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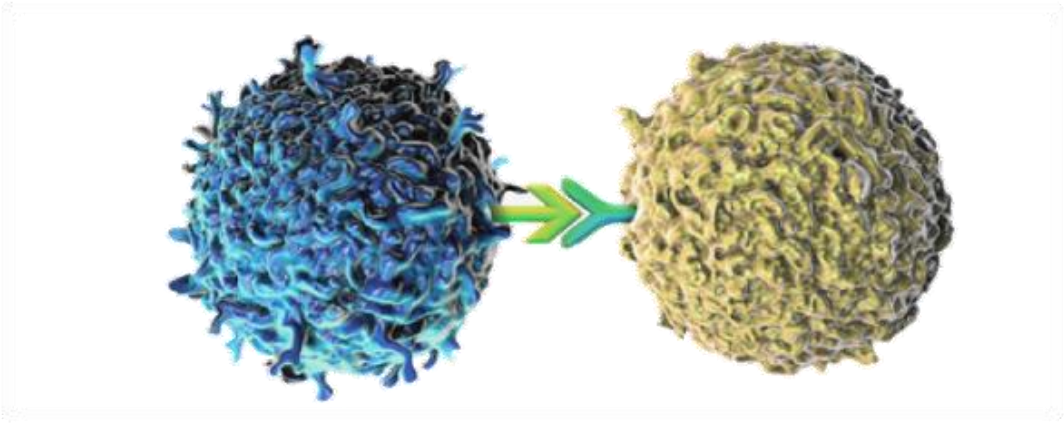
International MSc
Molecular Biomedicine

Master Thesis:

Investigation of the Role of Cancer Associated Fibroblasts in the Generation of Tumor-Specific T Regulatory (Treg) Cells that Promote Tumor Development



A thesis submitted for the Degree of the International Master of Science (M.Sc) Program of the National and Kapodistrian University of Athens: "Molecular Biomedicine"

Author:
Athina Varveri, B.Sc.

Thesis Supervisor:
Dr. Panayotis Verginis

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Supervisor: Panayotis Verginis, PhD, Researcher C' (BRFAA)

Three-member committee of inquiry:

- D. Boumpas, Professor at the National Kapodistrian University of Athens (NKUA), Medical School
- A. Eliopoulos, Professor at the National and Kapodistrian University of Athens (NKUA), Medical School
- P. Verginis, Researcher C' (BRFAA)

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Abstract

T regulatory cells (T_{regs}), as the most abundant immune suppressive cells of the TME, have been associated with impeding potent anti-tumor immune responses and impairing the success of immunotherapies. To this end, inhibiting or eliminating them is considered an attractive option for cancer treatments, as targeting them may facilitate containment of the growing tumor. However, in order to eliminate only tumor-infiltrating T_{regs} without affecting the peripheral T_{regs}, it is an urgent need to find out the mechanisms and molecules responsible for their recruitment and expansion in the TME. A dominant component of the TME, whose role as potential regulators of the anti-tumor immunity has only recently started to be explored, is Cancer Associated Fibroblasts (CAFs). Mainly through their secretome, CAFs are capable of affecting the induction and accumulation of T_{regs} in the TME. However, the mechanisms behind CAF-mediated immune suppression remain elusive until today.

For the present Master Thesis, we aimed to directly assess the potential cross-talk between CAFs and cells of the anti-tumor immunity, with focus on T_{regs}. Herein, we demonstrate a significant enrichment of α-SMA^+ CAFs in the TME of mouse melanoma, while a clear α-SMA^+ population was detected in circulation in later tumor stages. Specific ablation of α-SMA^+ CAFs before melanoma induction resulted in significant regression of tumor growth, accompanied with elevated numbers of tumor infiltrating $CD45^+$ cells and increased numbers of intratumoral T_{regs}. Furthermore, following $CD4^+FoxP3^-$ naive T cells culture in T_{reg} induction conditions with TES vs $TES_{CAFs\Delta}$, we found Foxp3 expression levels to be significantly downregulated in the presence of $TES_{CAFs\Delta}$, while their proliferation was hindered as well.

Collectively, our data bring into focus CAFs as an important cell population with immune-suppressive properties, characterized by a potency to induce highly immunosuppressive populations, such as T_{regs}. Elucidation of how this role of theirs unfolds shall provide a better mechanistic insight of how CAFs hinder tumor immune responses and ultimately lead to development of more efficacious immunotherapeutic approaches.

Introduction

Cancer is the second leading cause of death globally according to the World Health Organization (WHO); however, despite years of research, deciphering the precise mechanisms with which it manifests is still a challenge for medical scientists. Although tumor genesis and progression are processes classically characterized as orchestrated by genetically transformed cells which are altered on a cellular, genetic or epigenetic level, acquiring hyper-proliferative and invasive capacities[1,2], it is evident today that the complex changes regarding regulation of tumor development can't be fully explained by the properties of cancer cells alone. In fact, the importance of the tumor microenvironment (TME) – the surrounding stroma in which cancer cells are embedded in – is well accepted today[1,3]; its components are considered important targets in emerging anti-cancer therapies and unraveling how they support cancer cells and facilitate malignant progression is of utmost importance.

The TME **(Figure 1)** is comprised of i) a soluble compartment including growth factors, chemokines, cytokines, matrix metalloproteinases (MMPs), etc[4,5,6,7] and ii) a cellular component which includes non-cancer cells, such as endothelial cells, immune cells, cancer associated fibroblasts (CAFs), adipocytes and pericytes[4,5,6,7]. Its significance as an important contributor of malignant progression has first been noted over 100 years ago, when Paget et al. proposed the theory of "seed and soil"[8], which suggests that the "soil" (the microenvironment of the tumor) is necessary for growth and survival of the "seed", which is the cancer cell population. However, the functional importance of the TME has been in general overlooked until recent years; how the components of the TME fully impact on tumor occurrence and development, as well as cancer cell therapeutic resistance remain unappreciated[3].

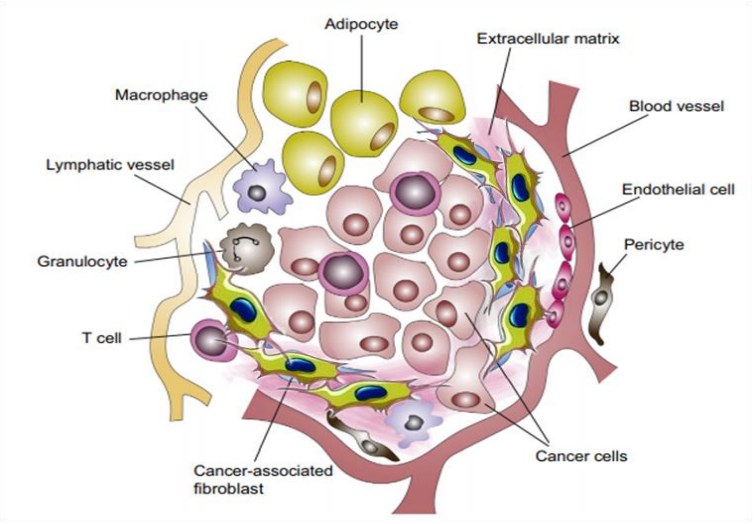


Figure 1 | Tumors have an "organ-like structure" and are comprised of many cell types which surround the cancer cells. Figure modified from Prajapati et al. (2016, J of Bone Oncology) and Ziani et al. (2018, Frontiers in Immunology)[9,10]

1. The anti-tumor immunity

1.1 Immune surveillance: an innate anti-cancer mechanism

Our immune system repels exogenous and endogenous threats, including tumor cells that may arise[11]. The existence of the anti-tumor immunity has been verified since the 19th century, when tumor infiltrating leukocytes (TILs) were first observed[12]; since then, experiments in immunodeficient mice have shown that they develop more often and faster spontaneous and carcinogen-induced tumors[13]. Despite all of these data pinpointing the importance of describing how the immune system affects tumors, it is only in recent years that immune-related mechanisms which prevent cancerous lesions from appearing and spreading have started to be unraveled[1,11,14]. Today, the theory of immune surveillance has been widely accepted.

