figure 1.1: *Characterization and isolation of CAFs from αSMA-RFP mice. Representative confocal images of melanoma tumor 15 days after the inoculation in αSMA-RFP mice.*

Furthermore, we used Fluorescence-Activated Cell Sorting (FACS) in order to analyze the profile of αSMA-RFP$^+$ cells. We isolated normal fibroblasts (NFs) from tail and lung tissues of naïve αSMA-RFP mice as well as CAFs derived from melanoma bearing mice (*Figure 2*).

In order to minimize the contamination of other cell population that may express the endogenous fluorescence but not belong to fibroblasts (i.e. fibrocytes, pericytes), we used as markers for negative selection antibodies for CD45 and CD31, which are not expressed by fibroblasts. So, we sorted fibroblasts as CD45$^-$CD31$^-$αSMA-RFP$^+$. To further analyze these cells, we used antibody for CD90.2, another commonly used marker for fibroblast characterization.

We observed that a great percentage of αSMA expressing cells, also expressed CD90.2 in naïve tail and melanoma tumor (αSMA-RFP$^+$CD90.2$^+$). According to this gating strategy, the CD45$^-$ CD31$^-$αSMA-RFP$^+$ cells were sorted from melanoma tumors and after one week of seeding exhibited the typical characteristics of CAFs (αMEM, 20% FBS): large, spindle-shape mesenchymal morphology of CAFs with a potential of planar polarity as it mentioned in the literature (Xing et., 2015; Kalluri et al., 2016).

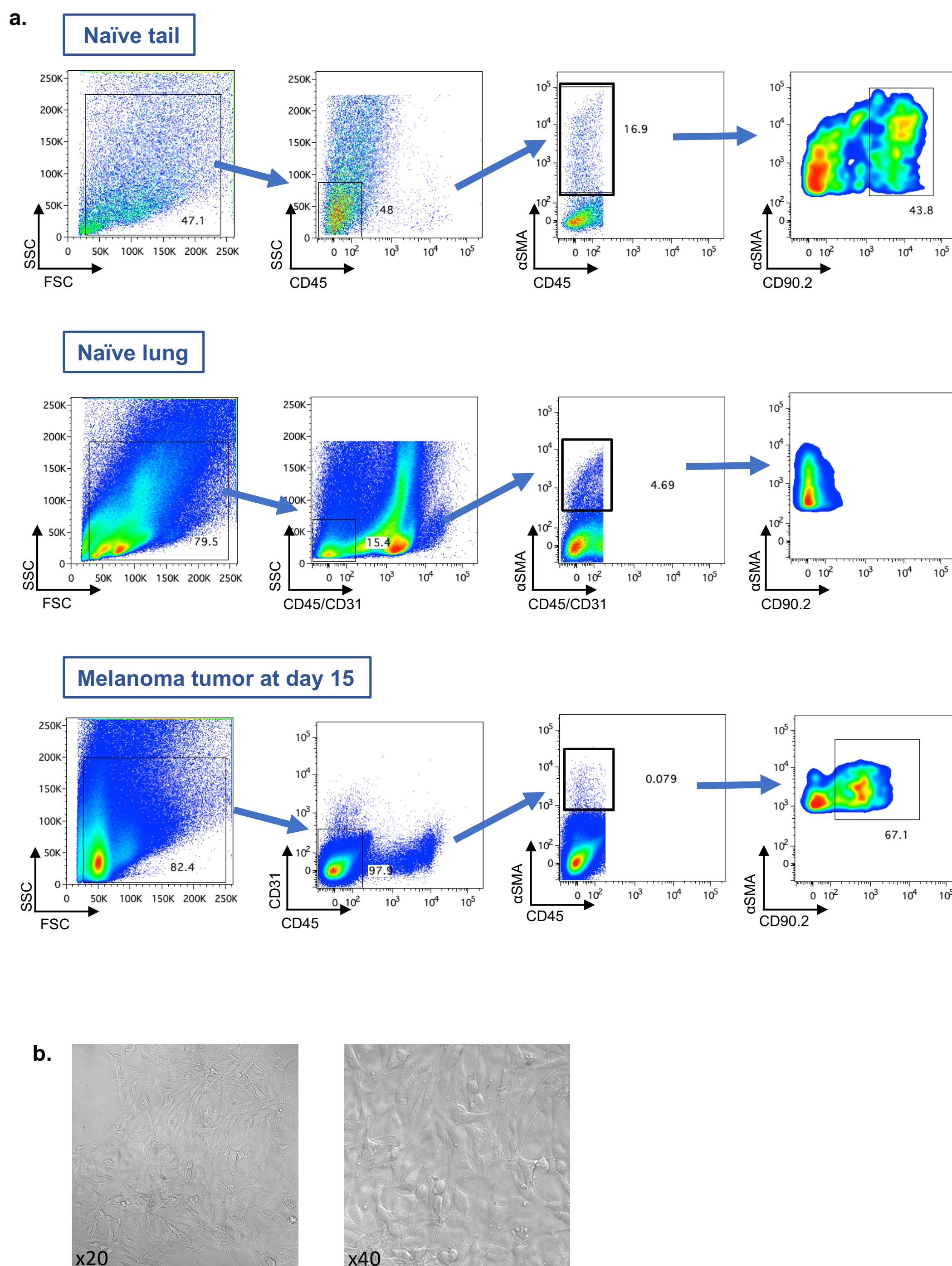


__Figure 1.2: a)__ Gating strategy for fibroblasts isolation from αSMA-RFP mice with FACS. Tail of naïve αSMA-RFP mouse, lung of naïve αSMA-RFP mouse, and B16-F10 melanoma tumor after 15 days of inoculation. __b)__ Representative images of CAFs (αSMA-RFP+) isolated at day 15 from B16-F10 mouse melanoma tumors and cultured for 7 days in α-MEM 20% FBS.

2. Ex vivo examination of CAFs autophagy

Moreover, the ex vivo evaluation of autophagy in those cells based on Confocal Microscopy experiments, provided evidence for activated autophagy (*Figure 2*). We observed functional formation of autophagolysosomes, based on the expression of the structural autophagosome protein LC3, the p62 protein that targets proteins for lysosomal degradation, and the lysosomal marker LAMP-1, which is essential for the fusion of autophagosomes with the lysosomes (Klionsky et al., 2016; Alissafi et al.,2018). The confocal immunofluorescence images demonstrated less p62 in CAFs from melanoma tumors compared with normal fibroblasts from tail, while the LC3 seemed to remain unchanged. Nevertheless, despite of the distinct fibroblast population determined by the FACS analysis, the ex vivo study of CAFs autophagy generated limitations that need troubleshooting, as the number of sorted cells was insufficient in order to be used for statistical analysis of the expressing proteins.

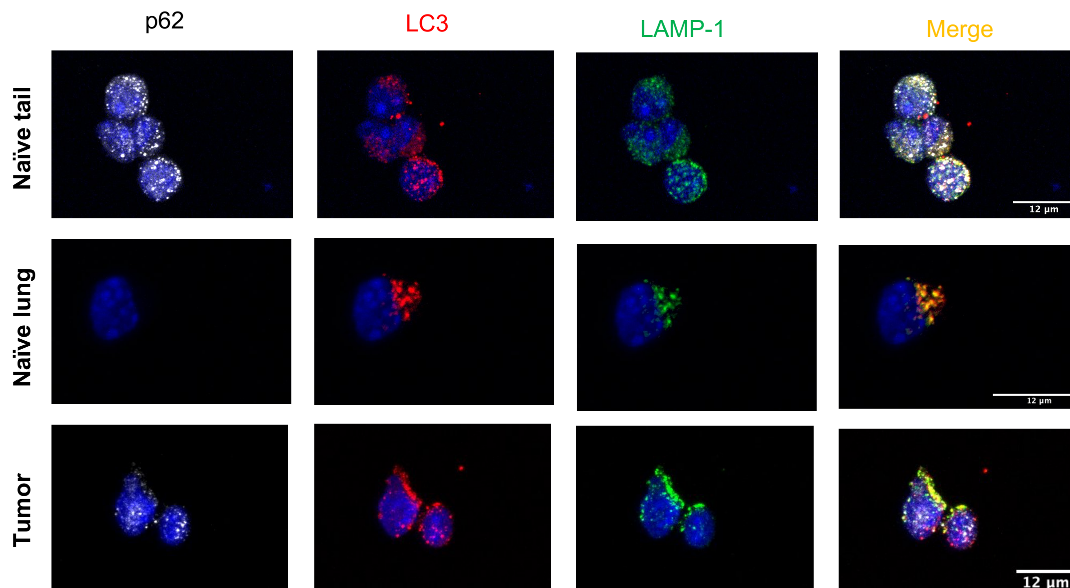


Figure 2: *Assessment of autophagy in sorted fibroblasts from naïve mice (tail and lung) and melanoma tumors: p62 (silver white), LC3 (red), LAMP-1 (green) and DAPI (blue).*

3. Establishment of a model for studying CAFs autophagy in vitro

The difficulties of CAFs autophagy evaluation ex vivo, generated the need to establish a model that could allow us to study CAFs autophagy in vitro. For this purpose, we cultured the mouse fibroblastic cell line, NIH/3T3, in a variety of different conditions, similar to those that dominate the tumor microenvironment, in order to establish the most relevant to cancer and address whether the autophagic pathway is upregulated.

3.1. Hypoxia

Firstly, we chose to culture fibroblasts in hypoxia, a predominant condition of the tumor microenvironment, capable to promote the induction of autophagy in many types of cells (Petrova et al., 2018; Jing et al., 2019). Autophagy is a highly dynamic process, that can be activated by a variety of stimuli, depending on the content and the cell type (Daskalaki et al., 2018). Even the attachment to the plastic bottom of the culture plate could be enough to activate this process. So, as the baseline of autophagy in NIH/3T3 cells is not clear, we chose to used two different controls in order to gain more precise conclusions.

We observed statistically significant decreased levels of p62 and increased levels of LC3 in hypoxic cells compared with normoxic, uncultured cells (control 1) and normoxic, cultured cells (control 2). Increased expression of LC3 denotes enriched autophagosome formation, while decreased expression of p62 denotes its degradation, both indicating upregulation of autophagy (*Figure 3.1.1*). According to our observations, autophagy is upregulated in CAFs under hypoxia.

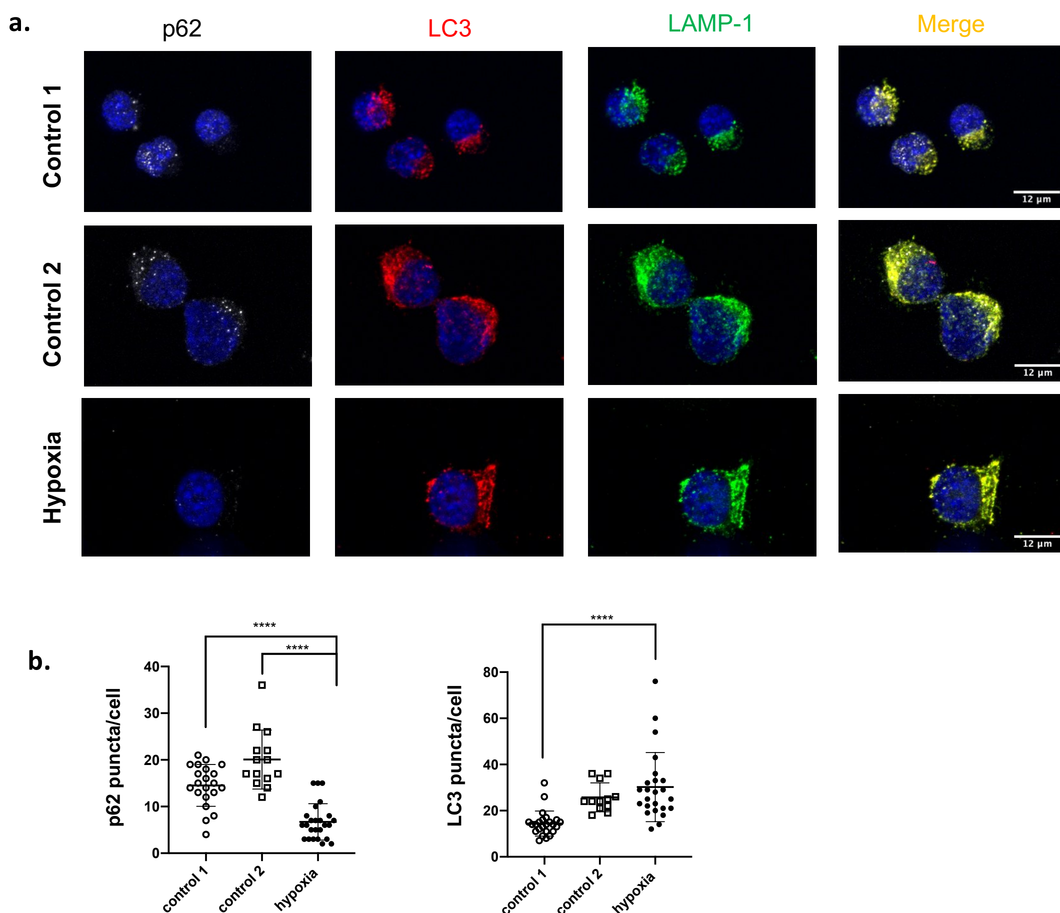


Figure 3.1.1: *Assessment of autophagy under hypoxia.* ***a)*** *Representative confocal immunofluorescence images of NIH/3T3 mouse fibroblasts in hy[...] white), LC3 (r[...] (blue).* ***b)*** *p62 puncta/cell and LC3 puncta/c[...] (***P < 0.0001 expressed as mean [...] (Images analyze[...] processing sc[...] uncultured normox[...], Control 2: cultur[...]s.*

PI3K
AKT
mTOR
S6K
4EBP1
Autophagy

Moreover, in order to determine the intracellular signaling pathway that could drive the upregulation of CAF autophagy in this experimental setting we examined the activation of PI3K/AKT/mTOR axis, which is one of the best-characterized regulators of autophagy. Activation of PI3K leads to activation and phosphorylation of AKT, which in turn, activates its downstream effector mTOR and subsequently drives the suppression of autophagy. Activated mTOR phosphorylates many protein targets, among them, S6k (p70 ribosomal S6 kinase) and 4EBP1 (initiation factor 4E Binding protein 1). In order to study this pathway, we assessed the levels of the phosphorylated proteins AKT (pAKT), mTOR (pmTOR), 4EBP1 (p4EBP1) and S6 (pS6) in hypoxic and normoxic cells with flow cytometry analysis. We observed downregulated expression of pAKT and pS6 in hypoxic cells, while pmTOR and p4EBP1 seemed to remain in the same levels (*Figure 3.1.2.*). These observations could indicate the implication of alternative pathways driving the activation of autophagy under the hypoxic environment.

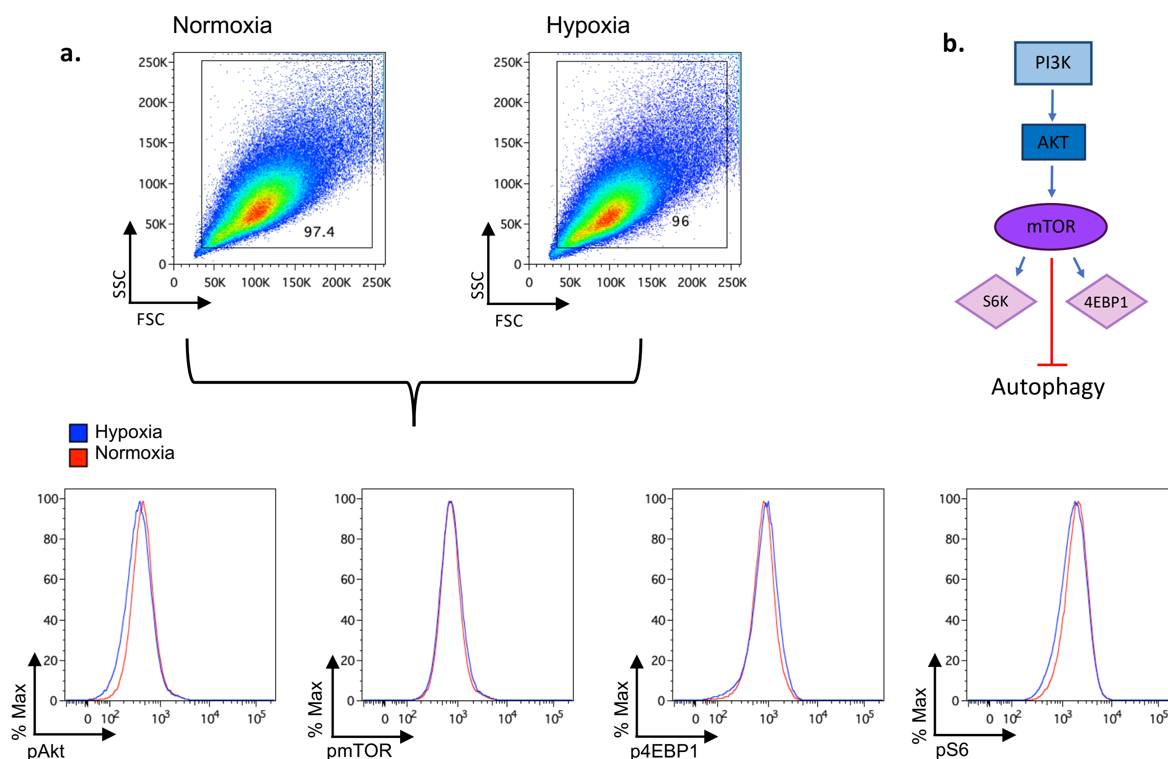


Figure 3.1.2: a) *mTOR pathway in hypoxic cells.* ***b)*** *Schematic illustration of PI3k/Akt/mTOR axis regulating autophagy*

3.2. Starvation

Expect from hypoxia, another dominant condition of the TME is nutrient deprivation. In a second setting of experiments, we studied fibroblasts cultured in starvation, the most potent known physiological inducer of autophagy. Under starvation NIH/3T3 cells exhibited increased expression of LC3, indicating activation of autophagy. Unexpectedly, these cells also exhibit increased levels of p62 compared to control cells (*Figure 3.2.*). Upregulation of LC3 along with upregulation of p62 could be correlated with deregulated autophagic activity and incomplete autophagy (Mizushima et

al., 2015). However, p62 is a multifunctional protein that participates in many biological processes inside the cells, that could be responsible for this upregulation (Moulis et al., 2017). In the same context, western blot analysis for LC3-II lipidation and p62 degradation, demonstrated augmented LC3 and decreased p62 expression in starved cells compared with control groups (cultured and uncultured normal cells), indicating the upregulation of autophagy in CAFs upon starvation (*Figure 3.2.c*).These experimental approaches were performed in different batches of cells and that's why they generated conflicting results about p62. Nevertheless, the above observations designated that under starvation fibroblasts increased their autophagy levels.

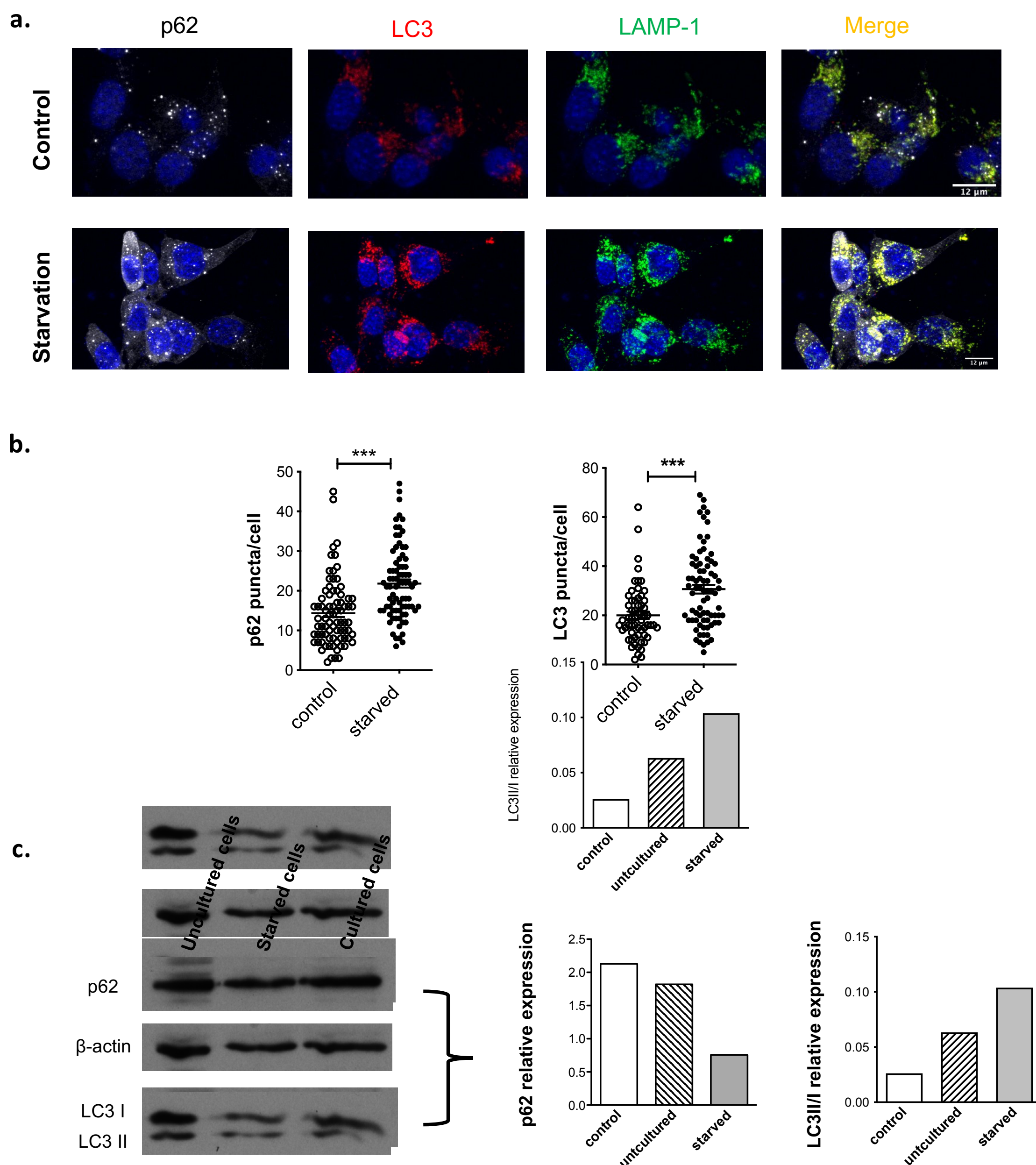


Figure 3.2: *Assessment of autophagy under starvation.* ***a)*** *Representative confocal immunofluorescence images of NIH/3T3* [illegible] *starvation. p62 (silver white), LC3 (red), LAMP-1 (green) and DAPI (blue).* ***b)*** *p62 puncta/cell and LC3 puncta/cell are depicted (***P < 0.0001, *P <0.05). Results are expressed as mean ± SD. (Images analyzed with the image processing software Fiji (SciJava).* ***c)*** *Western blot analysis*

of p62, LC3I/II and β-actin in total cell lysates from NIH/3T3 cells under starvation. Relative expression of p62 and LC3 II/I.

3.3 IFNα, LPS, TES treatment

To further investigate the conditions of the TME that could induce autophagy in CAFs, we treated NIH/3T3 cells with a variety of different stimuli, including LPS, IFNα and tumor explant supernatant (TES). The usage of TES is considered a good approach to study the conditions of cancer in vitro as it contains variant soluble factors produced by cancer cells and cells of the TME. In this experimental setting, we did not observe statistically significant differences neither when compared every circumstance with the control untreated cells or in comparison with each other. Nevertheless, cells treated with IFNα and TES seemed to have lower levels of p62 and higher (IFNα) or unchanged (TES) levels of LC3, compared with the untreated cells (*Figure 3.3*). These observations could provide indications for increased autophagy, but they do not lead to safe conclusion about the autophagic levels in CAFs.

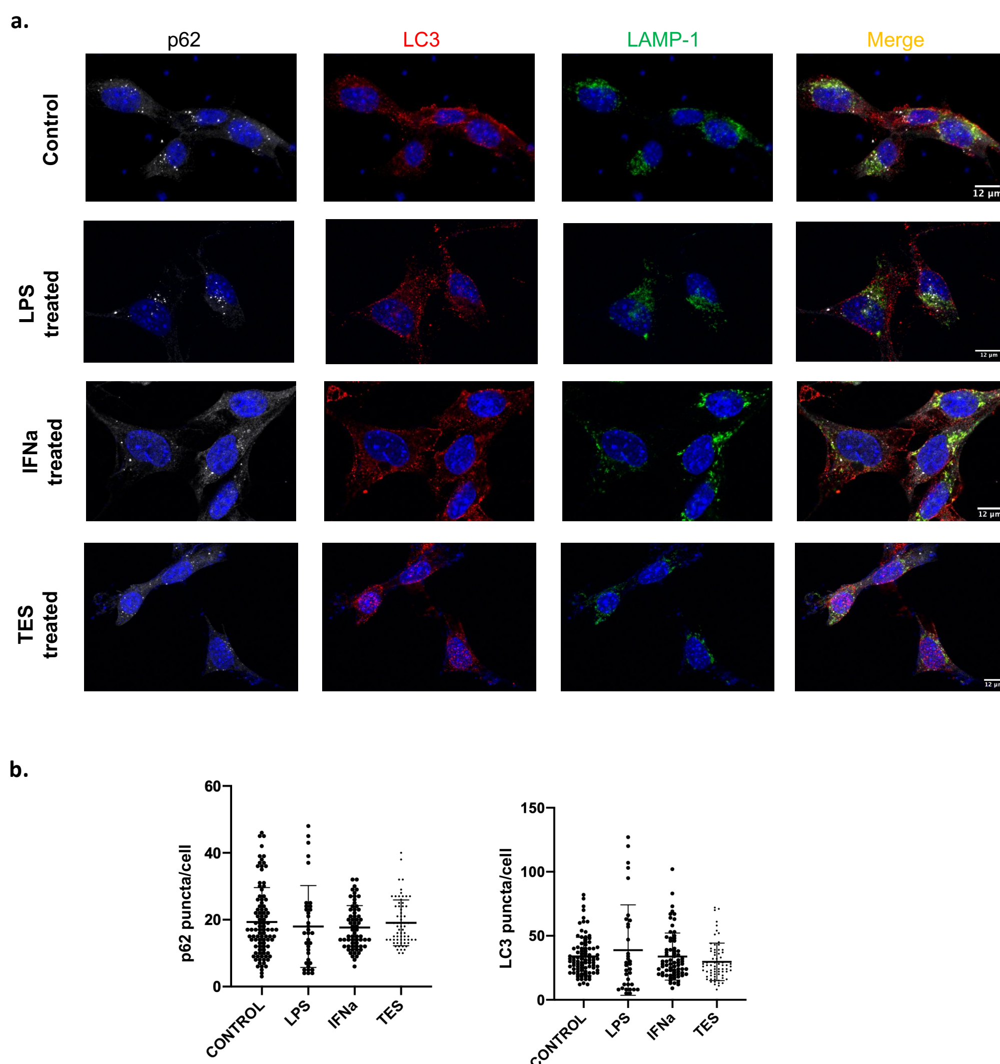


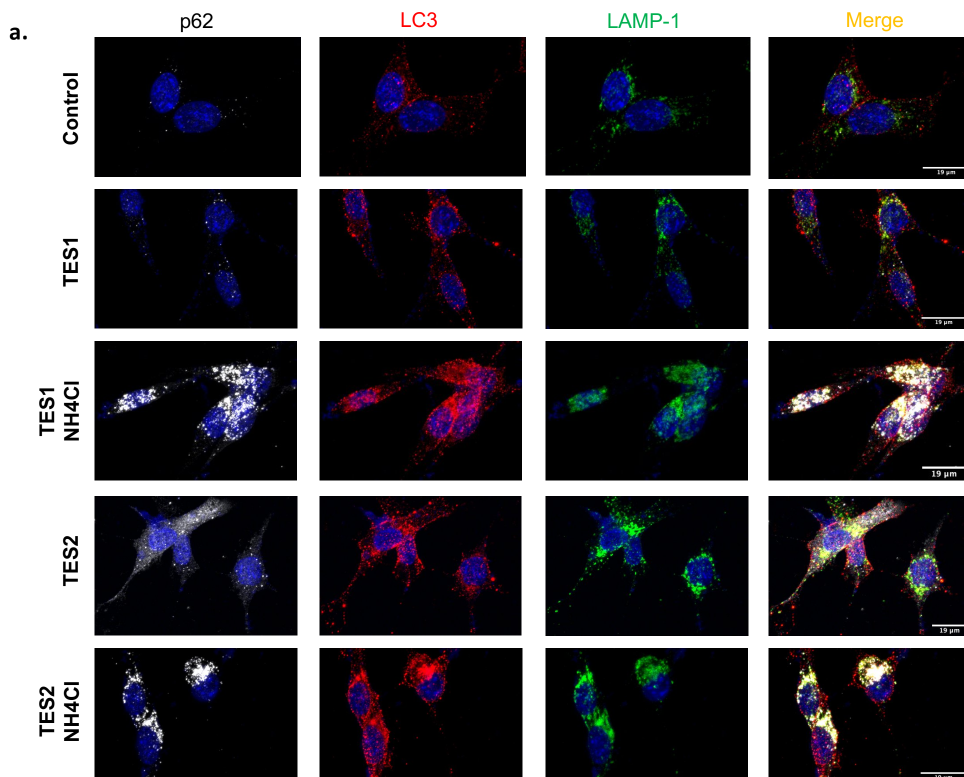
Figure 3.3: *Assessment of autophagy under different stimuli: LPS, IFNa, 30% TES.* ***a)*** *Representative confocal immunofluorescence images of NIH/3T3 mouse fibroblasts: p62 (silver white), LC3 (red), LAMP-1 (green) and DAPI (blue).* ***b)*** *p62 puncta/cell and LC3 puncta/cell are depicted (***P < 0.0001; *P <0.05).*

Results are expressed as mean ± SD. (Images analyzed with the image processing software Fiji (SciJava). TES: Tumor explant supernatant

3.4. Assessment of autophagic flux activity in TES treated cells

The usage of different TES (TES9, TES1, TES2, TES10) had variant effects on the expression levels of p62 and LC3 in TES-treated cells (vs untreated), according to observations derived from confocal microscopy and western blot experiments. The induction of autophagy did not occur in the same level and as a result, we weren't able to draw a valid conclusion. As the used TES derived from different melanoma tumors the variant effects observed in treated cells may have occurred due to the different circumstances that characterize each specific tumor.

In order to investigate the overall autophagic degradation rather than the autophagosome formation we tried to address whether there was a functional autophagic flux (Moulis et al., 2017; Klionsky et al., 2016; du Toit et al., 2018). We treated cells with different TES along with NH4Cl, which is a well-known autophagy inhibitor that alerts the lysosomal pH inducing the blockage of autophagic flux. Immunofluorescence microscopy experiments demonstrated that cells treated with TES and NH4Cl had significantly increased levels of both p62 and LC3, which were accumulated into the autophagosomes and were also highly co-localized. Western blot analysis revealed the same effect for p62 and LC3 expression of TES+NH4Cl treated cells compared with cells treated with TES alone (*Figure 3.4*). These observations, denoted the activation of functional autophagic flux in cells treated with TES.