The theory of immune surveillance proposes that the immune system constantly monitors the cells and tissues of the host, recognizing tumor cells by their genetic and cellular alterations, orchestrating their elimination and thus maintaining immune homeostasis[1,15]. Both the innate and the adaptive compartments of the immune system are involved in this process, having as a result the prevention of the evolution and development of malignancies[1,11]. More specifically, once an immune cell recognizes a cancer cell, the "Cancer-Immunity Cycle" is initiated[16] **(Figure 2)**, resulting in elimination of the latter and an amplified immune response locally to ensure that any remaining cancer cells are eradicated as well.

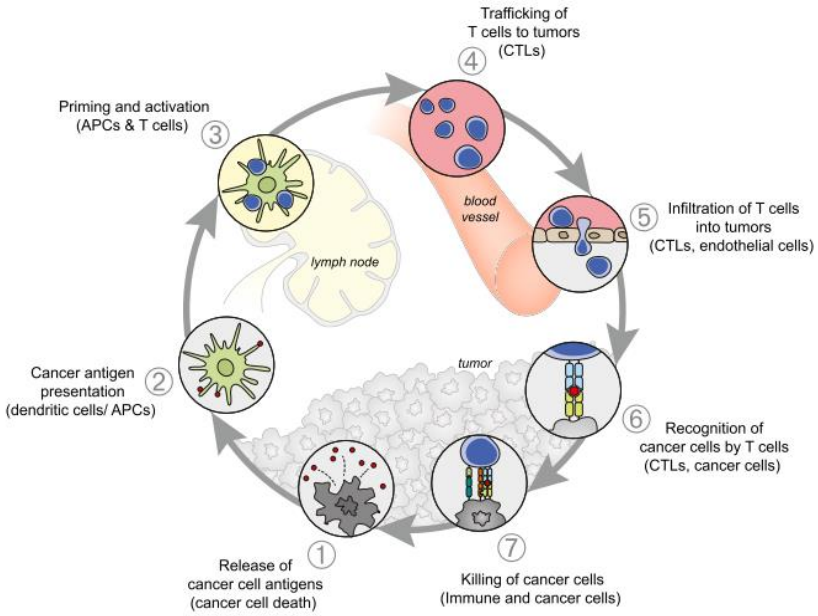


Figure 2 | The "Cancer-Immunity Cycle", as proposed by Chen & Mellman (2013, Immunity).[16]

The cancer-immunity cycle consists of seven main steps. Briefly, a cancer antigen is captured and processed by antigen presenting cells (APCs), which present it to T lymphocytes, leading to activation of the latter. The recognition of cancer cells by T cells has as a result their elimination and release of more cancer-associated antigens, amplifying the anti tumor responses and re-initiating the cycle.

However, on some occasions cancer cells may manage to avoid detection by the immune system or evade immune eradication; as a result, solid tumors do appear[1,16,17]. The concept of cancer immunoediting has been proposed in order to describe how tumor cells interplay with immune cells, which is separated in three main phases[13,17]:

- **Phase 1 – Elimination:** Phase 1 is repeated every time cancer cells arise due to failed tumor suppressor mechanisms and includes their successful recognition and elimination by the immune system.
- **Phase 2 – Equilibrium:** Tumor cells that survived Phase 1 enter a temporary equilibrium with the host immune system which is only enough to control (but not exterminate) the developed tumor bed. On this stage, the tumor cells contained evolve by accumulating further changes, providing them with reduced immunogenicity. Phase 2 is considered to be the longest phase of the three.
- **Phase 3 – Escape:** The tumor cell clones that have been generated in the phase of equilibrium and that are resistant to immune recognition or can suppress the anti-tumor immune response are selected. Thus, the immune system can no longer contain the development of the cancerous lesion.

Following the phase of the escape, cancer cells are able to avoid elimination by the anti-tumor immunity by an affluence of mechanisms **(Table 1)**, allowing for expansion of the cancerous lesion. However, cells of the immune system still manage to kill cancer cells or inhibit their functions, through cell to cell interactions or production of soluble factors such as growth factors, cytokines, chemokines and reactive oxygen and nitrogen species[1,12]. Emerging anticancer therapies attempt to boost these capabilities of the immune system, by either promoting effector cell functions or inhibiting the immune suppressor mechanisms that cancer cells utilize.

Table 1 | Mechanisms cancer cells utilize to avoid recognition and destruction by the immune system.[1,14,18]

Mechanism	Effect
cell surface alterations (eg. loss/gain of antigen expression)	reduced recognition by immune cells
mutation in antigen presentation pathways	reduced recognition by immune cells
defects in death-receptor signaling pathways or expression of antiapoptotic signals	hampered immune destruction
creation of physical barriers	hampered immune infiltration
production of immunosuppressive soluble factors (eg. TGF-β, IL-10)	suppression of immune effector cells
expression of immune-checkpoint proteins (eg. CTLA-4, PDL1)	suppression of immune effector cells
recruitment of cell populations with immunosuppressive functions (eg. the T regulatory lymphocytes and the myeloid-derived suppressor cells)	suppression of immune effector cells

1.2 T regulatory lymphocytes: Basic characteristics and their importance regarding immune suppression in cancer

Among the mechanisms that growing tumors utilize in order to inhibit effector responses is the recruitment and expansion of T regulatory cells (T_{regs}), which comprise the dominant immunomodulatory cell population of the immune system[15,19,20]. The main function of T_{regs} is to regulate T cell-mediated responses towards self antigens (eg. tumor cells) and non self antigens

(eg. microbes)[15,19,20,21,22]. Furthermore, their regulatory functions affect many cells of the immune system, including dendritic cells, macrophages, neutrophils, γδ T cells, natural killer cells and innate lymphoid cells[20,23,24,25,26]. Undeniably, the existence of a balanced, functional population of these cells is crucial for the prevention of autoimmune manifestations and chronic inflammatory diseases, by ensuring peripheral tolerance and stopping excessive immune responses when they prove to be life-threatening for the host.

One of the cardinal features of T_{regs} is the expression of CD25 on the cell surface, which is the high affinity chain of the interleukin-2 receptor[27,28,29,30]. Nonetheless, up to date, the most definite marker characterizing Tregs is the transcription factor Foxp3 (forkhead box P3) [15,22,31,32], which orchestrates Treg development and function[15,20]. Foxp3 is essential for $CD4^+CD8^-$ T cells to differentiate in thymus to T_{reg} lineage, while induced Foxp3 expression is potent of conferring suppressive activity to non-T_{regs} in the peripheral organs **(Figure 3)**. As a consequence of the aforementioned, T_{regs} are divided in two main subpopulations: i) natural T_{regs} (nT_{regs}) which arise in thymus in early life and comprise 5-10% of total peripheral T cells in mice and humans[15,21,24,33,34] and ii) induced T_{regs} (iT_{regs}), which are generated in the periphery from naïve $CD4^+FoxP3^-$ cells through upregulation of Foxp3 transcription factor.[15,20,35,21,36,37,38] Although their separate functions have not been clearly elucidated until today, it is undisputable that those two distinct subtypes do exert different, yet complementary roles; more specifically, nT_{reg} cells seem to have a prominent role in recognizing self-antigens, while iT_{regs} are implicated in establishing tolerance to non-self antigens (e.g. gut commensal bacteria and innocuous antigens present in food)[39,40,41]. The mechanisms underpinning the development and antigen specificities of the two subtypes probably differ and lead to a global promotion of peripheral tolerance.

The importance of Foxp3 expressing T_{regs} is highlighted by the fact that mutations in the gene that codes for this transcription factor (*FOXP3* gene) lead to development of devastating autoimmune disorders in male humans and mice. More specifically, mutations of the human Foxp3 cause the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome in men (IPEX syndrome), while mutations of the mouse Foxp3 cause the Scurfy syndrome; both syndromes are characterized by hyperactivation of $CD4^+$ T cells and overproduction of proinflammatory cytokines[15,20,38,42,43]. The phenotype of these disorders is the result of failed T_{regs} differentiation into effective immune suppressors and outlines the importance of this transcription factor for the normal development and function of T_{regs}.