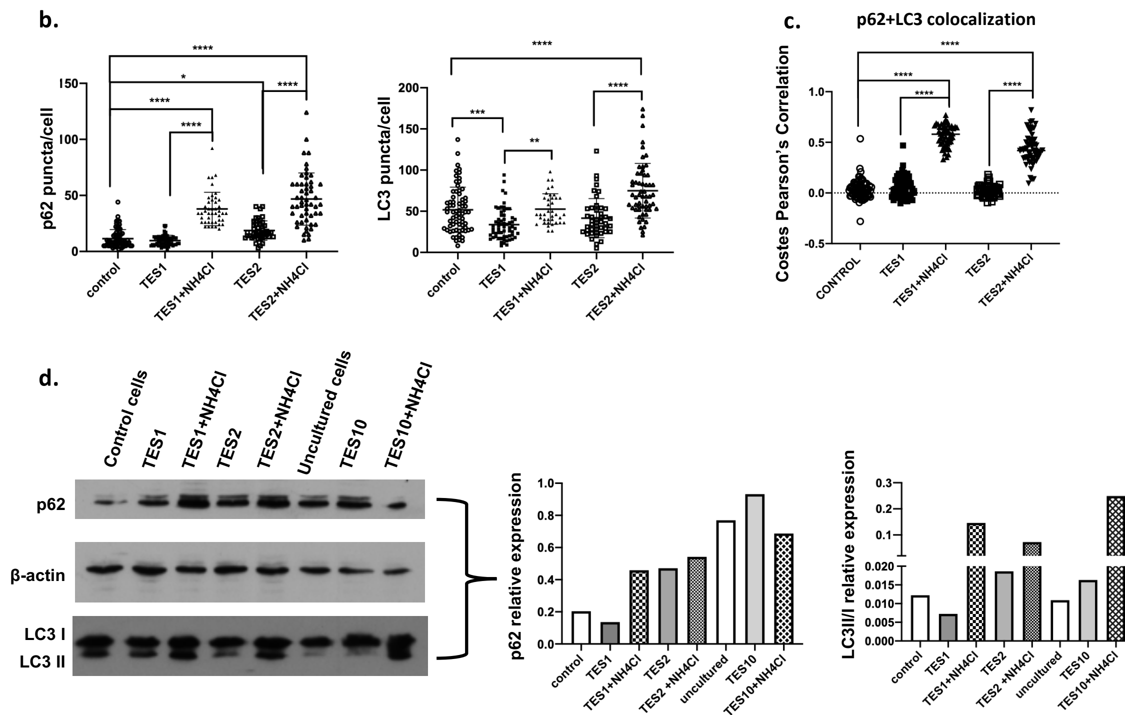


Figure 3.4: *Autophagic flux assays upon NH4Cl in TES-treated cells.* ***a)*** *Representative confocal immunofluorescence images of NIH/3T3 mouse fibroblasts for evaluation of autophagy upon treatment with 30% TES (TES1 and TES2) and TES with NH4Cl that inhibits autophagy compared with control cells (untreated cultured cells). p62 (silver white), LC3 (red), LAMP-1 (green) and DAPI (blue).* ***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).****c)*** *Pearson's correlation of LC3 versus p62 (***P < 0.0001) in NIH/3T3 fibroblastic cells (TES-treated, TES-NH4Cl and control cells).* ***d)*** *Western blot analysis of p62, LC3I/II and β-actin in total cell lysates from NIH/3T3 cells treated with TES, TES and NH4Cl, control untreated cultured cells and control uncultured cells. Relative expression of p62 and LC3II/I.*

3.5. TGFβ treatment

3.5.1 Upregulation of LC3 and p62 expression in response to TGFβ treatment

In the last setting of in vitro experiments, we investigated the effect of TGFβ in CAFs autophagy. TGFβ is one of the most abundant cytokines in the tumor environment, which can both be expressed by CAFs and drive their activation and proliferation. TGFβ can be produced by a variety of cells including tumor cells and several immune populations of the TME (Suzuki et al., 2010; Batlle et al., 2019). We treated NIH/3T3 cells with TGFβ and examined the levels of autophagy with confocal microscopy. The confocal microscopy analysis revealed increased expression of p62 and LC3 in cells treated with TGFβ for 24hr compared with untreated cells (*Figure 3.5.1*). Moreover, p62 with LC3 and LAMP-1 co-localization indicated that TGFβ treated cells exhibited formation of functional autophagolysosomes. These results are reproducible and consistent, as they are representative from three independent experiments, indicating increased activation of the

autophagic flux. As mentioned above, p62 expression is cell type and context dependent and it is a multifunctional protein involved in many biological procedures. In order to clarify, if the elevated expression of p62 is correlated with increased transcriptional activity, we performed Real Time PCR analysis (*Figure 3.5.1.c*). We examined the expression of SQSTM1 gene in comparison with the housekeeping gene, HPRT. These results, revealed that TGFβ treated cells had decreased expression of SQSTM1 gene compared with untreated cells, demonstrating that the greater protein expression in p62 was not related to transcriptionally upregulated p62 expression and that there was decreased p62 expression.

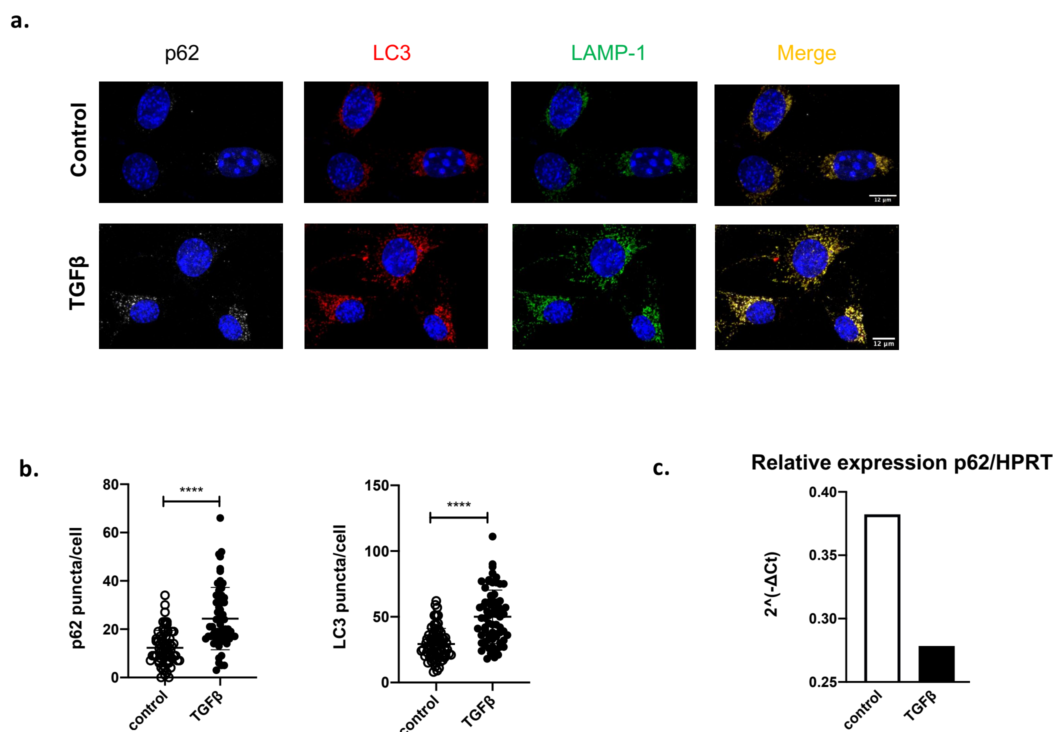


Figure 3.5.1: *NIH/3T3cells treated with TGFβ for 24hr compared with control untreated cultured cells.* ***a)*** *Representative confocal immunofluorescence images of three independent experiments for autophagy assessment: p62 (silver white), LC3 (red), LAMP-1 (green) and DAPI (blue).* ***b)*** *p62 puncta/cell and LC3 puncta/cell are depicted (***P < 0.0001; *P <0.05). Results are expressed as mean ± SD. (Images analyzed with the image processing software Fiji (SciJava).* ***c)*** *RT-PCR analysis of p62 relative expression compared with HPRT housekeeping gene.*

3.5.2 Upregulation of PI3K/AKT/mTOR axis upon TGFβ treatment

To further examine if the increased LC3 expression and the induction of autophagy occurs through the PI3K/AKT/mTOR axis, we assessed the levels of the phosphorylated proteins AKT (pAKT), mTOR (pmTOR), 4EBP1 (p4EBP1) and S6 (pS6) in TGFβ treated cells with flow cytometry. We observed that the PI3K/AKT/mTOR pathway was upregulated upon the TGFβ treatment (*Figure 3.5.2.*). The elevated levels of these subunits were not necessarily correlated with suppression of autophagy, on the contrary, as PI3K/AKT/mTOR signaling axis participated in many cellular procedures, its upregulation by TGFβ could be implicated in other processes. The upregulation of

autophagy in fibroblasts could occur though a different signaling pathway, in PI3K/AKT/mTOR-independent manner.

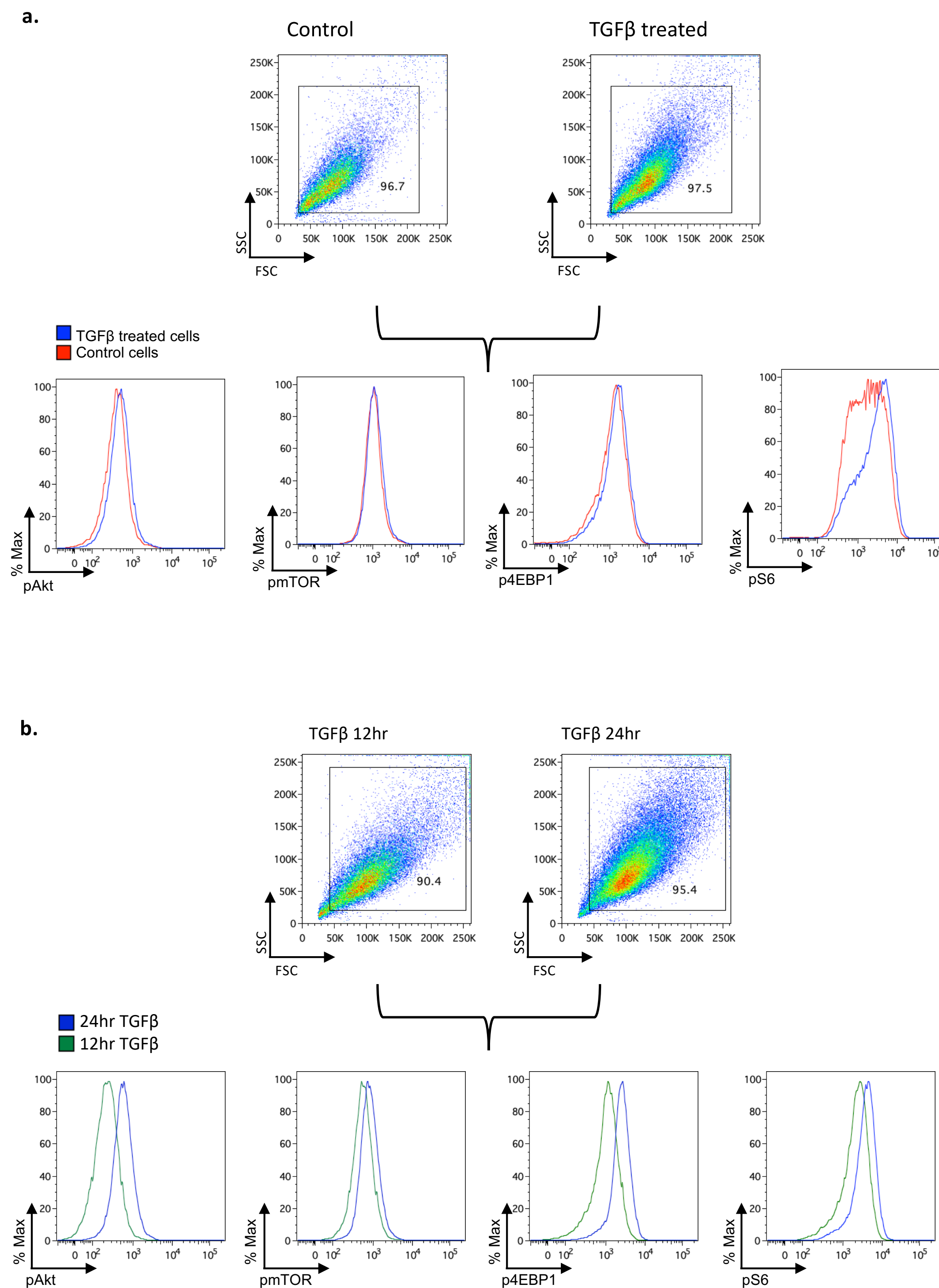


***Figure 3.5.2:** Upregulation of PI3K/Akt/mTOR pathway in TGFβ treated cells. **a)** Representative plots of three independent experiments of mTOR axis phosphorylation in TGFβ treated cells for 24hr and control cells. **b)** Representative timepoint experiment of mTOR axis phosphorylation in TGFβ treated cells for 12hr and 24hr.*

Conclusively, despite the difficulties we addressed in the investigation of a good in vitro experimental setting for studying CAFs autophagy, taken all the observations together they

provided evidence for autophagy activation in CAFs due to the formation of autophagolysosomes, the increased expression of LC3 and the functional autophagic flux. According to the above observations, we decided that the best in vitro model for studying CAFs autophagy is hypoxia.

4. Characterization of αSMA-creAtg5$^{fl/fl}$ mice

To study the effect of CAFs autophagy in vivo we generated for the first time the transgenic mice αSMA-creAtg5$^{fl/fl}$, where the activated fibroblasts that express αSMA exhibit impaired autophagic pathway due to the depletion of the essential autophagy gene, ATG5 (Santana et al., 2017). These mice were generated by crossing αSMA-cre mice with Atg5$^{fl/fl}$ mice, using the cre-LoxP system (*Figure 4.1.*).

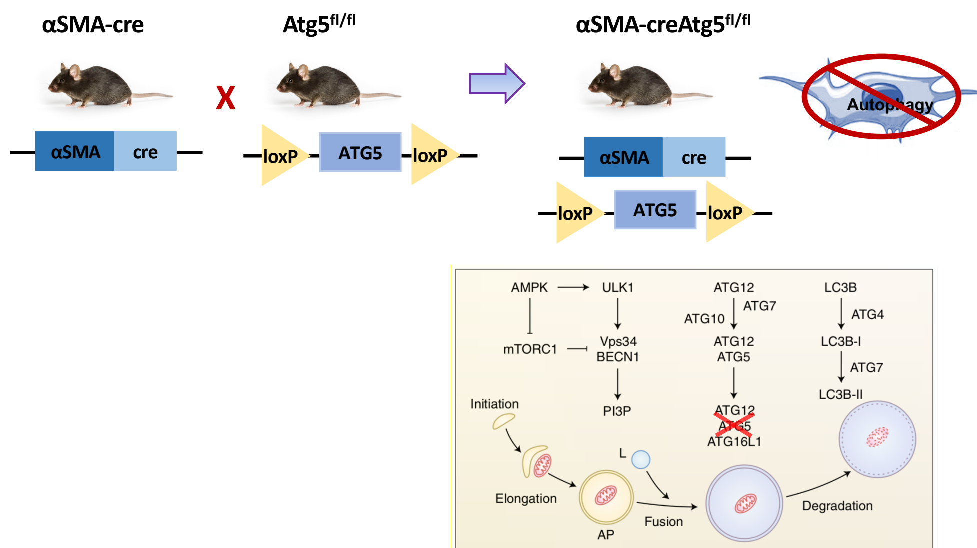
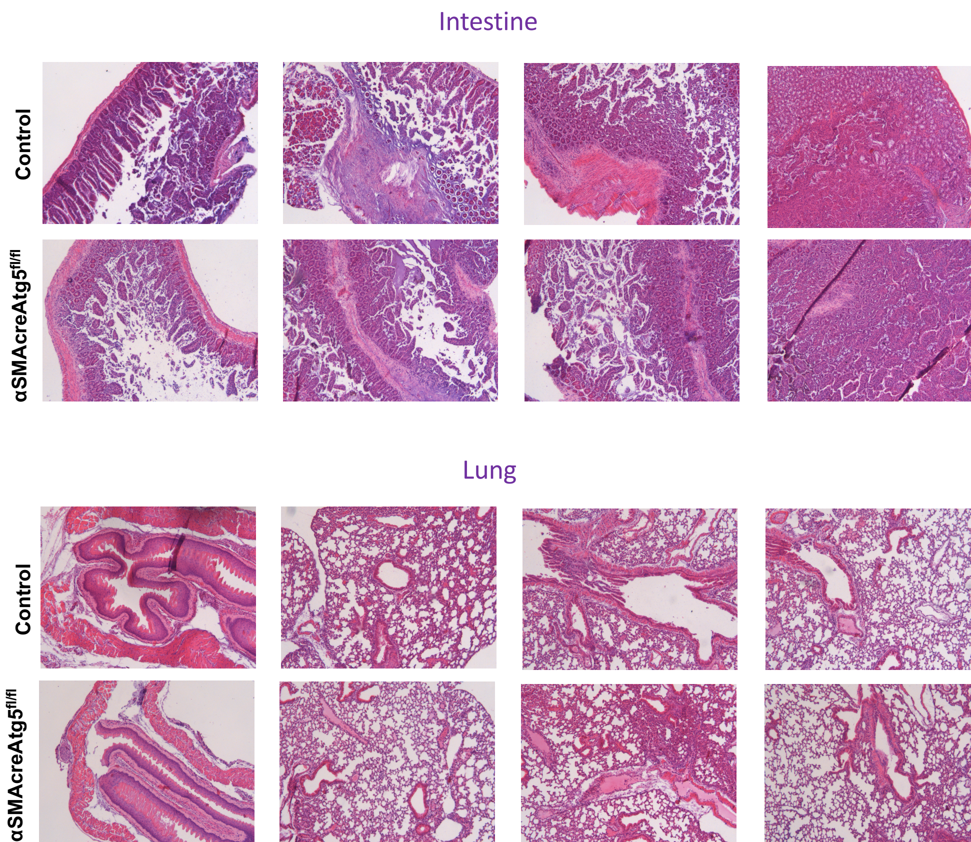


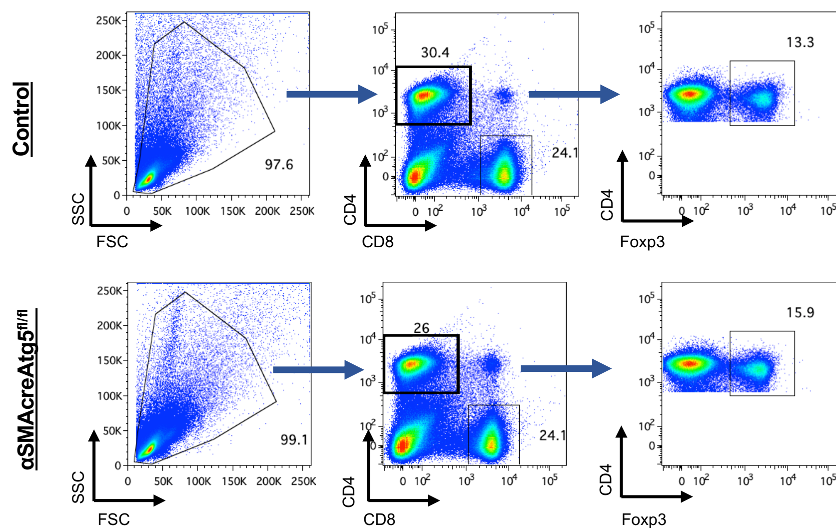
Figure 4.1: αSMA-creAtg5$^{fl/fl}$ mice. *Generation of the transgenic αSMA-creAtg5$^{fl/fl}$ mouse model with the cre-LoxP system, by crossing αSMA-cre mice with Atg5$^{fl/fl}$ mice. The cre recombinase is expressed under the control of acta2 gene promoter, while the exon 3.1 of ATG5 gene is flanked by the LoxP sites containing neomycin resistant cassette. The generated mice have impaired autophagy in αSMA expressing cells.*

As these mice have never been generated before, we aimed to characterize their immune phenotype in steady state. For this purpose, we isolated a variety of tissues including total lymph nodes, spleen, bone marrow, lungs and intestine from two αSMA-creAtg5$^{fl/fl}$ male mice and we compared them with those from two control Atg5$^{fl/fl}$ mice. Hematoxylin and eosin (H&E) staining of paraffin embedded sections from lungs and intestines did not reveal differences in αSMA-creAtg5$^{fl/fl}$ tissues compared to normal mice, as we did not observe lesion or inflammation (*Figure 4.2.a*). Even so, a more detailed examination of other organs could further confirm these indications. Moreover, we used Fluorescence-Activated Cell Sorting (FACS) to analyze the profile of immune populations of lymph nodes, spleen and BM. In lymph nodes we examined CD4$^+$ and

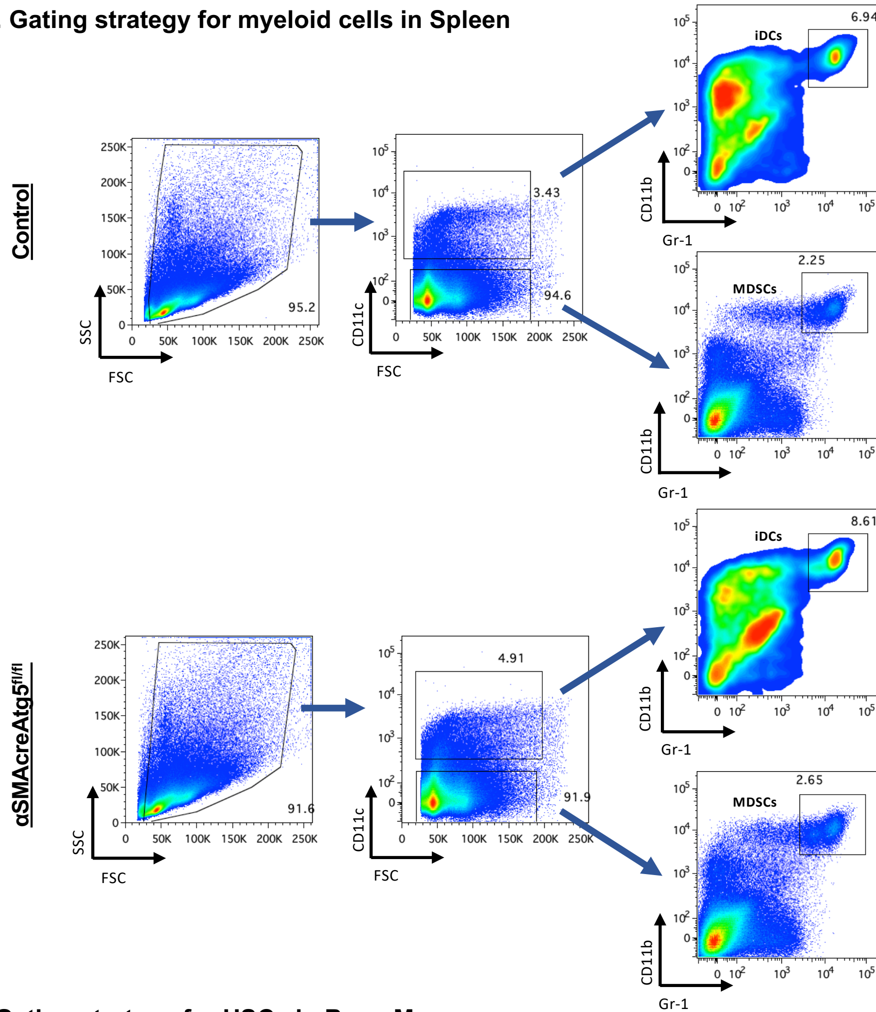
CD8$^+$ T cells, as well as, T regulatory cells (CD4$^+$Foxp3$^+$). In spleen we examined the myeloid lineages inflammatory Dendritic cells (iDCs) as CD11c$^+$CD11b$^+$GR1$^+$ and Myeloid-derived suppressor cells (MDSCs) as CD11c$^-$CD11b$^+$GR1$^+$. Next, from bone marrow we analyzed hematopoietic stem cells HSCs as Lin$^-$Sca1$^+$cKit$^+$ (*Figure 4.2 b, c, d*). No differences were observed among the groups.



a. Gating strategy for T cells in Lymph nodes



b. Gating strategy for myeloid cells in Spleen



c. Gating strategy for HSCs in Bone Marrow

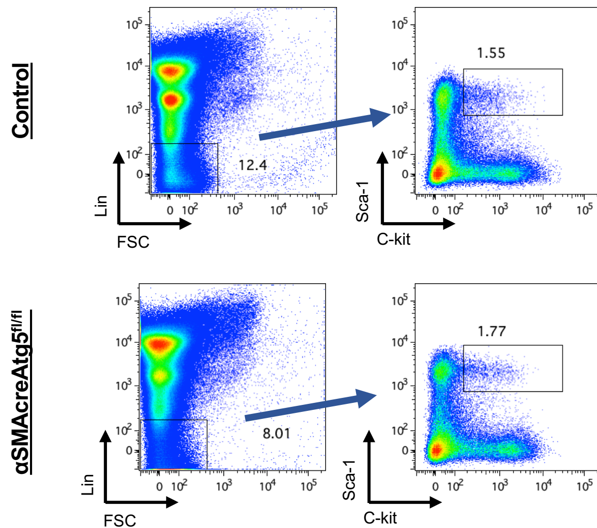


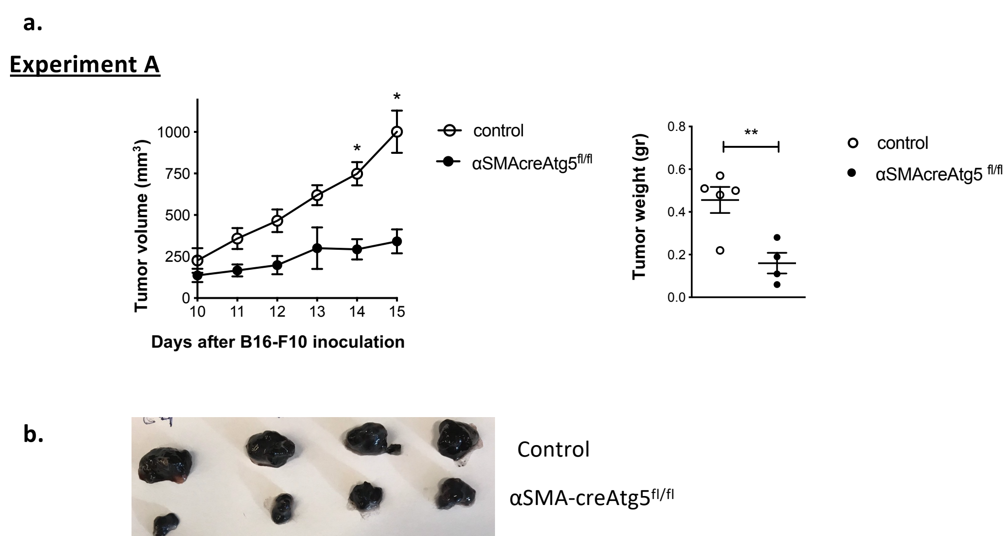
***Figure 4.2:* Characterization of αSMA-cre;Atg5fl/fl mice**. **a)** Hematoxylin and eosin (H&E) staining of intestine and lung derived from naïve αSMA-creAtg5$^{fl/fl}$ male mice (n=2) compared with control Atg5$^{fl/fl}$ (n=2). **b)** Gating strategy for T cells in lymph nodes. **c)** Gating strategy for myeloid cells in spleen **d)** Gating strategy for HSCs in bone marrow.

5. Tumor inoculation experiment in αSMA-creAtg5$^{fl/fl}$ mice

To investigate the role of CAFs autophagy in tumor growth and its potential regulatory role in the immune contexture of the TME, we performed tumor inoculation with B16-F10 mouse melanoma cells in αSMA-creAtg5$^{fl/fl}$ mice and control mice. In the absence of autophagy in CAFs, we monitored the tumor growth and the anti-tumor immune response by analyzing the infiltrating immune populations with flow cytometry. Even though we repeated this experiment several times using different controls, we addressed difficulties to reach our goals and draw conclusive results, due to variant and inconsistent observations. We used both Atg5$^{fl/fl}$ and αSMA-cre female mice as controls, as in some transgenic mice the expression of cre recombinase itself may influence the phenotype of the mice, depending on the context and the cell type, and we also performed the experiments in male mice, as the gender may influence the effect in the immune populations. Tumor curves and immune analysis of three representative, independent experiments (A, B, C) are introduced below.

5.1. Tumor growth in αSMA-creAtg5$^{fl/fl}$ mice

We monitored the tumor growth during day 10 until day 15 and we measured tumor weight in the final stage of the tumor at day 15. In experiment A, the αSMA-creAtg5$^{fl/fl}$ female mice exhibited significant decreased tumors compared to the control group of Atg5$^{fl/fl}$ mice, indicating that the depletion of autophagy in CAFs could drive tumor reduction. H&E staining did not reveal differences between the tumor tissues of these groups (*Figure 5.1.1*). In experiment C, by using male mice we also observed similar effect in tumor growth. However, in some of the experiments the differences in tumor volume were not so obvious, while the tumor weight in the final stage remained unchanged between the groups as indicating by experiment B (*Figure 5.1.2.*). Despite the inconsistency in tumor weight among the experiments, the trend in tumor volume was still obvious between the groups, demonstrating that CAFs autophagy may feature a regulatory role in tumor growth.



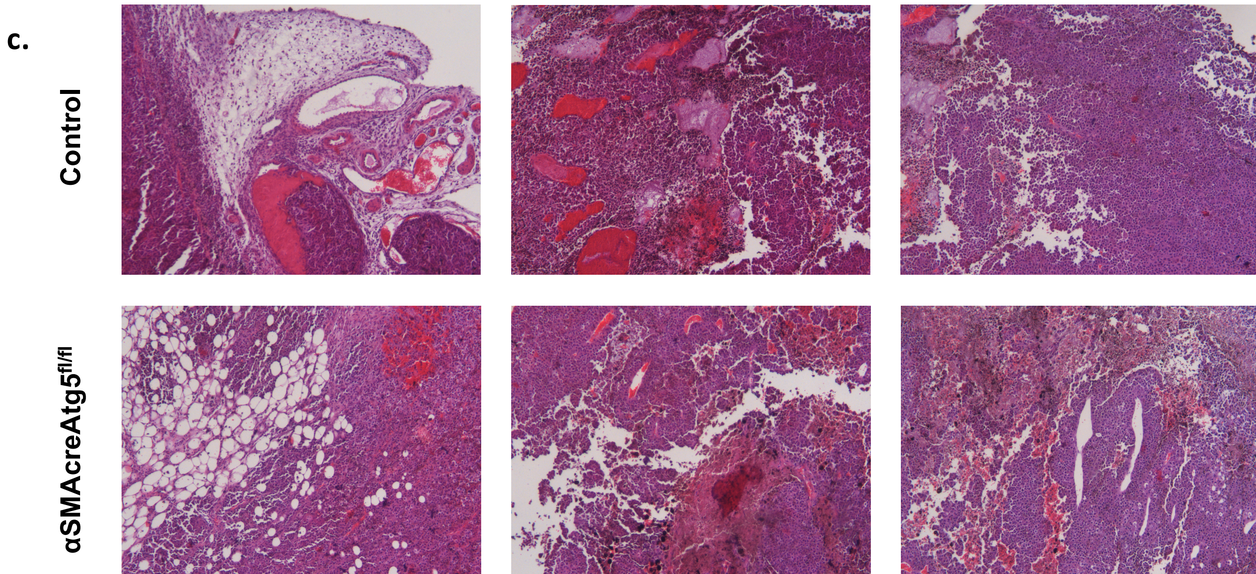
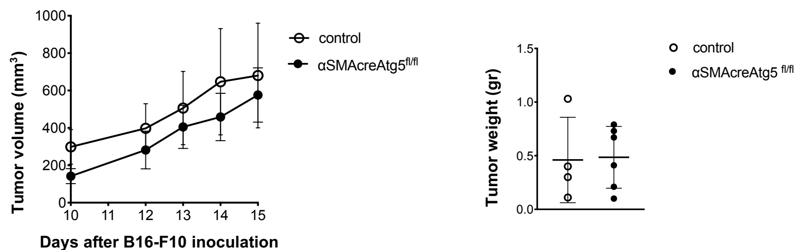


Figure 5.1.1: **B16-F10 inoculation in αSMA-creAtg5$^{fl/fl}$ mice of experiment A**. **a)** Tumor volume (mm^3) from day 10 to 15 and tumor weight (gr). αSMA-creAtg5$^{fl/fl}$ (n=4) and Atg5$^{fl/fl}$ control (n=5) female mice. **b)** Representative image of excised tumors of B16-F10 inoculated αSMA-creAtg5$^{fl/fl}$ mice and Atg5$^{fl/fl}$ control mice at day 15 from experiment A. **c)** Representative images of H&E staining of excised B16-F10 mouse melanoma tumors at day 15 after inoculation.