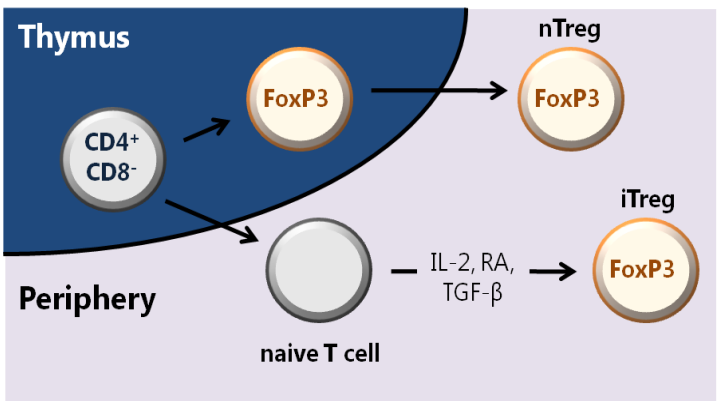


Figure 3 | Differentiation of $CD4^+CD8^-$ precursor cells to nT_{regs} in thymus or later to iT_{regs} in peripheral organs.
Following expression of Foxp3 transcription factor, the expression of other T_{reg}-associated molecules is upregulated as well, such as CD25, CTLA-4 (cytotoxic T cell associated antigen-4) and GITR (glucocorticoid-induced TNF receptor family-related gene/protein)[20]. nT_{reg}, natural T_{reg}; iT_{reg}, induced T_{reg}; RA, retinoic acid

FoxP3$^+$ T_{regs} are found in increased frequencies in tumors, where they mediate the formation of a tolerogenic microenvironment, functioning as powerful inhibitors of the anti-tumor immunity[15,20,37,44,45]. They affect almost all immune cells present in the TME **(Figure 4)**, exerting their immunosuppressive functions mainly through cell-to-cell interactions, secretion of suppressive factors and immune cell nutrient deprivation[1,20,44,46,47]. As a result, they disrupt immune homeostasis towards an immunosuppressive state, thereby promoting immune evasion of cancer cells. Their functions importantly contribute to the progressive features of cancer cells, increasing the need for unraveling the mechanisms and the cell populations mediating their recruitment and expansion in the TME in order to combat cancer more effectively.

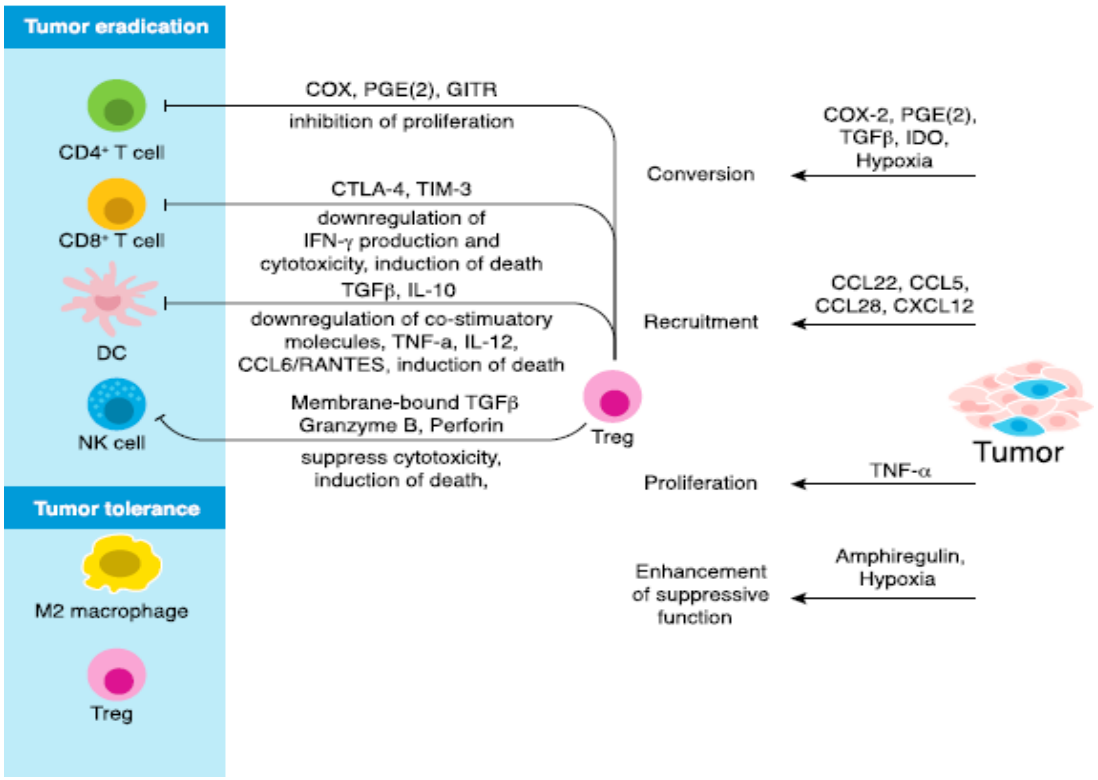


Figure 4 | Tumor cells promote immunosuppression by inducing the presence of T_{regs} in the TME.

Soluble factors secreted by tumor cells and the components of the TME affect the enhanced presence of T_{regs} in the TME. T_{regs} in turn affect almost all cells of the immune system, creating an immunosuppressive TME which allows tumor cells to evade immune destruction. Figure modified from Hatzioannou et al. (2017, Oncoimmunology).[15]

T_{reg} cell numbers are significantly increased within the TME of various tumor types in humans and mice[48,49,50,51]. The mechanisms behind intratumoral T_{reg} cell aggregation aggregation are not fully described; experimental evidence implicates their recruitment through chemokines produced by tumor or host cells (e.g. CCL22, CCL5, CCL28, CCL2, and CXCL12), local spread of nT_{regs} and the immense resistance of Tregs to reactive oxygen species (ROS) extensively produced in the TME[52,53,54,55,56,57,58,59,60,61,62,63,64]. Increased presence of T_{regs} in malignant tissues has been associated with reduced patient survival, increased likelihood for metastasis and advanced-stage disease in

many types of cancer, including melanoma, pancreatic ductal adenocarcinoma (PDAC) and non small-cell lung, gastric and ovarian cancer[55,56,65,66,67].

Besides their generation and accumulation in the tumor mass, the phenomenon of T_{reg} induction is also quite intensified in the TME; a significant percentage of tumor infiltrating $CD4^+FoxP3^-$ T cells upregulate Foxp3, quite possibly due to molecules secreted in abundancy by cancer cells and other cell types of the TME; this conversion confers conventional T cells with all the characteristics of T_{regs}[15,38,68]. Moreover, studies in colorectal and prostate cancer have demonstrated that Tregs present in the TME proliferate, in contradiction to their anergic natural state[15,69,70]. Finally, T_{regs} levels have also been noted to be significantly elevated in the peripheral blood of cancer patients on many occasions[71,72,73,74,75], outlining their potential as a marker of poor disease prognosis. However, the exact mechanisms that determine their recruitment, induction and proliferation in the TME, their entrance in peripheral blood, as well as the cell populations that mediate these processes remain unidentified.

1.3 Targeting tumor-infiltrating T_{regs}: A paradigm shift in cancer treatment

Due to the major role that the immunosuppressive characteristics of the TME play in dictating tumor progression and metastasis, emerging approaches aim to boost the effectiveness of the anti-tumor immune responses, either by targeting the components that facilitate the immune escape of cancer cells or by enhancing the cytotoxic capabilities of immune effector cells, while simultaneously avoiding the development of autoimmune disorders[15,18,36]. Immunotherapy approaches are considered a breakthrough in cancer therapeutics; the 2018 Nobel Prize Award in Physiology or Medicine was awarded to the two researches-pioneers of the field whose work led to the advent of checkpoint blockade immunotherapies.[15,18,76]

As the main immunosuppressors of the tumor bed, inhibiting or eliminating tumor-infiltrating T_{regs} is considered an attractive option for cancer treatments; targeting them may facilitate containment of the growing tumor[20,37]. Emerging immune-based therapies are focused on molecules expressed by T_{regs}, such as LAG3, OX-40, CD25, GITR, CTLA-4 and PD-1 **(Figure 5)**.