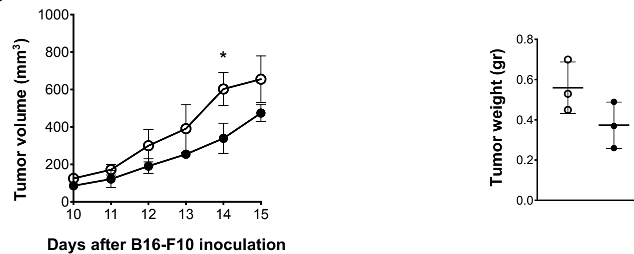


Figure 5.1.2: **B16-F10 inoculation in αSMA-creAtg5$^{fl/fl}$ mice**. Tumor volume (mm^3) from day 10 to 15 and tumor weight (gr). Experiment B: αSMA-creAtg5$^{fl/fl}$ (n=3) and Atg5$^{fl/fl}$ control (n=3) female mice, and Experiment C: αSMA-creAtg5$^{fl/fl}$ (n=3) and Atg5$^{fl/fl}$ control (n=3) male mice.

5.2. Immune response in αSMA-creAtg5$^{fl/fl}$ tumors

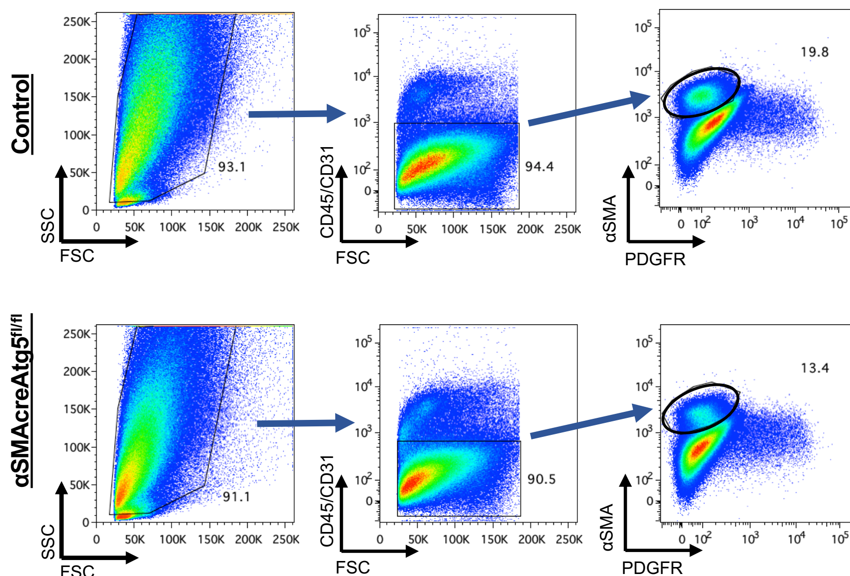
To examine how the CAFs autophagy deficiency affects the anti-tumor immune response we analyzed several populations with essential role in anti-tumor immunity. More specifically, with flow cytometry we analyzed the infiltration of the immune counterparts of CD45 hematopoietic cells and cells from myeloid and lymphoid lineages. Nevertheless, we addressed difficulties to determine a specific immune phenotype due to the great variation among the groups and the conflicting results of the experiments, that would be discussed extensively below.

5.2.1. CAFs in αSMA-creAtg5$^{fl/fl}$ mice

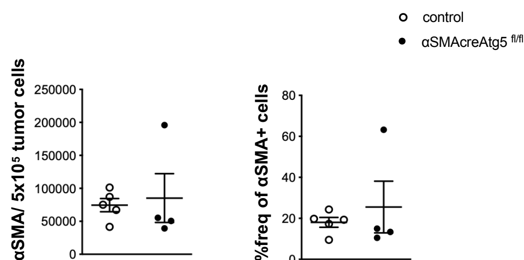
Firstly, we assessed whether the CAFs population exhibit differences among the groups in experiment A. We gated CAFs as CD45$^-$CD31$^-$αSMA$^+$ cells and we also examined the expression of PD-L1 in these cells. CAFs population remained similar among the groups, however, PD-L1 expression in CAFs of αSMA-creAtg5$^{fl/fl}$ was reduced compared to the control group (*Figure 5.2.1*). This denoted that autophagy may have an important role in PD-L1 expression in CAFs.

Experiment A

a. Gating strategy for CAFs



b.



c.

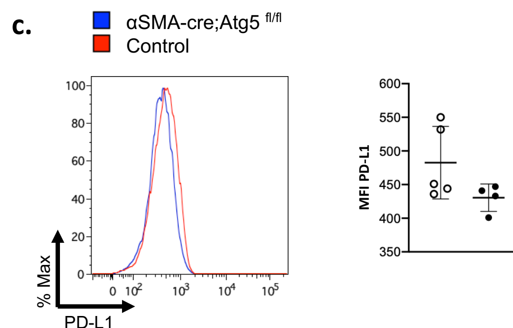


***Figure 5.2.1:* Cancer-associated fibroblasts in αSMA-creAtg5$^{fl/fl}$ tumors**. **a)** Representative gating strategy for CAFs (CD45-αSMA+) in experiment A. **b)** Frequencies of CAFs (CD45$^-$CD31$^-$αSMA$^+$) in

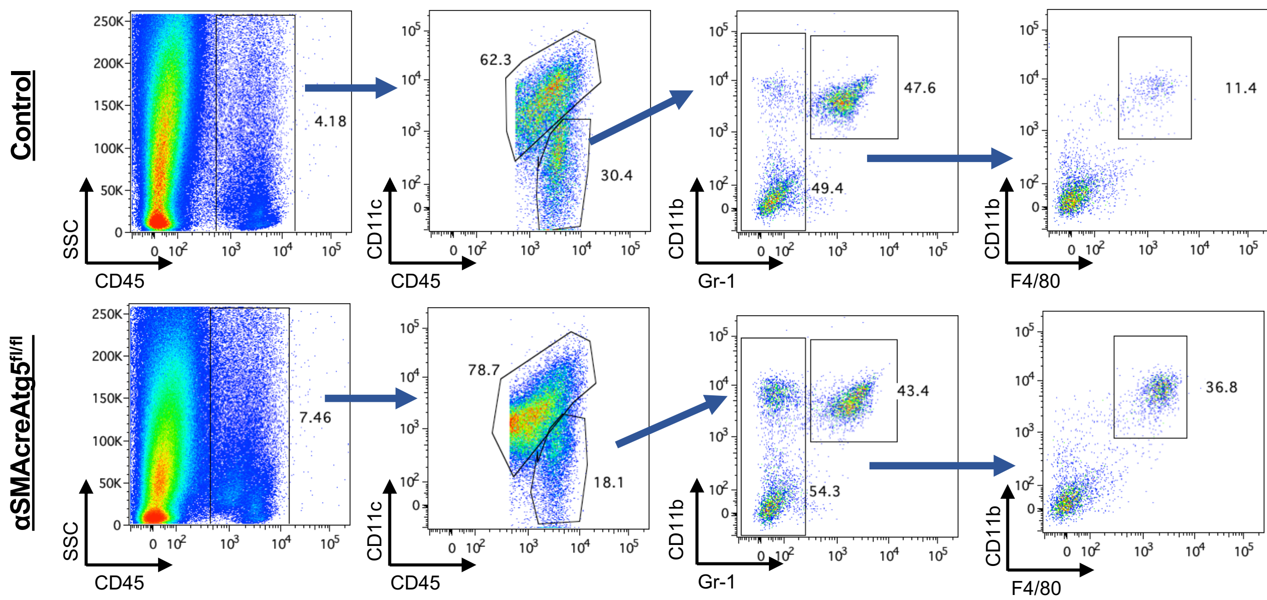
αSMA-creAtg5$^{fl/fl}$ and Atg5$^{fl/fl}$ control mice. **c)** Representative histogram and MFI of PD-L1 expression in CD45^{-}αSMA^{+} cells. Representative results expressed as mean ± S.D. **P<0.01, *P<0.05.

5.2.2. Myeloid cells in αSMA-creAtg5$^{fl/fl}$ tumors

For the analysis of the immune populations that dominate the tumor microenvironment, we first assessed the differences between αSMA-creAtg5$^{fl/fl}$ tumors and controls groups in CD45^{+} infiltrating leukocytes. In experiment A, CD45 cells were significantly increased in αSMA-creAtg5$^{fl/fl}$ tumors indicating increased immune infiltration compared to the control group (*Figure 5.2.A*). However, in experiment B, the opposite effect was observed as αSMA-creAtg5$^{fl/fl}$ mice had lower immune infiltration due to significantly decreased CD45 cells (*Figure 5.2.B*). On the other hand, in experiment C, CD45 frequencies had big variations (*Figure 5.2.C*), indicating that there were no differences between the groups. These contradictory observations did not provide sufficient evidence for drawing a conclusion about the CD45 infiltration in as αSMA-creAtg5$^{fl/fl}$ tumors. Further, we examined the infiltrating myeloid populations including Dendritic cells (DCs) gated as CD45^{+}CD11c^{+} cells, Macrophages (MΦs) gated as CD45^{+}CD11c^{-}CD11b^{+}F4/80^{+} cells and Myeloid-derived suppressor cells (MDSCs) gated as CD45^{+}CD11c^{-}CD11b^{+}GR-1^{+} (*Figures 5.2.2 A, B, C*). Frequencies DCs and MΦs followed the effect of CD45 cells, in all three experiments. More specifically, in experiment A CD11c and MΦs frequencies were significantly increased in αSMA-creAtg5$^{fl/fl}$ tumors. On the other hand, in experiment B these frequencies were significantly decreased αSMA-creAtg5$^{fl/fl}$ tumors while in experiment C, they did not exhibit significant differences. Frequencies of MDSCs had no differences among the groups, in all the experiments, however in experiment B MDSCs/ 500000 tumor cells were significant decreased in αSMA-creAtg5$^{fl/fl}$ mice. Similarly, the inconclusive results did not lead to a sufficient conclusion about the infiltration of myeloid cells.

Experiment A

a. Gating strategy for myeloid cells



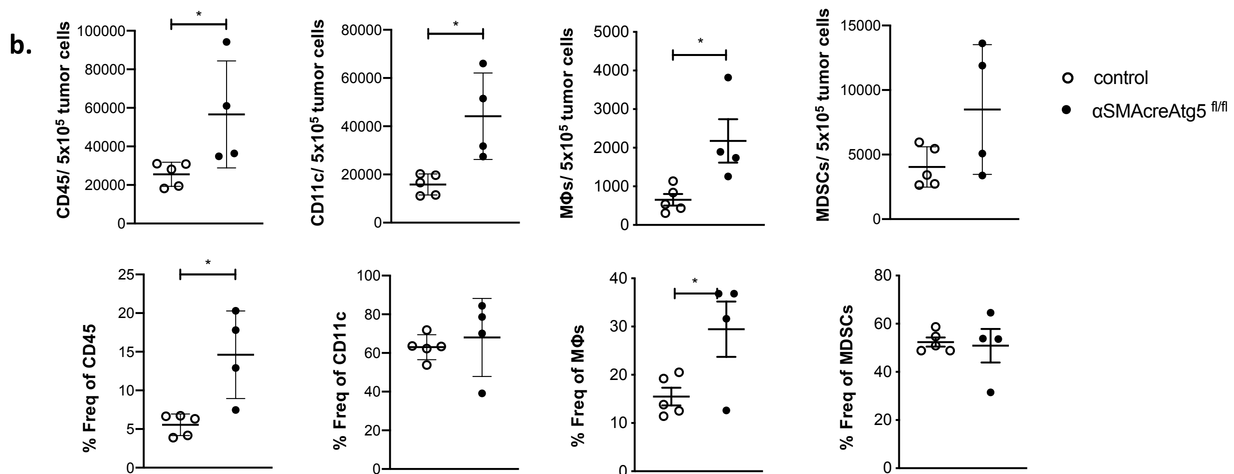
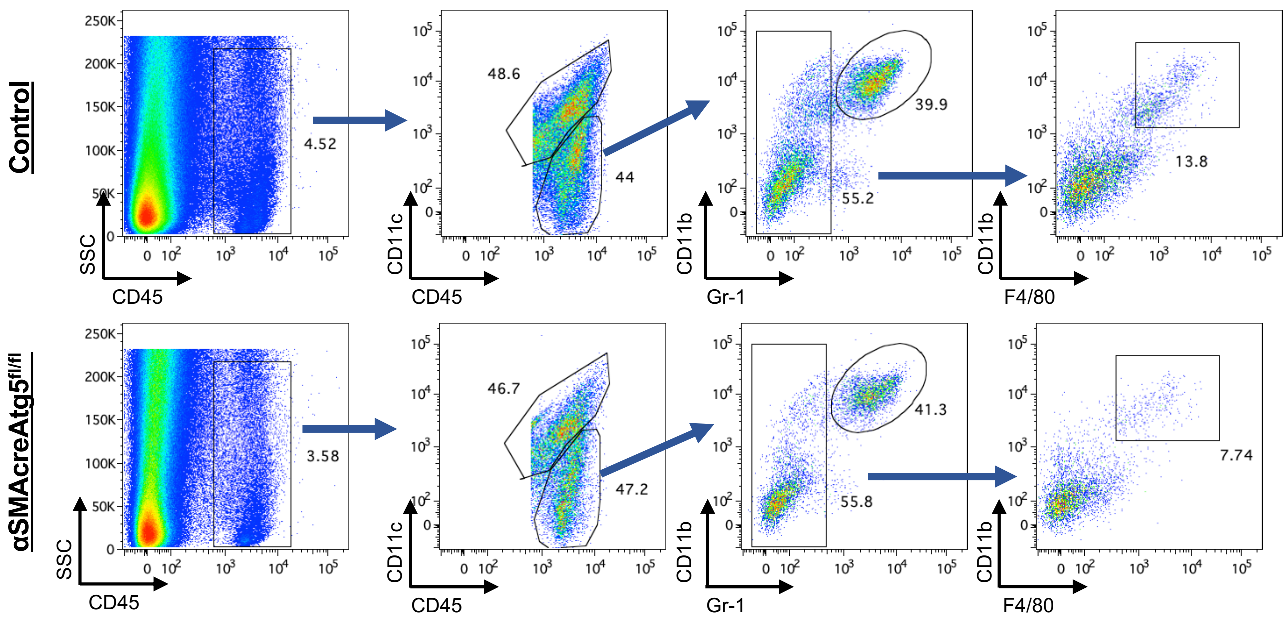


Figure 5.2.2 A: *Myeloid cell populations in αSMA-creAtg5$^{fl/fl}$* tumors. *a)* *Representative gating strategy of flow cytometry analysis and* ***b)*** *frequencies of CD45 cells, Dendritic cells (CD11c), Macrophages(MΦs) and Myeloid-derived suppressor cells (MDSCs) in αSMA-creAtg5$^{fl/fl}$ and control mice in Experiment A. Representative results expressed as mean ± S.D. **P<0.01, *P<0.05.*

Experiment B

a. Gating strategy for myeloid cells



b.