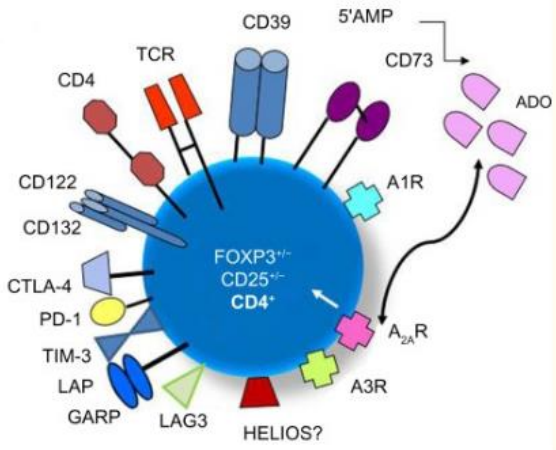


Figure 5 | Cell surface molecules expressed on T_{regs} are utilized as immunotherapy targets.

Figure adapted from Whiteside et al. (2015, Immunotargets and Therapy)[61].

CTLA-4, cytotoxic lymphocyte antigen-4; PD-1, programmed death-1; TIM-3, T cell immunoglobulin mucin-3; LAP, latency-associated protein; GARP, glycoprotein A repetitions predominant; LAG-3, lymphocyte activation gene-3; TCR, T cell receptor

An excellent implementation of immunotherapies targeting T_{regs} in cancer is the therapeutic antibody ipilimumab (known as Yervoy), which has been approved by the Food and Drug Administration (FDA) and is currently used for melanoma treatment.[76] Ipilimumab targets the checkpoint molecule CTLA-4 which is an inhibitory T cell costimulatory molecule abundantly expressed by T_{regs}, thereby preventing blockage of T cell function **(Figure 6)**. Indeed, by ipilimumab administration, the anti-tumor immunity is enhanced in cancer patients and immune cells are able to attack the tumor successfully.

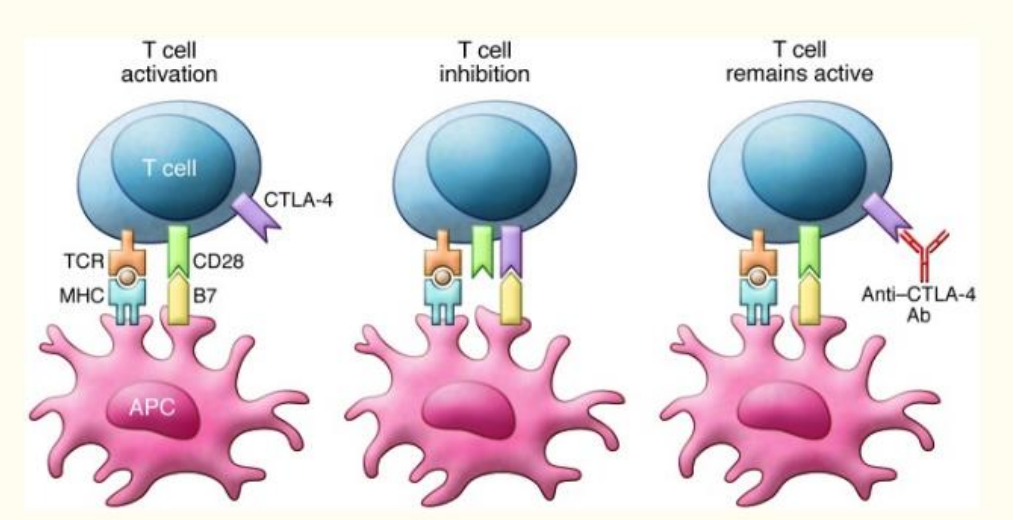


Figure 6. Ipilimumab mechanism of action. Figure adapted from Hurst (2015, JCI)[76]

(Left; Center): If CTLA-4 is binds to a B7 family member in the surface of antigen presenting cells (APCs), T cell function is inhibited and the anti-tumor immune response is halted.
(Right): The anti-CTLA-4 antibody binds to CTLA-4, expressed abundantly on the cell surface of T_{regs}. Therefore, inhibition of T cell function is prevented.

Nonetheless, existing regimens are associated with low response rates in a sizable portion of cancer patients[15,18]. Furthermore, despite enhancing anti-tumor responses, such therapies also inhibit the peripheral T_{reg} population, which is crucial for tolerance maintenance, occasionally leading to secondary autoimmune manifestations (IRAEs; immune related adverse events)[15,77]. Until today, it still remains a challenge to eliminate only tumor-infiltrating T_{regs} without affecting the peripheral T_{regs}. Current research aims to find out how they are specifically recruited and expanded in the TME, aiming to target the mechanisms and molecules dictating their tumor-promoting function.

2. Cancer Associated Fibroblasts

Among the major cell constituents of the TME are cancer associated fibroblasts (CAFs), a highly abundant and heterogeneous population of cells[3,4,5,78,10,79,80,81,82,83]. CAFs have been many times characterized as tumor promoters, having been implicated in almost all of the hallmarks of cancer[1,84] **(Figure 7)**. However, the precise mechanisms with which they might facilitate cancer initiation, progression and metastasis have not been fully deciphered yet; thus, studying their biology and means of interaction with both the cancer cells and the other components of the TME will undoubtedly lead to new information that shall ultimately lead to development of more efficacious and enduring cancer therapies.

2.1 Basic characteristics of fibroblasts – Irreversible activation of fibroblasts to CAFs in the TME

Fibroblasts in normal tissues **(normal fibroblasts or NFs)** constitute one of the most abundant cell populations of the connective tissue[85]. They are characterized by an elongated spindle-like shape; in fact, their appearance is similar to mesenchymal cells and smooth muscle cells[85]. They usually remain in a quiescent state in the epithelium stroma, exhibiting little metabolic or transcriptomic activity[85,86].

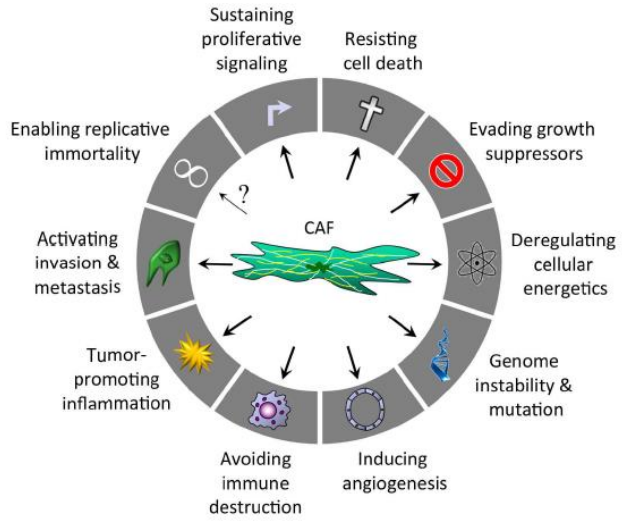


Figure 7 | Almost all hallmarks of cancer are regulated by CAFs. (Figure adapted by Tommelein et al., 2015, Frontiers in Oncology)

Once NFs receive certain stimuli (mainly during wound healing, inflammation and senescence[85,87]) they get activated, acquiring a series of different features such as the expression of different cell surface and intracellular markers (more notably α-SMA or FAP), a rapid proliferation rate, increased cell contractility and development of stress fibers, enhanced collagen production and an enhanced transcriptional and secretory program[85,87]. Overall, their activated phenotype reminisces that of smooth muscle cells. Through these changes, their main role is to maintain tissue homeostasis by remodeling the extracellular matrix (ECM), recruiting immune cell

populations and forming the basement membrane[85,87], all necessary to heal a wounded tissue. When the stimulus that caused their activation ceases to exist, they usually revert to their original quiescent state or undergo apoptosis[85].

In the context of the TME, fibroblasts also get activated, therefore acquiring all aforementioned characteristics; however two major differences are that their activation is irreversible and they do not get removed by apoptosis. This process seems to be mainly mediated by cancer cells, which secrete high levels of growth factors (notably TGF-β, PDGF and FGF), resulting in fibroblast activation and initiation of the phenomenon known as cancer fibrosis[78,80,88]. In fact, CAFs are constantly exposed to never-ending stimuli, which promote an exaggerated secretory and ECM remodeling phenotype and is thought to require epigenetic modifications (for example, CAFs gain different methylation patterns compared to NFs)[6,85]. As a result, tumors are considered as "wounds that do not heal"[89], characterized by chronic tissue injury and a continuous repair response. Although the role of activated fibroblasts regarding tissue healing is well described, many of their functions in the TME once they become irreversibly activated remain unidentified.

2.2 CAFs heterogeneity in the TME

CAFs have been attributed with complex and at many times contradictory roles. To study how CAFs function in cancer progression and metastasis, one must consider their intrinsic heterogeneity and multiple possible origins **(Figure 8)**. The different cellular sources that give rise to the population of CAFs in the TME lead to a great diversity regarding the cell surface and intracellular markers they express. This characteristic of theirs makes studying and isolating them is quite an arduous task; this fact is among the main reasons why CAFs functions in tumor initiation, progression and metastasis haven't been extensively studied until today.

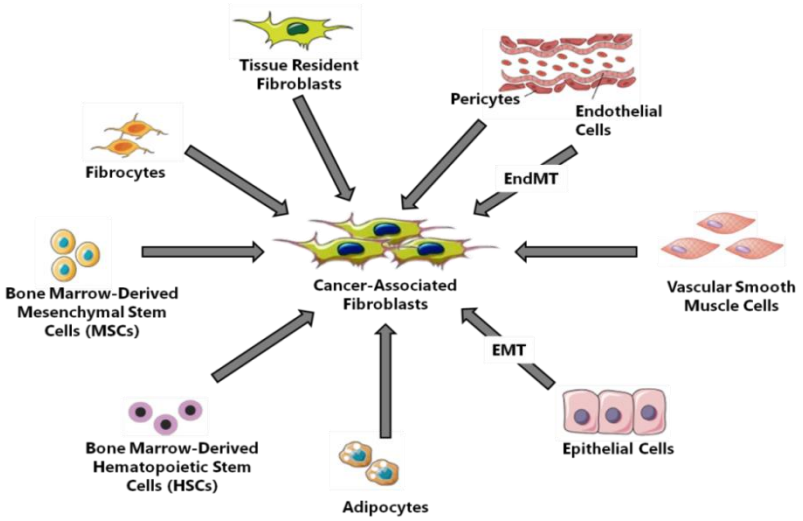


Figure 8 | Multiple CAFs origins indicate diversity within the fibroblast population and contribute to the difficulty to distinguish them from other cell types expressing similar markers.[5,6,79,80,87,90,91,92] **Figure modified from Alkasalias et. al (2018, Int. J. Mol. Sci.); Shiga et. al (2015, Cancers); Ziani et.al (2018, Front. Immunol). EndMT, endothelial to mesenchymal transition; EMT, epithelial to mesenchymal transition.**

Besides the cell type of origin, other factors may enhance the heterogeneity of CAFs, including the tumor type, the tissue type in which the tumor grows and the local paracrine environment[5,90]. As a result, CAFs display varying gene signatures and protein expression patterns. Furthering their plasticity, until today there is a lack of CAF-specific markers; most of the markers used to isolate and study them are either expressed in other cell types (including cancer cells, smooth muscle cells, stem cells, etc), or they are also expressed in normal (hence, not activated) fibroblasts[5,6,90]. Moreover, different CAFs subpopulations seem to appear even in the same tumor stroma, adding one more degree to their complexity; recent data even propose a distinct function regarding the different subpopulations of the TME[78,92,93]. Collectively, the very definition of CAFs remains controversial, pinpointing the need for description of specific criteria that characterize this heterogeneous cell population of the TME.

Experimentally, CAFs are defined based on i) their spindle-like shape, ii) the absence of endothelial (eg CD31), epithelial (eg EpCAM or cytokeratin) and inflammatory (eg CD45) markers and iii) the presence of markers for activated fibroblasts, most notably alpha smooth muscle actin (α-SMA), fibroblast activation protein (FAP), fibroblast specific protein (FSP-1), platelet-derived growth factor-α receptor (PDGFR-α), platelet-derived growth factor-β receptor (PDGFR-β), and vimentin **(Table 2)**[5,6,86,87,90,91,92,93,94,95,96,97,98]. As already mentioned though, these markers are not CAF-specific and are not expressed in all existing CAF subpopulations.

Table 2 | Markers typically used to distinguish CAFs.[5,6,86,87,90,91,92,93,94,95,96,97,98]

Positive Markers	Negative Markers
alpha smooth muscle actin (αSMA) fibroblast activating protein (FAP) fibroblast specific protein (FSP-1 or S100A4) platelet-derived growth factor-α receptor (PDGFR-α) platelet-derived growth factor-β receptor (PDGFR-β) vimentin tenascin-C periostin neuron glial antigen-2 (NG-2) desmin podoplanin paladin vimentin endosialin integrin B1/CD29 caveolin-1	CD31 (endothelial marker) cytokeratin (epithelial marker) EpCAM (epithelial marker) CD45 (marker of cells of hematopoietic origin)

2.3 Studying CAFs holds great potential for anticancer therapies

In agreement with the above, CAFs are either recruited or expanded in the TME. There, they exert their many functions, modulating the characteristics of the tumor bed, through their mechanical properties, or through the molecules they secrete and the cell interactions they

mediate. As expected, multiple roles have been attributed to them, most of them supporting malignacy **(Table 3)**. Recent studies highlight tumor-suppressive properties in CAFs, underlying the heterogeneity of different CAFs subsets that may play opposing roles in tumor development.

Studying the properties of CAFs holds great potential for anticancer therapies. In fact, CAFs constitute on many occasions the most prevalent cell population of the TME[5,85,92,99] and have been found to mediate resistance to chemotherapy and radiotherapy[6,87,91, 95,97]. The fact that they are highly heterogeneous, having multiple origins and being characterized by a lack of CAF-specific markers makes the task of isolating and studying them quite difficult. As a result, despite their abundance in the TME, their tumor-promoting capabilities haven't been fully explored yet.

CAFs in general exhibit the functions of activated fibroblasts, having increased contractility, enhanced metabolic activity and modifying the surrounding ECM. Therefore, they promote cancer cell transformation, proliferation, angiogenesis, invasion, and metastasis via secretion of various growth factors, cytokines, chemokines, and degradation of extracellular matrix (ECM) proteins, impacting on all cell types present in the TME[1,4,5,80,91,94,95]. Furthermore, CAFs tend to surround cancer cells, being the first to invade through adjacent normal tissues[100], while they have also been found to exchange miRNAs with cancer cells and other cell types of the TME (possibly through gap junctions or exosomes)[6,80,91]. To design effective anti-cancer therapies, more information regarding CAFs is required and the mechanisms with which CAFs mediate their functions need to be unraveled.

2.4 CAFs and the anti-tumor immunity

In later years, the interactions between CAFs and infiltrating cells of the anti-tumor immunity have started to be explored. Despite the fact that this field is still at its infancy, data so far demonstrate an overall immunosuppressive role of CAFs; through their secretome they are capable of modulating the characteristics of immune cells, therefore potentiating immune evasion of cancer cells[33,10,80,91,96,101,102]. Their role in tumor-enhancing inflammation must be fully characterized, since they can be key players in the creation of the highly immunosuppressive TME, and therefore they could be among the main reasons behind failed immunotherapeutic approaches.