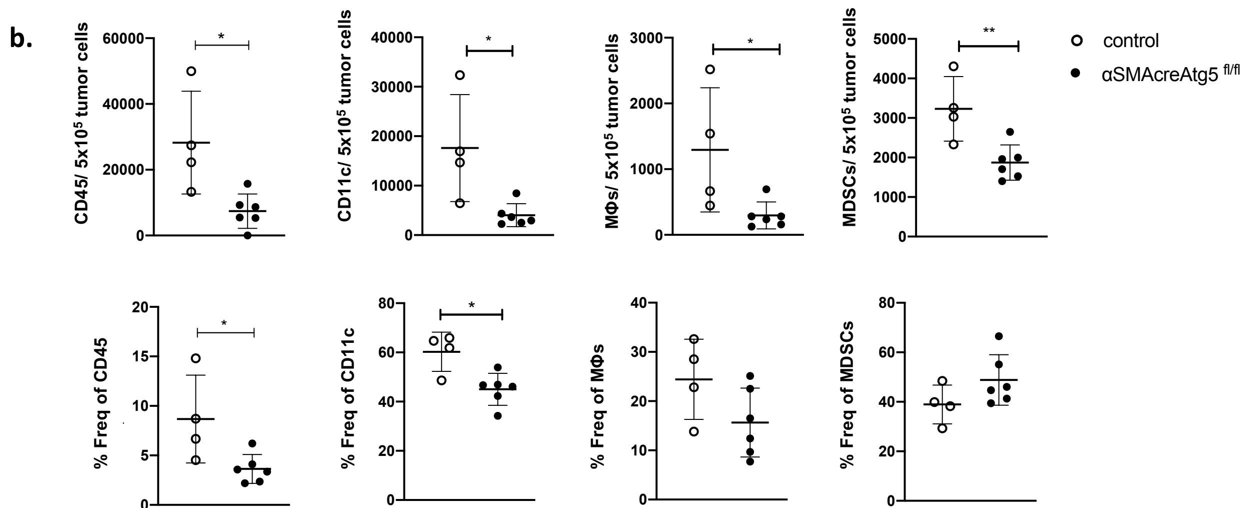
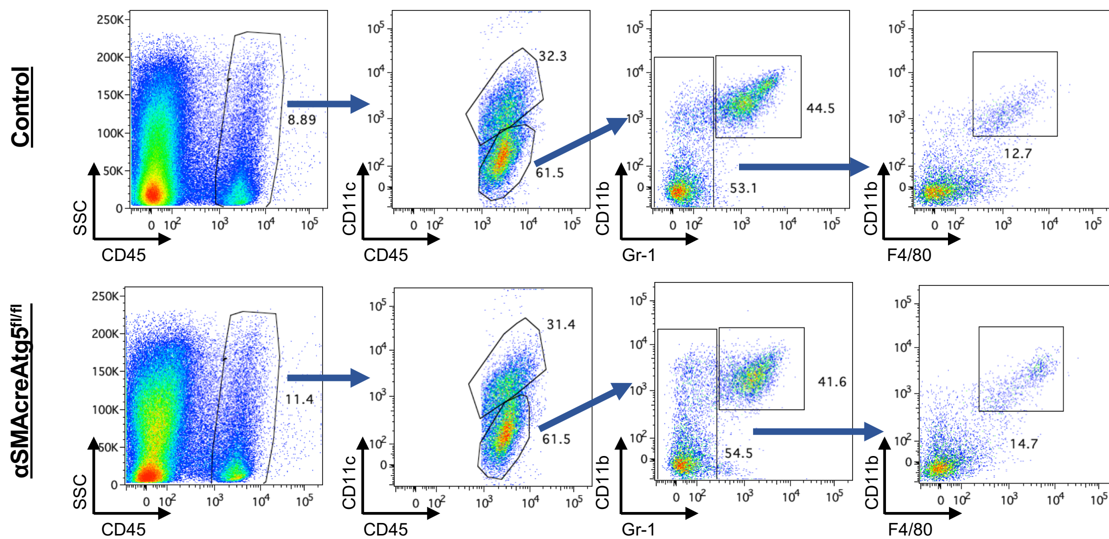


Figure 5.2.2 B: ***Myeloid cell populations in αSMA-creAtg5$^{fl/fl}$* tumors.** ***a)*** *Representative gating strategy of flow cytometry analysis and* ***b)*** *frequencies of CD45 cells, Dendritic cells (CD11c), Macrophages(MΦs) and Myeloid-derived suppressor cells (MDSCs) in αSMA-creAtg5$^{fl/fl}$ and control mice in Experiment B. Representative results expressed as mean ± S.D. **P<0.01, *P<0.05.*

Experiment C

a. Gating strategy for myeloid cells



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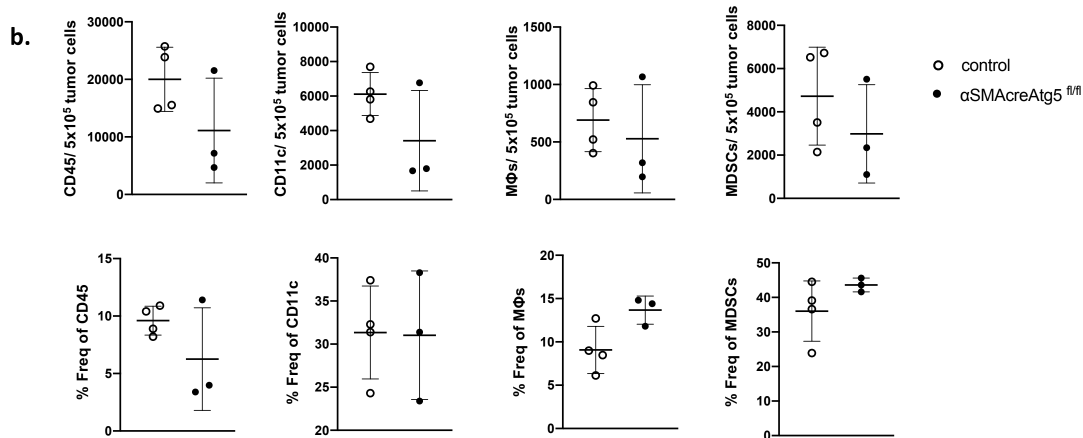


Figure 5.2.2 C: *Myeloid cell populations in αSMA-creAtg5$^{fl/fl}$* tumors. *a)* *Representative gating strategy of flow cytometry analysis and* ***b)*** *frequencies of CD45 cells, Dendritic cells (CD11c), Macrophages(MΦs) and Myeloid-derived suppressor cells (MDSCs) in αSMA-creAtg5$^{fl/fl}$ and control mice in Experiment C. Representative results expressed as mean ± S.D. **P<0.01, *P<0.05.*

5.2.3. MHCII and PD-L1 expression in αSMA-creAtg5$^{fl/fl}$ mice

For a better understanding of how the inhibition of CAFs autophagy could determine the immune profile of αSMA-creAtg5$^{fl/fl}$ mice we assessed the expression of MHC II and PD-L1 in myeloid cells of experiment C. MHC II expression was slightly elevated in CD45, CD11c and MDSCs of αSMA-creAtg5$^{fl/fl}$ tumors, while there was significant increased expression in MDSCs. Regarding the PD-L1 expression, there was a trend for increased expression in myeloid population of αSMA-creAtg5$^{fl/fl}$ mice, however, the samples experimental group exhibited great variation compares to the control group.

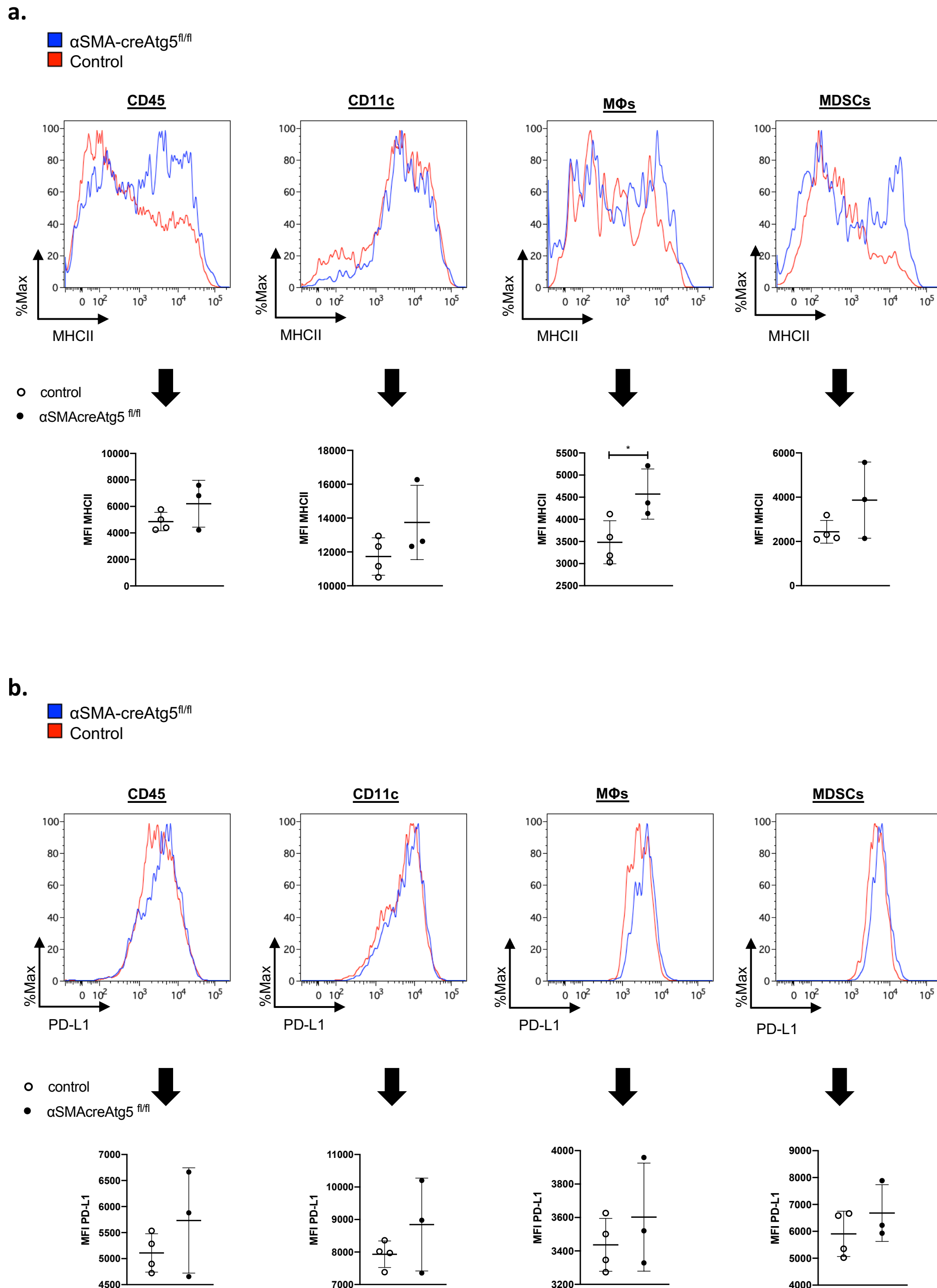


Figure 5.3.: MHCII and PD-L1 expression in myeloid cells (CD45, DCs, MΦs, MDSCs) in αSMA-creAtg5$^{fl/fl}$ male mice from experiment C. a) *Representative histograms and MFI of MHCII.* ***b)*** *Representative histograms and MFI of PD-L1.*

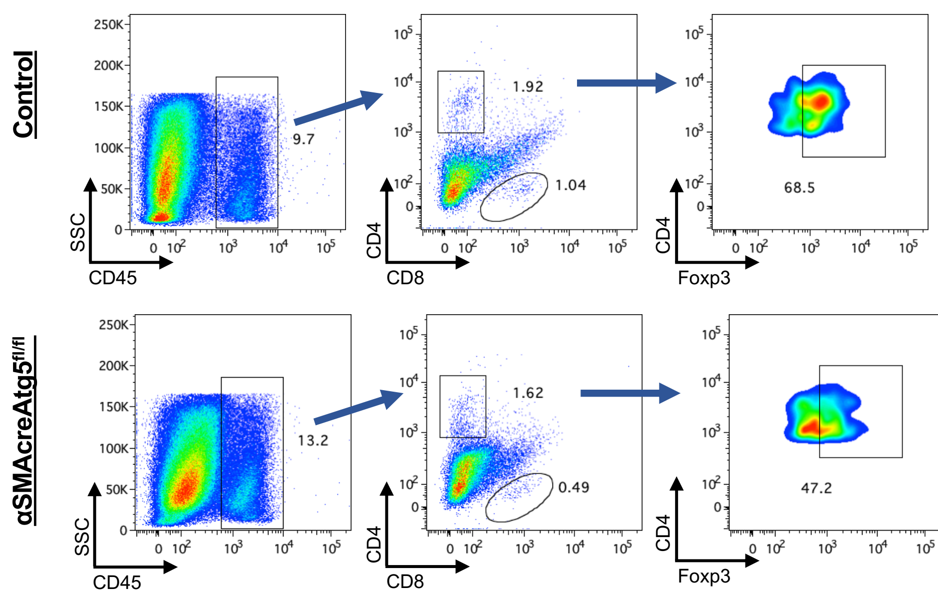
5.2.4. T cells in αSMA-creAtg5$^{fl/fl}$ mice

To further assess the immune profile of αSMA-creAtg5$^{fl/fl}$ mice we examined the infiltrating population of lymphoid lineage. In experiment A, analysis of CD4, CD8 and Tregs frequencies revealed slightly increased frequencies of CD4 T cells and decreased CD8 T cells, while Tregs frequencies remained the same between the αSMA-creAtg5$^{fl/fl}$ mice and the control group. However, αSMA-creAtg5$^{fl/fl}$ group exhibited great variation that did not allow us to draw a safe

conclusion. In experiment B, the infiltrating populations had no differences between the groups with great variations among the samples of each group, especially in the control group. In experiment C, decreased infiltration of CD4 and CD8 T cells was observed in αSMA-creAtg5$^{fl/fl}$ male mice compared to the control group, while Treg numbers were significant decreased inside the tumor cells but their frequencies remained unchanged. The examination of T cells populations, also provided inconsistent results (*Figure 5.2.4 A, B, C*).

Experiment A

a. Gating strategy for T cells



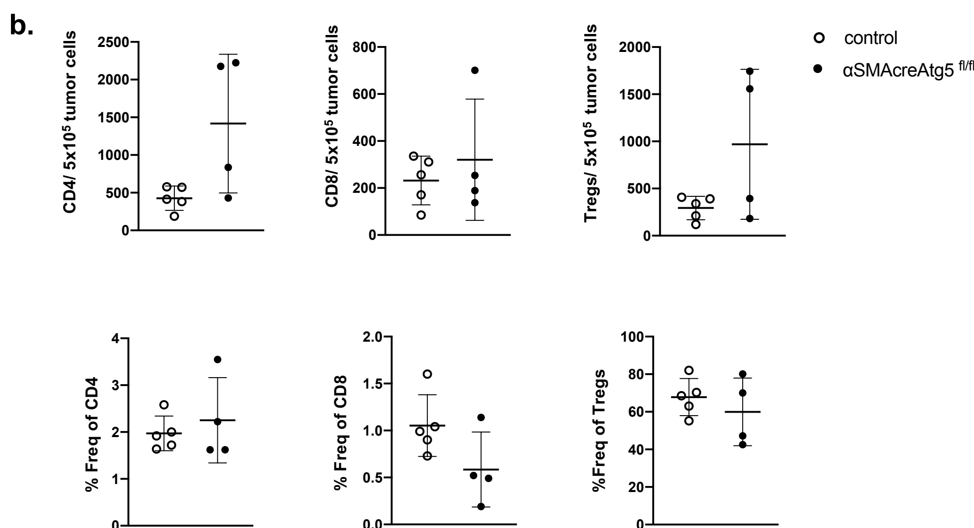
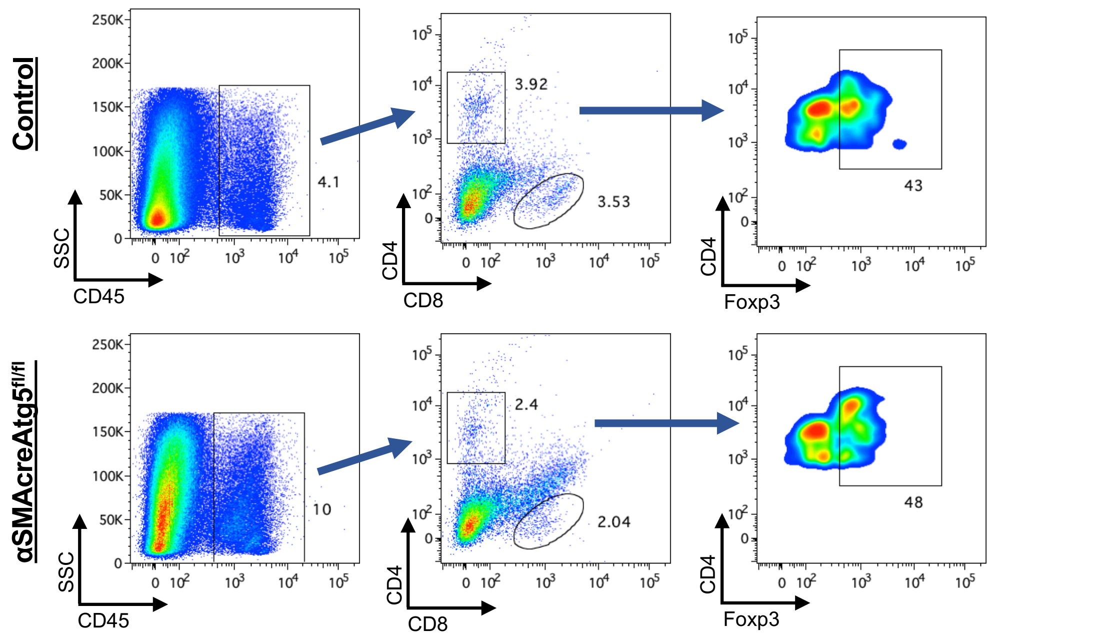