I. CAFs effect on the innate immunity

As a major source of immunomodulatory cytokines and chemokines in the TME, CAFs are capable of affecting almost all infiltrating immune cells of the innate immunity **(Figure 9)**. In 2012 and 2013, two independent groups demonstrated for the first time that co-expression of CAFs and M2 macrophages markers (FSP-1, α-SMA and FAP for CAFs; CD163, DC-SIGN for M2 macrophages) is associated with a poor outcome in patients with colorectal cancer and oral squamous cell carcinoma.[103,104] Since then, CAFs have been documented to mediate recruitment

and expansion of several immune cell populations in the TME (such as monocytes, mast cells and neutrophils), mainly exerting this function of theirs with their secretome.[10]

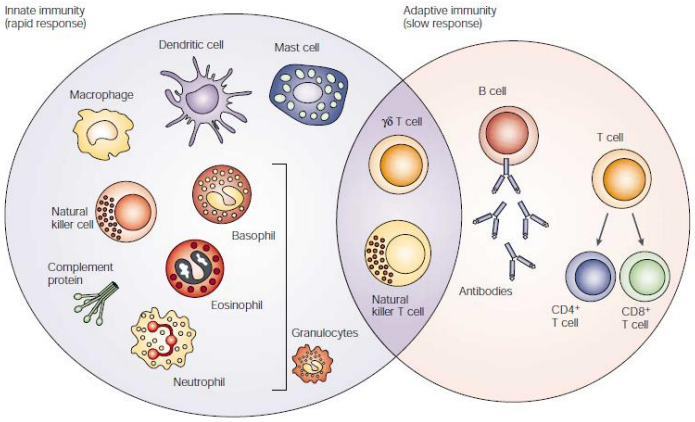


Figure 9 | Cell types of the innate and adaptive immunity (Figure adapted by Dranoff et al., 2004, Nature Reviews in Cancer).[105]

The innate immunity comprises of soluble factors (such as the complement proteins) and a number of different cvells types such as macrophages, mast cells, dendritic cells, natural killer cells and granulocytes. The adaptive immunity comprises of B lymphocytes (and the antibodies they produce) and T lymphocytes (widely divided as $CD4^+$ T cells and $CD8^+$ T cells). Finally, a number of cytotoxic cell types, most notably the natural killer cells and the γδ T cells are considered to belong between the two groups of immunity.

Notably, through IL-6 secretion, CAFs efficiently restrict DC maturation and redirect monocytes towards macrophage differentiation[80]. Furthermore, by producing a number of chemokines and other proteins (such as Chitinase 3, CXCL12/SDF1, M-CSF, IL-6 and CCL2/MCP-1), they recruit monocytes in the TME and promote their differentiation into M2 macrophages (which are highly immunosuppressive, inhibiting cytotoxic $CD8^+$ T cell-mediated responses in the TME).[10,80,96,101] Finally, through the expression and secretion of TGF-β and MMPs, CAFs can potentially attenuate the activation of natural killer cells and their cytotoxic activities.[10,102]

II. CAFs effect on the adaptive immunity

CAFs seem to mediate tumor-enhancing inflammation[33,10,97,106,107,108] and to interfere with the adaptive anti-tumor immune response as well (Figure 1.2.3), leading to a disruption of T cell cytotoxic function by secreting factors that result in T-cell anergy and apoptosis or hardening T-cell proliferation and functions[1,10,109]. Furthermore, via their secretory molecules they favor T_H cell polarization towards the tumor-enhancing T_{H2} and T_{H17} subtypes, at the expense of the protective T_{H1} subtype, while via IL-6 expression they are capable of differentiating monocyte precursors into the highly immunosuppressive MDSCs.[10,91,96] By CAFs-derived molecules (such as TGF-β and VEGF), the expression of MHC II molecules and of the co-stimulatory molecules CD40, CD80, and CD86 in antigen presenting cells (most notably DCs) is downregulated, thus abrogating effective antigen presentation and facilitating immune evasion.[10] Finally, CAFs have been observed on limited

documented cases to express PDL-1 and PDL-2, two cell surface molecules which engage their receptor programmed death receptor 1 (PD1) expressed on T-cells, strongly counteracting TCR signaling and CD28-costimulation, resulting in the inhibition of T cell activation, proliferation, and functions.[10,109,110,111]

As a major source of TGF-β, CAFs are capable of modulating directly the cell types of the adaptive immunity. Among the many functions of this particular growth factor in tumor progression, TGF-β promotes death of T effector cells and inhibits the cytotoxic function of the $CD8^+$ T cells (regarding the latter, TGF-β is known to reprogram the metabolism of T effector cells, as well as downregulate the expression of perforin, granzymes A and B, Fas ligand, and IFN-γ).[10] TGF-β further contributes to creation of the immunosuppressive TME, by enhancing FoxP3 upregulation in naive $CD4^+$ T cells, and thus facilitating Treg induction in the tumor stroma.[5,10]

Concluding, mainly through their secretome, CAFs are capable of affecting the anti-tumor immune responses, potentially mediating the observed T effector cell anergy and tumor cell immune evasion, therefore facilitating tumor growth and metastasis. Clearly, further investigations are required in order to determine exactly the mechanisms behind immunosuppression induced by CAFs in vivo; a clear elucidation of how the aforementioned processes unfold will provide a better mechanistic insight of how CAFs influence anti-tumor immune responses, ultimately leading to establishment of new approaches for cancer immunotherapy.

Table 3 | Multifaceted roles of CAFs in cancer

TME Modulation and Epithelial-to-Mesenchymal Transition CAFs secrete plasminogen activators, MMPs and other proteins (eg fibronectin, collage, hyaluronan, lysyl-6-oxidase or LOX) which degrade and remodel the ECM and therefore increase the stiffness of the matrix.[80,112] Furthermore, through their paracrine function, this cell subpopulation cleaves cell adhesion molecules, potentiating increased motility and finally EMT (eg MMP-2 and MMP-9 secretion in prostate cancer leads to downregulation of E-cadherin and thus EMT).[80]
Tumorigenesis Through growth factors (notably TGF-β, HGF) that CAFs secrete, CAFs facilitate tumor initiation.[85] Their characteristics enhance epithelial cell transformation and immortalization.[80,85]
Epithelial Transformation and Proliferation CAFs secrete paracrine factors (cytokines, chemokines, growth factors and other soluble molecules) which affect normal epithelial cells as well as cancer cells[4,91]. For example, by H_2O_2 secretion, they mediate epithelial cell transformation and enhance cancer cell aggressiveness[91]. Through their secreted molecules (by contributing various well known mitogens for epithelial cancer cells, such as HGF, EGF, b-FGF and cytokines such as SDF-1 and IL-6), they facilitate tumor cell proliferation, as well as promote tumor growth.[80]
Tumor Metabolism CAFs in contact with cancer cells produce lactate and pyruvate through aerobic glycolysis, as well as energy-rich metabolites. This fact helps cancer cells to overcome depletion of nutrients, O_2 and pH.[80]

Provide cancer cells with nutrients

Through soluble factors, nutrient-rich exosomes, etc.[112]

Cancer Cell Invasion and Metastasis

Through matrix-remodeling enzymes (eg. MMPs) and other soluble factors that CAFs secrete (eg chemokines/cytokines/growth factors), they promote tumor invasion and metastasis.[85,91] Of note, through their mechanical properties, they are also capable of creating gaps in the basement membrane, thus allowing for cancer cell invasion without MMPs production[80,112]. Furthermore, CAFs have been shown to increase the stiffness of the TME and to create structures which facilitate tumor cell invasion and metastasis.[5,80] Finally, on limited occasions CAFs have been documented to travel with cancer cells in the circulation[91,113,114,115], ensuring cancer cell survival and preparing formation of the metastatic niche.

Angiogenesis

Through VEGF, FGF2, SDF-1 that CAFs secrete, they promote angiogenesis. [80,85,90]

ECM Remodeling

CAFs are responsible for the synthesis, deposition and turnover of the ECM.[4]

Drug Resistance

The complex and intricate signaling network induced by CAFs has been found to promote drug resistance and the survival of cancer cells. Moreover, the ability of CAFs to support cancer stem cells, which remain in a quiescent state and are resistant to chemo- and radiotherapy, further reducing the vulnerability of the tumor to treatment.[95]

Notable examples include:

1) By activating the PDGF-C pathway they bypass anti-VEGF treatment.[87]

2) Through HGF secretion they promote cancer cell resistance to RAF inhibitors.[87]

3) By secretion of glutathione and cysteine, CAFs antagonize platinum-based chemotherapy, an effect which can be reversed by effector T cells in the TME boosted by a synergistic immunotherapy.[91]

4) Cisplatin treatment leads to IL-11 secretion by CAFs, inducing survival and anti-apoptotic signals in tumor cells.[91]

5) DNA damage in CAFs (induced by chemotherapy or radiation) leads to production of WNT16B, enhancing cancer cell proliferation and invasion.[6]

6) By secreting MMP-1 and carbonic anhydrase IX following treatment with cetuximab, CAFs induced EMT.[95]

7) By overexpressing BCL-XL CAFs protect prostate cancer cells from the cytotoxic effect of sorafenib.[95]

Tumor Suppression

CAFs have been found to inhibit the growth of cancer cells in vitro and xenograft tumors by secretion of tumor-suppressive molecules (eg. Wnt3a)[5].

2.5 Future of research in CAFs – Switching to T_{regs} biology

As already described, one of the most interesting characteristics for CAFs is their enhanced secretory function[33,10,80,91,96,101,102]; importantly, they are a major source of TGF-β and other immunosuppressive cytokines. Through their properties, they are acknowledged as being completely potent in recruiting and inducing T_{regs}[33,10], which are indeed the major immunosuppressive immune cell subpopulation in the TME. However, the correlation between Tregs and CAFs has not been thoroughly investigated, despite data from the literature depicting a direct association between these two subpopulations.

An important early observation regarding the potential interplay between T_{regs} came in 2005 from the work of Sharma et al, who published their results depicting that expression of COX-2 by CAFs in lung or pancreatic cancers leads to their secretion of PGE-2, which is known to induce Foxp3 expression[116]. Furthermore, in 2009 Liao et al focused on FAP^+ CAFs and showed that their depletion in mammary tumors is associated with reduction of T_{regs}[117], while in 2013 Kinoshita et al found that the presence of T_{regs} coexisting with CAFs is correlated with poor outcomes in lung adenocarcinoma patients[118]. Finally, in 2014, Ozdemir et al depleted the α-SMA^+ CAFs in mouse models of pancreatic cancer and found that their absence is associated with an increase in the number of $CD4^+FoxP3^+$ T_{regs}[119].

Collectively, all of the aforementioned data suggest that CAFs might enter to a cross-talk with T_{regs} in the TME, favoring and/or leading to Treg recruitment and differentiation. Delineation whether CAFs are directly implicated in inducing immunosuppression in the TME through an interplay with T_{regs} and unraveling the specific mechanisms which define this process will undoubtedly provide a better understanding of the global regulation of the anti-tumor immune response.

3. Aim of our project

The advent of checkpoint blockade immunotherapy has revolutionized cancer therapeutics, leading to the 2018 Nobel Prize Award in Physiology or Medicine. Yet, existing regimens are associated with low response rates in a sizable portion of cancer patients. The immunosuppressive networks of the TME impede the elicitation of potent anti-tumor immune responses and impair the success of immunotherapy. Among the constituents of the TME, CAFs represent a highly heterogeneous yet abundant cell population, quite resistant to chemo/radiotherapy. Although their tumor-promoting function is acknowledged, their role in shaping the anti-tumor immune response remains elusive.

The accumulation of immunosuppressive lymphocytes in the TME, mainly represented by T_{regs} whose normal function is to suppress autoreactive T cells to maintain immunological self-tolerance and inhibit autoimmunity, is associated with advanced tumor growth and a poor outcome in several types of malignant tumors. The correlation between tumor-infiltrating T_{reg} cells and CAFs that express immunoregulatory cytokines has not been thoroughly investigated. Importantly, current research focusing on the role of CAFs in the anti-tumor immunity has bypassed how exactly they exert their immunosuppressive functions; therefore, there is an increasing need to delineate the exact mechanisms that shape this characteristic of theirs. Undoubtedly, future research on antitumor treatments should include a viable strategy that disrupts the relationship between CAFs and Tregs.

Based on the aforementioned data, for the present Master Thesis we formed the hypothesis that CAFs are required for the induction and expansion of tumor-specific FoxP3$^+$ T regulatory (T_{reg}) cells, leading to tumor development and hampering the effectiveness of immunotherapies. Specifically, data from bibliography led us to believe that CAFs directly affect Tregs recruitment, proliferation and induction, thus being key players in the formation of the highly immunosuppressive TME.

To dissect our hypothesis, we set out to work with induced melanoma models and directly assess the interplay between CAFs and T_{regs}. Specifically, first we aimed to see how CAFs accumulate in mouse melanomas and second to find out their functional role regarding the anti-tumor immunity. Among our primary objectives was to monitor the infiltration of T_{regs} upon absence of CAFs in the tumor, as well as how the secretome of CAFs impacts on the phenomenon of T_{reg} induction. Ultimately, our goal was to focus our research on how prohibiting CAFs from interacting with T_{regs} affects tumor development, and can be therefore utilized for therapeutic purpose.

It is expected that the outcomes of this Master Thesis will broaden our knowledge on the involvement of CAFs in establishing immune suppression and the present studies will help establish novel therapeutic targets.

Cell Lines

The melanoma cell line B16-F10 was kindly provided by Dr. Aris Eliopoulos (Medical School, University of Crete, Greece) and the B16-F10 cell line stably expressing ovalbumin with gfp (B16-F10-OVA.GFP) was kindly provided by Dr. Caetano Reis e Sousa (The Francis Crick Institute, London, United Kingdom). Cells were maintained in RPMI Medium (Gibco) supplemented with 10% fetal bovine serum (FBS, StemCell Technologies Inc), β-mercaptoethanol (Gibco) and a mix of Penicillin/Streptomycin (P/S, Gibco).

Mice

Mice **(Table 1)** were maintained in the animal facility of the Biomedical Research Foundation of the Academy of Athens (BRFAA). C57BL/6 mice were purchased from the Jackson laboratory; αSMA-TK mice (C57BL/6 background) were kindly provided by Dr. Raghu Kalluri (Department of Cancer Biology, Division of Basic Sciences, MD Anderson Cancer Center, Texas, USA); Foxp3gfp.KI mice (C57BL/6 background) were kindly provided by Dr. Alexander Rudensky (Department of Immunology, Memorial Sloan-Kettering Cancer Center, New York, USA). All procedures were in accordance to institutional guidelines and were approved by the Greek Federal Veterinary Office. All mice used in the experiments were 8-10 weeks old.

Table 1 | Mice strains used for all experiments

Strain	Description
C57BL/6	Commonly used as a general purpose strain and background strain for the generation of congenics carrying both spontaneous and induced mutations. **(jax.org)**
αSMA-TK	These transgenic mice express a truncated *TK*, herpes simplex 1 virus thymidine kinase (HSV TK) gene under control of an extended 5.2kb mouse *Acta2* (alpha smooth muscle actin) gene promoter that includes the *Acta2* promoter plus exon 1, intron 1, and part of exon 2. This strain allows Ganciclovir-inducible ablation of proliferating cells (myofibroblasts) that express *Acta2*. **(jax.org)**
Foxp3gfp.KI	These transgenic mice co-express EGFP and the regulatory T cell-specific transcription factor Foxp3 under the control of the endogenous promoter. **(jax.org)**

Tumor Induction

For melanoma induction **(Figure 1)**, mice were injected subcutaneously (s.c.) in the back with $3x10^5$ B16-F10 melanoma cells (or with $3x10^5$ B16-F10-OVA.GFP cells, accordingly). Tumor volume was monitored from day 9 to day 15 following melanoma cells inoculation by measurement of two perpendicular diameters (d) of the tumor by caliper every day; tumor growth was calculated using

the equation (d1∗d2∗d2)/2. On day 15 following melanoma cells inoculation the mice were sacrificed and analyzed; upon sacrifice tumor weight was assessed.