Figure 5.2.4.A: ***T cells populations in αSMA-creAtg5$^{fl/fl}$* tumors.** ***a)*** *Representative gating strategy of flow cytometry analysis and* ***b)*** *frequencies of CD8 cells, CD4 cells and Tregs (CD4+Foxp3+) in αSMA-creAtg5$^{fl/fl}$ and control mice in Experiment A. Representative results expressed as mean ± S.D. **P<0.01, *P<0.05.*

Experiment B

i. Gating strategy for T cells



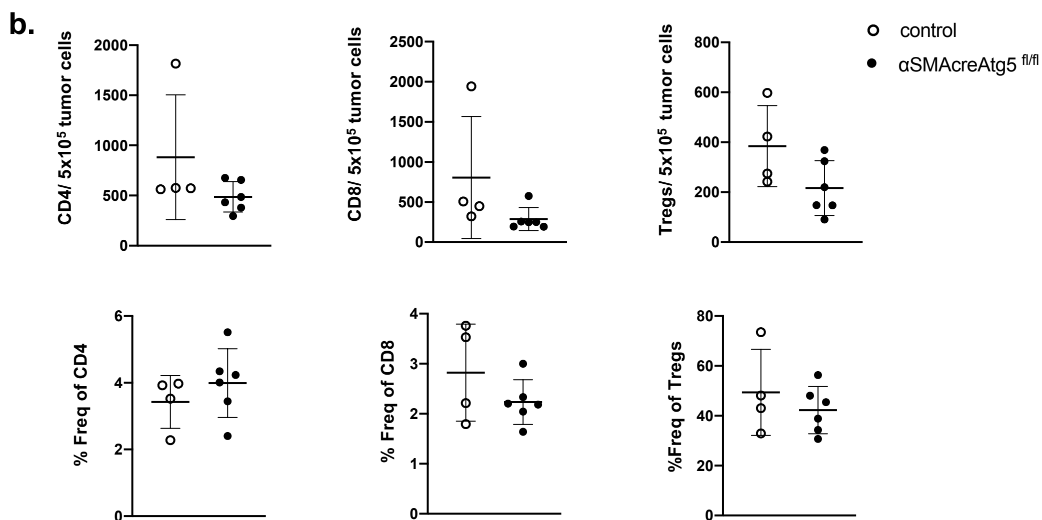
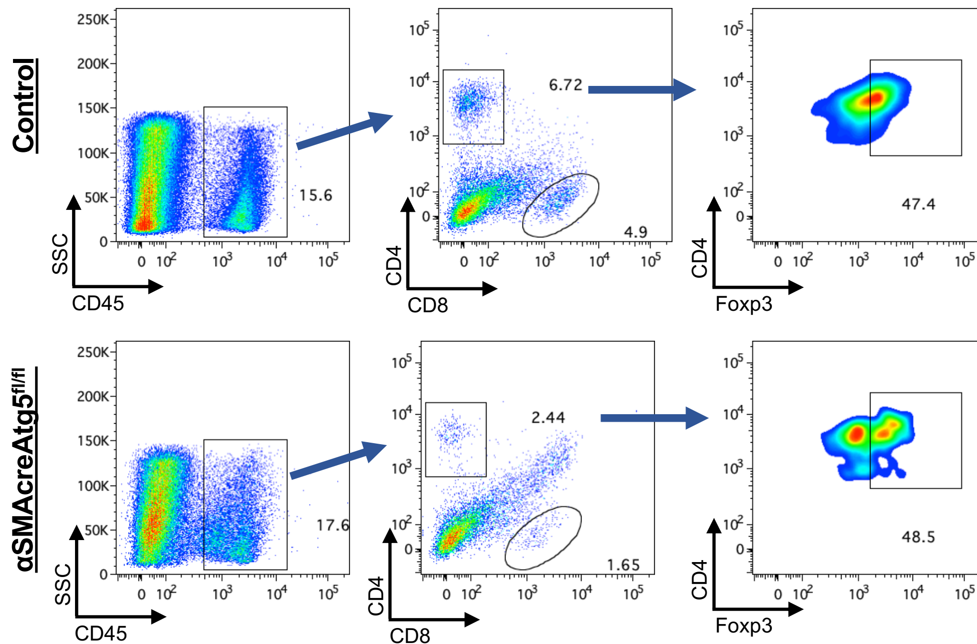


Figure 5.2.4 B: ***T cells populations in αSMA-creAtg5$^{fl/fl}$*** **tumors.** ***a)*** *Representative gating strategy of flow cytometry analysis and* ***b)*** *frequencies of CD8 cells, CD4 cells and Tregs (CD4+Foxp3+) in αSMA-creAtg5$^{fl/fl}$ and control mice in Experiment B. Representative results expressed as mean ± S.D. **P<0.01, *P<0.05.*

Experiment C

a. Gating strategy for T cells



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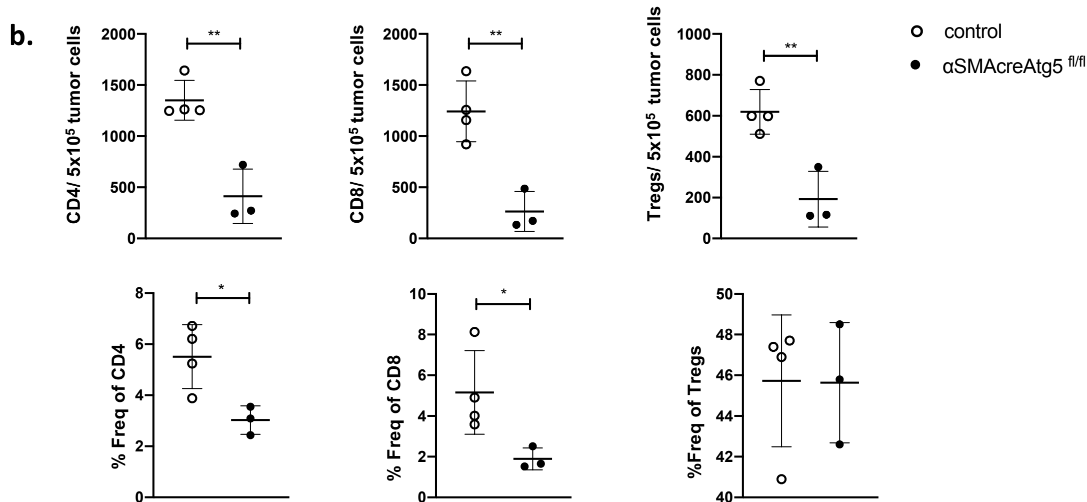


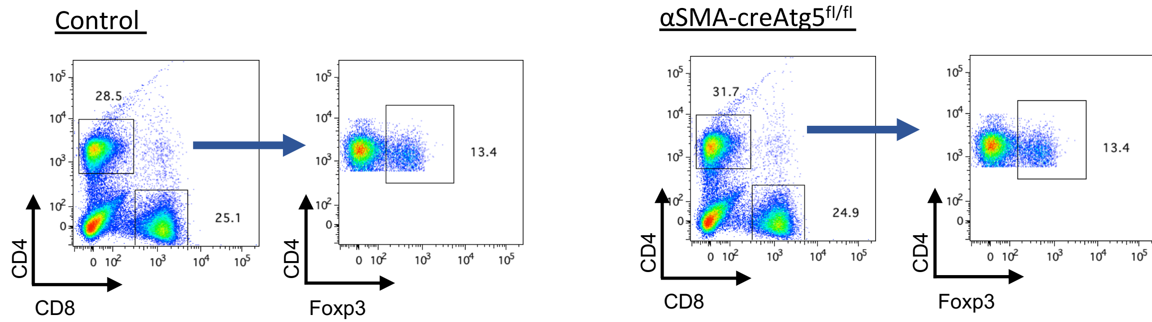
Figure 5.2.4 C: ***T cells populations in αSMA-creAtg5$^{fl/fl}$* tumors.** ***a)*** *Representative gating strategy of flow cytometry analysis and* ***b)*** *frequencies of CD8 cells, CD4 cells and Tregs (CD4+Foxp3+) in αSMA-creAtg5$^{fl/fl}$ and control mice in Experiment C. Representative results expressed as mean ± S.D. **P<0.01, *P<0.05.*

5.2.5. Draining lymph nodes in αSMA-creAtg5$^{fl/fl}$ mice

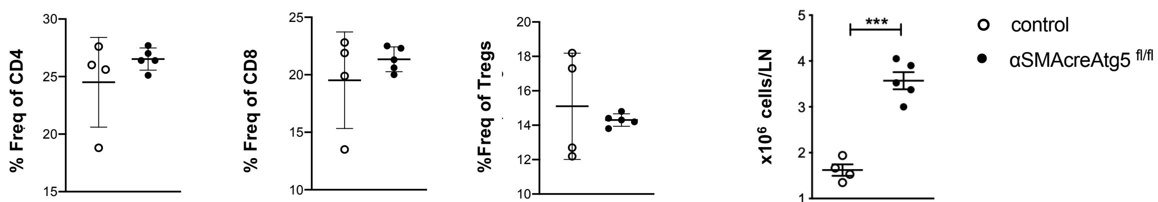
Lastly, the evaluation of draining lymph nodes did not reveal statistically significant differences in these experiment regarding the frequencies of CD8 and Tregs, while in experiment B CD4 were increased in αSMA-creAtg5$^{fl/fl}$ mice. According to the total lymph node cellularity, in experiment A

αSMA-creAtg5$^{fl/fl}$ mice had increased cell numbers compared to control, however, this difference was not observed in the following experiments (*Figure 5.5*).

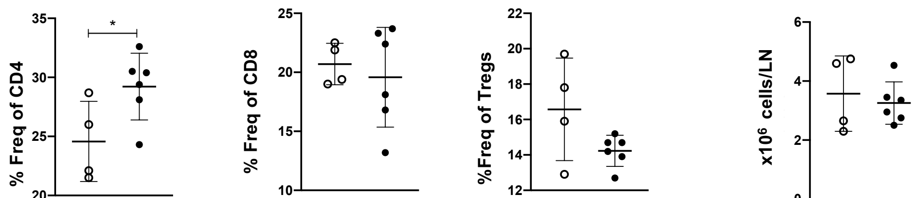
Gating strategy for drained lymph nodes



Experiment A



Experiment B



Experiment C

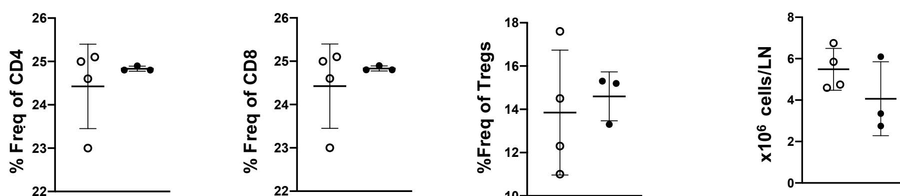


Figure 5.5.: Drained lymph nodes in *αSMA-creAtg5$^{fl/fl}$*. Representative gating strategy of flow cytometry analysis and frequencies of CD8 cells, CD4 cells and Tregs (CD4+Foxp3+) in *αSMA-creAtg5$^{fl/fl}$* and control mice in Experiment A, B, C. Representative results expressed as mean ± S.D. **P<0.01, *P<0.05.

Taken together, the great variation among the groups, the inconsistency of the results and the modest number of mice in each group did not allow us to reach our goals and drive a safe conclusion regarding the impact of CAFs autophagy deficiency in the anti-tumor immunity. Nevertheless, the great heterogeneity that characterize the infiltrating populations may indicate

that the immune alterations occur as a secondary phenomenon of CAFs autophagy depletion. For a better understanding of the regulatory role of CAFs autophagy we would isolate CAFs from αSMA-creAtg5$^{fl/fl}$ and control tumors and we would perform transcriptomics analysis with RNA-sequencing.

6. Phagosome purification

Another approach to further investigate the functional role of CAFs, as well as, the changes that take place during the biogenesis of autophagosomes in CAFs includes the analysis of their phagocytic contexture.

The internalization of cargo into single-membrane organelle (phagosome) and its sequential maturation that terminates in fusion with lysosomes and cargo degradation, called phagocytosis. Several compartments of the autophagy pathway have been linked to phagosome maturation, in a process called LC3-associated phagocytosis (LAP), where autophagy machinery conjugates LC3 directly onto the phagosomal membrane and promotes lysosome fusion (Cemma et al., 2016; Lai and Devenish 2012). Many components are implicated in the process of phagosomal maturation, among the most essential proteins are the Rab-family GTPases. Different Rab proteins are localized to the phagosome at different time points depending on the maturation stage (Kinchen et al., 2008). In their final stages, the phagosome is fused with a lysosome to produce a phagolysosome where the cargo degradation occurs (Cemma et al., 2016; Lai and Devenish 2012).

NIH/3T3 fibroblasts do not normally engulf cells or particles, as they are not considered as professional phagocytotic cells (such as macrophages or dendritic cells), however under specific stimuli they are capable to phagocytose. In this notion, we used magnetic beads to isolate phagosomes from NIH/3T3 cells treated with stimuli relevant to the tumor environment, according to previously described protocols (Guermonprez et al., 2003; Cebrian et al., 2011), in order to examine the phagocytic content of CAFs. We identified the purity of purified phagosomes according to the expression of the phagosomal marker Rab7 with western blot analysis. The recruitment of Rab7 occurs during the later stages of phagosomal maturation, so it is considered as marker for late phagosome formation. We treated the cells for different timepoints to determine the stage of phagosomal maturation, while we also examined whether the purified phagosomes were fused with lysosomes or autophagosomes with the usage of antibodies for LAMP-1 and LC3 (Kinchen et al., 2008; Swanson et al., 2008). The presence of LAMP-1 in 16hr treatment with LPS, demonstrated the fusion with the lysosomes and the formation of phagolysosomes, which occurs in the later stages of maturation. On the other hand, in TGFβ treated cells the absence of LAMP-1 and LC3 indicated that the isolated organelles were not phagolysosomes or LC3-decorated phagosomes (*Figure 6*).

In future experiments we would use this method to evaluate the phagocytotic contexture of CAFs with proteomics analysis. This approach could provide great insights about the proteome implicating in CAFs regulatory functions.

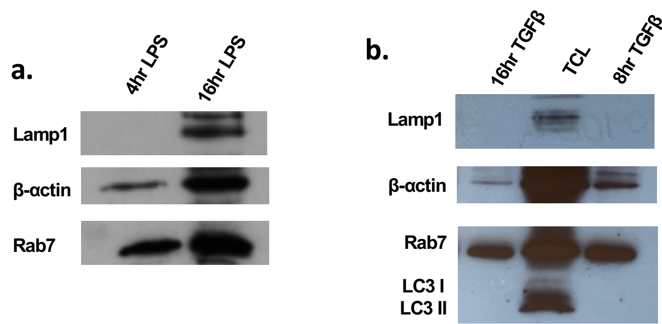


Figure 6: Phagosome purification. *Western blot analysis of purified phagosomes derived from NIH/3T3 cells treated with* ***a)*** *LPS and* ***b)*** *TGFβ, for different timepoints. TCL: Total Cell Lysate*

Future directions

We managed to establish hypoxia in NIH/3T3 cells as a good in vitro model for studying CAFs autophagy, so this experimental setting would be used for the future experiments. Regarding the existing evidence that the immune checkpoint inhibitor PD-L1 is expressed by NIH/3T3 cells, we would treat the hypoxic NIH/3T3 cells with anti-PD-L1 in order to address how the immunotherapy may affect autophagy in CAFs in vitro. In this notion, we would study the effect of immunotherapy in CAFs autophagy in vivo.

Experiment A of tumor inoculation experiments in αSMA-creAtg5$^{fl/fl}$ mice indicated that CAFs with depleted autophagy had decreased expression of PD-L1 compared to control. Therefore, we would treat mice with anti-PD-L1 in order to address how immunotherapy in combination with the specific depletion of autophagy in αSMA-expressing cells could affect tumor development in vivo.

In an effort to better understand the functional role of CAFs autophagy in tumor progression we isolated CAFs with CD90.2 tumor-associated fibroblasts isolation kit from αSMA-creAtg5$^{fl/fl}$ tumors and αSMA-cre tumors of female mice at day 10 upon tumor inoculation. In future experiments, we would perform transcriptomic analysis with RNA-sequencing in these cells, to examine the differentially expressed genes between the two groups, to identify transcripts that could be responsible for functional properties of CAFs and to deeply investigate the molecular mechanisms, signaling pathways and biological processes of those cells.

Moreover, to clarify the molecular signaling pathway that drives autophagy in CAFs, we would use αSMA-RFP mice in order to study the PI3K/AKT/mTOR axis during the progression of melanoma tumors with flow cytometry.

Lastly, in another setting of in vitro experiments, according to our observations about the purified phagosomal compartment, we would perform proteomics analysis in isolated phagosomes of hypoxic CAFs that have previously phagocytosed apoptotic cancer cells. This experimental setting would provide evidence about the expression protein patterns and the implicated protein networks in these cells, revealing insights for CAFs regulatory functions.

Discussion

Over the last years, the extent investigation of the complexity of cancer biology has provided evidence about the crucial regulatory role of the tumor microenvironment (TME) in tumor progression and metastasis, while it generates opportunities for the development of novel anti-cancer therapies (Hanahan et al., 2000; Gonda et al., 2010; Östman et al.,2009; Micke et al., 2004). Regarding to its regulatory role in immune system, TME can influence the anti-tumor immune response and promotes resistance both in conventional drug therapy and immunotherapy (Polanska et al., 2013; Oliver et al.,2018). However, the determination of the underlying mechanisms that drive the TME regulatory functions remains elusive.

Cancer-associated fibroblasts (CAFs) constitute the most abundant cell population inside the TME, with a major regulatory function in tumor progression (Castells et al.2012; Hammer et al.2017; Tommelein et al.2015; Ziani et al.,2018) and anti-tumor immunity. CAFs can shape an immunosuppressive phenotype (Kalluri et al., 2016; Polanska et al.,2013; Ngabire et al., 2017; Kakarla et al.2012; Paulsson et al. 2017) by recruiting immune cells and driving their differentiation, while they feature central role in the complex interactions that characterized tumors (Augsten et al.,2014; Capparelli et al.,2012; Kalluri et., 2006; Kalluri et al.,2016; Ziani et al., 2018). Over the last years, many studies have focused their interest on the tumor promoting functions of CAFs regarding their interactions with the cancerous compartments, however, less are known about the underlying mechanisms and pathways that provoke their regulatory effect on the immune response.

TME is characterized by highly hypoxic and low-deficient conditions as well as by the great presence of inflammatory factors (Daskalaki et al., 2018; Petrova et al., 2018). Autophagy is a fundamental pathway activated under these pathological conditions that dominate the TME enabling the adaption and survival of cancer and stroma cells (Daskalaki et al., 2018; Ngabire et al., 2017; Kalluri et al., 2016) . The pathway of autophagy has been related to multiple aspects of tumorigenesis, while it is considered as a cancer hallmark (R. Amaravadi et al. 2011; Wang et al., 2018). Many studies provide evidence for autophagy upregulation in a variety of human cancers, including primary human breast cancer, melanoma, esophageal cancer, while the presence of autophagy markers is correlated with tumor progression, evasion and metastasis as well as with early disease recurrence and poor prognosis (Espina et al., 2016; Galavotti et al., 2013; Whelan et al. 2017; Mowers et al.,2018).

The role of autophagy in cancer is complex and context-dependent, since it can function as a tumor suppressor (Wilkinson et al., 2010; Ngabire et al., 2017) when it is induced in early stages of tumorigenesis or as tumor promoter when is activated in cancer cells of established tumors (Gewirtz et al., 2016; Amaravadi et al., 2011; Ngabire et al., 2017; Levy et al.,2017; Wang et al.,

2017). The role and functions of autophagy in cancer cells has been studied extensively in many types of cancers, due to its importance, however, the evidence about its regulatory functions in other compounds of TME remains limited. Very recently the role of autophagy in the non-cancerous stromal compartments has gained great interest, and in this notion, several studies have focused on the investigation of the autophagic pathway in CAFs (Capparelli et al., 2012; Chauhan et al., 2013; Levy et al., 2017).
Recent studies indicate autophagy upregulation in CAFs (Chaudhri et al. 2013; New et al. 2017; Wang et al., 2017) and contribution in their tumor promoting functions (Sousa et al., 2016). Autophagy inhibition in a mouse xenograft model resulted in significant decreased tumors (New et al., 2017). While, increased levels of CAFs autophagy in human tissues of luminal breast cancer is associated with more aggressive disease and poorer prognosis (Zhao et al., 2017). Moreover, ATG5 depletion in CAFs significantly decreased tumor growth, indicating the tumor promoting function of autophagy in CAFs. Nevertheless, the impact of CAF autophagy in the anti-tumor immune response remains unknown.

Considering all the above, we generate the hypothesis that CAFs upregulated autophagy is involved in their tumor promoting and immunosuppressive functions. In this project, we try to address questions like how CAFs autophagy affects the tumor growth and anti-tumor immunity, which are the underling mechanisms that drive CAFs autophagy, how the specific depletion of autophagy in CAFs can influence the tumor development and anti-tumor immune responses and which is the impact of immunotherapy in CAFs autophagy.

CAFs consist one of the most heterogenous stromal cell populations that may arise by multiple origins. The lack of reliable and unique markers for their characterization (Sun et al., 2015) is the greatest limitation in studying CAFs in vivo. For this purpose, we used the transgenic αSMA-RFP mice in order to identify the activated fibroblasts, according to the expression of α-smooth muscle actin (αSMA). In mouse melanoma tumors, we identified CAFs by the classical characteristics of their large and spindle-shape morphology (Xing et., 2015; Kalluri et al., 2016), while their spatial presence both scattered among the cancer cells and by creating a peripheral “barrier”, align to their tumor supporting functions and their feature to create immunosuppressive environment. Next, we isolated fibroblasts derived from αSMA-RFP normal tissues and melanoma tumors in order to study the process of autophagy in these cells.

The ex vivo assessment of autophagy in αSMA-RFP CAFs with confocal microscopy experiments, provided evidence for activated autophagy and formation of functional autophagolysosomes according to the presence of autophagolysosomal proteins (LAMP-1, p62 and LC3) (Klionsky et al., 2016; Moulis et al., 2017). However, the small amounts of isolated cells did not allow a

statistical analysis of the expression levels of these proteins among the groups. Therefore, the establishment of an in vitro system for studying CAFs autophagy was necessary.

In this notion, we used the mouse fibroblastic cell line NIH/3T3 and cultured them under conditions relevant to those that dominate the tumor microenvironment. More specifically, we investigated the induction of autophagy in fibroblasts under hypoxia, starvation and treatment with TES, IFNα and TGFβ regarding to the expression of LC3, p62 and LAMP-1. LC3 is considered as an indicator of autophagosome formation and is correlated with the number of autophagosomes, thus increased LC3 expression demonstrates increased autophagic activity. On the other hand, p62 is another marker for measuring autophagy, as it binds to LC3 and facilitates the delivery of autophagic cargo to the degradation machinery, as long as it is degraded itself. In general, upon autophagy activation p62 quantities are decreased, whereas accumulation of p62 occurs due to inhibition of autophagy. LAMP-1, participates in the fusion of autophagosomes with lysosomes and the generation of autophagolysosomes (Klionsky et al., 2016; Moulis et al., 2017).

NIH/3T3 cells under hypoxia exhibited elevated LC3 expression and reduced p62 expression compared to the control groups, indicating upregulation of autophagy. The evaluation of the best-characterized regulator of autophagy, PI3K/AKT/mTOR axis (Alissafi et al., 2018) revealed decreased expression of the phosphorylated substrates of Akt and S6 in hypoxic cells, while pmTOR and P4EBP1 expression remained the same. These observations did not reveal strong evidence about the leading signaling pathway of autophagy activation, indicating that alternative implicated pathways other than mTOR could drive the activation of autophagy in hypoxic CAFs.

Next, induction of autophagy in fibroblasts was also observed under serum starvation, as indicated by the increased LC3 expression in confocal microscopy experiments and western blot analysis. In this experimental setting, p62 expression was also observed to be increased. Normally, p62 undergoes degradation during the early phases of autophagy, so we would expect decreased expression in cells with upregulated autophagy. Upregulation both in LC3 and p62 expression are indications for deregulated, incomplete autophagy. However, p62 expression is a cell type and context -specific protein according to the literature. For this reason, in many cell types, upon prolonged serum starvation p62 expression tend to be rescued due to the compensatory upregulated transcription of SQSTM1 gene (Mizushima et al., 2015). As a result, the elevated levels of p62 in starved cells are not correlated with deregulated autophagic flux, but probably with transcriptionally upregulation of p62 gene as a response to the long-term amino acid deficiency. Therefore, there is an upregulation in autophagy upon starvation in NIH/3T3 cells.

The same effect regarding the p62 and LC3 expression was observed in TGFβ treated fibroblasts, while this upregulation in the protein levels of p62 was not correlated with transcriptionally

upregulation of SQSTM1 gene. As mentioned previously, the expression of p62 depends on the cell type and the biological context, while its upregulation could be implicated in many biological procedures, including extensive activation of proteasome (Moulis et al., 2017).
Further, in this experimental setting the investigation of the driving intracellular signaling pathway revealed upregulation in PI3K/AKT/mTOR pathway. A recent study demonstrated that TGFβ can drive the activation of PI3K/Akt/mTOR signaling axis leading to increased cell proliferation and collagen synthesis in human lung fibroblasts (Woodcock et al., 2019). Account of this, the upregulation of mTOR signaling in our experimental setting may not lead to suppression of autophagy, but in a variety of cellular processes necessary for fibroblasts functions. On the other hand, another study provided evidence for activation of autophagic pathway by TGFβ through AMPK/ULK1 axis, another major regulatory pathway of autophagy (W. Liu et al., 2018; Wong et al., 2013; Heras-Sandoval et al., 2014).
In the context of evaluation of autophagic flux activity, TES treated cells were cultured with NH4Cl, which is considered as an autophagy inhibitor as it blocks autophagic flux by changing the lysosomal pH. Significantly increased expression and co-localization of both LC3 and p62 in cells treated with TES and NH4Cl revealed accumulation of these proteins due to the blockade of autophagic flux, indicating activation of functional autophagic flux in the absence of the inhibitor (Moulis et al., 2017; Toit et al., 2018).

Overall, despite the fact that in some of the mentioned experimental settings it wasn't clear which is the underlying signaling pathway driving this activation and the which is the responsible source of p62 upregulation, they still provided evidence that in conditions relevant to tumor microenvironment, NIH/3T3 fibroblasts could activate their autophagic procedure as observed by the formation of autophagolysosomes and the augmented expression of LC3. We concluded that the best in vitro model for studying CAFs autophagy in vitro is hypoxia, as it exhibited the strongest evidence for upregulation of autophagy and it remains the most relevant condition to cancer.

To study the role of CAFs autophagy in vivo, we generated for the first time the transgenic mouse model αSMA-creAtg5$^{fl/fl}$, where autophagy is impaired in αSMA expressing cells. The characterization of these mice in steady state did not reveal differences in the immune populations compared to tissues from Atg5$^{fl/fl}$ mice.
The potential regulatory role of CAFs autophagy in tumor progression and anti-tumor immune response was investigated with tumor inoculation experiments in αSMA-creAtg5$^{fl/fl}$ and control mice. This experiment was repeated several times due to the inconclusive results and the great variations among the groups. Analysis of tumor growth demonstrated decreased tumor volume in αSMA-creAtg5$^{fl/fl}$ compared to the control, as indicated by the three representative experiments (A,

B, C). The differences in tumor weight in the final stage were not so obvious among the groups. Despite of the inconclusive results regarding the tumor weight, there was still a trend for decreased tumor volume in mice with depleted autophagy in CAFs, providing evidence for the regulatory role of CAFs autophagy in tumor progression. These observations aligned to the existing literature that demonstrated that ablation of autophagy in mouse xenografts model lead to significant reduced tumor volumes (New et al., 2017) and that ATG5 depletion in CAFs significantly decreased tumor growth (Zhao et al., 2017).

Concerning the analysis of the infiltrating immune populations in αSMA-creAtg5$^{fl/fl}$ and control tumors, ambiguous results were generated due to the incontinency of the experiments and the great variations among the groups. In experiment A, CAFs derived from αSMA-creAtg5$^{fl/fl}$ tumors had decreased expression of PD-L1 compared to the control group, indicating that autophagy in CAFs could regulate its expression. Moreover, the significant reduction on tumor growth was accompanied by greater infiltration of hematopoietic CD45 cells, dendritic cells and macrophages in αSMA-creAtg5$^{fl/f}$ tumors compared to control, while the infiltration of T cells did not reveal significant differences among the groups. A possible explanation could be that autophagy in CAFs has a critical role in the suppression of the anti-tumor immunity of myeloid lineage, and this effect was reversed upon its ablation. On the other hand, in experiment B, significantly diminished levels of myeloid cells (CD45, DCs, MΦs and MDSCs) compared to control group were observed in αSMA-creAtg5$^{fl/f}$ mice, suggesting an opposing enhancing role for CAFs autophagy in immune cells. In experiment C, there were great variations regarding the frequencies of myeloid populations, however, CD4 and CD8 T cells were significantly decreased in αSMA-creAtg5$^{fl/fl}$ tumors. The reduced infiltration of lymphoid lineage cells could indicate that CAFs autophagy feature a regulatory role in the interplay between CAFs and T cells influencing the recruitment of T cells.

Nevertheless, none of these explanations could be considered sufficient in driving a safe conclusion due to the great inconsistency between the experiments, the conflicting results and the lack of literature about the role of CAFs autophagy in the regulation of the immune system. These observations revealed great heterogeneity among the infiltrating immune populations, which may indicate that these alterations occurred as a secondary effect upon depletion of CAFs autophagy. Overall, our results demonstrated upregulation of autophagy in CAFs in vitro, while the in vivo study of CAFs autophagy supported the idea that CAFs autophagy can play an essential role in tumor growth. The effect of CAF autophagy in the regulation of the immune response remained unclear due to the inconclusive results. As a result, further experiments are required to provide stronger evidence about the potential regulatory role of CAFs autophagy. In this notion, we would attempt to answer questions like which are the implicated signaling pathways that drive the activation of autophagy in vivo, how immunotherapy influences autophagy both in vitro and in vivo

and how CAFs autophagy depletion in combination with immunotherapy affect tumor progression and anti-tumor immunity, while the transcriptomic analysis of CAFs with depleted autophagy would provide insights about the implicating mechanisms and pathways. Moreover, proteomics analysis of CAFs phagocytotic compartment containing apoptotic tumor cells, would reveal protein expression patterns and implicated protein networks, facilitating the understanding of CAFs regulatory role.

This project, focus on the role of autophagic pathway of cancer-associated fibroblasts inside the tumor microenvironment. Autophagy is a fundamental cellular procedure with critical role in cancer survival, progression and resistance to therapy, so the understanding of the functional role of autophagy in CAFs could provide novel insights about the mechanisms implicated in tumor progression and produce prognostic or therapeutic procedures for the generation of a variety of solid cancers.

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