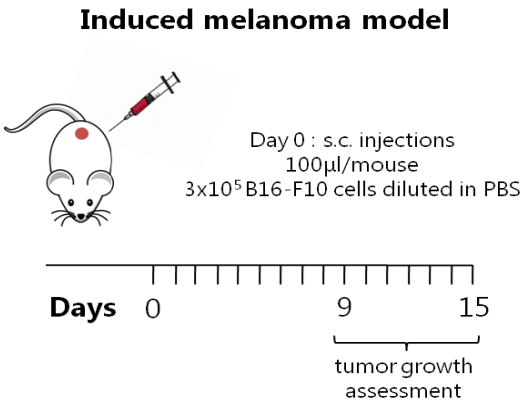


Figure 1 | Protocol for melanoma induction in mouse models (the dot represents the site of the injection).

Mouse PBMCs Isolation

For mouse peripheral blood mononuclear cells (PBMCs) isolation, approximately 500µl of heparinized blood were collected from the retro-orbital sinus and diluted 1:1 with 1x phosphate buffer saline (PBS; Gibco). 500µl of Ficoll Histopaque® 1077 (Sigma Life Science) were overlayed with the diluted blood (overlay is conducted slowly, to avoid mixing blood with ficoll) and then centrifuged for 30' at 1800rpm in room temperature (RT) with brakes. After the centrifugation, the plasma was discarded and the layer containing the PBMCs, located over the ficoll layer, was collected **(Figure 2)**. PBMCs were washed with PBS and centrifuged (10', 1800rpm, 4°C). Finally, pellets were resuspended in PBS containing 5% fetal bovine serum (FBS).

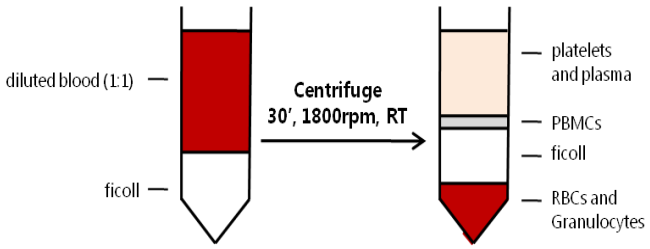


Figure 2 | PBMCs isolation with ficoll.

Ficoll is a polysaccharide which allows for PBMCs isolation based on the difference in their density. After centrifugation, the layers created contain (top to bottom): i) platelets & plasma (the least dense cells), ii) PBMCs, iii) ficoll, iv) RBCs and granulocytes, which are dense enough to cross the ficoll.

Tumor Cells Isolation

For tumors analysis, melanoma tissues were excised and cut into the smallest possible fragments by using an ophthalmic scissor. The minced tissues were afterwards incubated for 45 min at 37ºC in RPMI medium containing 0.128 mg/ml DNase I (Sigma-Aldrich), and 1 mg/ml collagenase D (Roche); then, they were homogenized and strained through a nylon filter with a pore size of 40µm (BD Falcon). Cells were pelleted by centrifugation (1650rpm, 10 minutes, 4ºC) and finally cell suspensions were prepared (cells were resuspended in PBS/5%FBS buffer).

Lung Cells Isolation

Lungs were excised and cut into the smallest possible fragments by using an ophthalmic scissor. The minced tissues were afterwards incubated for 45 min at 37ºC in RPMI medium containing 0.128 mg/ml DNase I (Sigma-Aldrich), 2 mg/ml Collagenase D (Roche) and 2 mg/mL Dispase (Gibco); then, they were homogenized and strained through a nylon filter with a pore size of 40µm (BD Falcon). Cells were pelleted by centrifugation (1650rpm, 10 minutes, 4ºC) and then incubated for 2' in erythrolysis medium (containing 155mM NH_4Cl, 10mM $KHCO_3$, 0,1mM EDTA, pH 7,3). Following, they were washed and centrifuged. Finally cell suspensions were prepared (cells were resuspended in PBS/5%FBS buffer).

Lymph Node Cells and Splenocytes Isolation

Mouse lymph nodes (either total lymph nodes or draining lymph nodes) and spleens were isolated **(Figure 3)** and passed through a nylon filter with a pore size of 40µm (BD Falcon). Cells were pelleted by centrifugation and then incubated for 2' in erythrolysis medium. Following, they were washed and centrifuged. Finally, pellets were resuspended in PBS/5%FBS buffer.

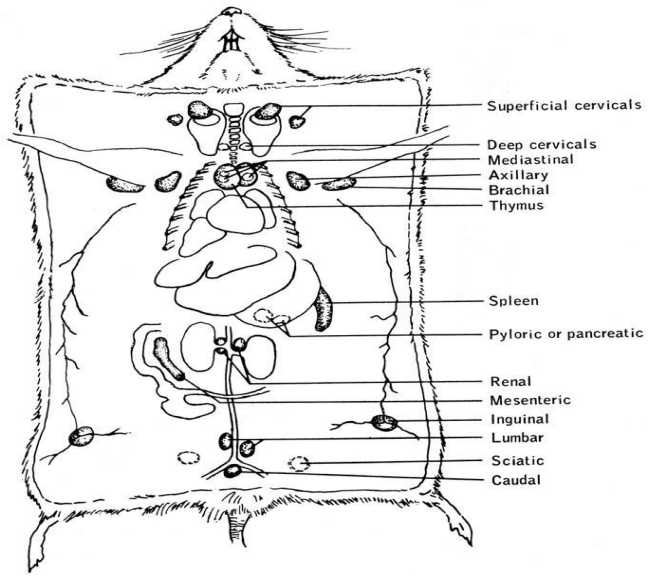


Figure 3. Diagrammatic illustration of the location of the spleen, the thymus and the principal lymph nodes of a healthy mouse (Adapted from Dunn et al, 1954, J. Nat. Cancer Inst.).[120]

Flow Cytometry and Cell Sorting

For extracellular staining, single cell suspensions were incubated for 20 minutes at 4°C with conjugated antibodies to mouse: PD-L1 (clone 10F.9G2), CD45 (30-F11), CD31 (390), CD140α (APA5), EpCAM (G8.8), CD4 (GK1.5), CD8 (53-6.7), NK1.1 (PK136), CD11c (N418), CD11b (M1/70), GR1 (RB6-8C5), Neuropilin-1 (3E12), Helios (22F6). All antibodies were purchased by BioLegend and were used in concentration 1/200 (diluted in PBS/5%FBS). For α-SMA intracellular staining, anti α-SMA (clone 1A4; purchased by Abcam) was used. Cells were fixed and stained using the eBioscience Intracellular (IC) Fixation Buffer (ThermoFisher) and the eBioscience Permeabilization Buffer (ThermoFisher), according to manufacturer instructions. For Foxp3 intracellular staining, anti-Foxp3 (clone 150D; purchased by BioLegend) was used. Cells were fixed and stained using the eBioscience FoxP3 Staining Buffer Set (ThermoFisher), according to manufacturer instructions. All intracellular antibodies were used in concentration 1/50 (diluted in buffer provided by the staining set).

Following the staining, cells were washed with PBS and then resuspended in PBS/5%FBS. $CD45^-CD31^-PDGFR\alpha^+PD\text{-}L1^-$, $CD45^-CD31^-PDGFR\alpha^-PD\text{-}L1^+$, $CD45^-CD31^-PDGFR\alpha^-PD\text{-}L1^-$, $CD45^+CD31^+$ cells were sorted from B16-F10 tumors of C57BL/6 mice; $CD45^-CD31^-PDGFR\alpha^+$ cells were sorted from lungs of C57BL/6 mice; $CD4^+FoxP3^-$ cells were sorted from total lymph nodes and spleen of Foxp3gfp mice on a FACS ARIA III (BD Biosciences). Cell purity was above 95%.

Flow cytometry data were analyzed with FlowJo Software (Tree Star).

GCV Treatment

Ganciclovir (GCV; Cymevene®, Roche) was utilized to obtain a suicide effect in $\alpha SMA\text{-}TK^+$ cells. GCV is an analog of deoxyguanosine used as a substrate, which is converted into GCV-triphosphate (GCV-TP) by cellular thymidine kinases. GCV-TP is incorporated in DNA and leads to premature termination of DNA synthesis and subsequently in cell death.[121,119,122,123]

Mice received intraperitoneal (i.p.) injections with 50 mg/kg body weight of GCV diluted in 100μl PBS, every 48 hours. Mice serving as controls received 100μl PBS every 48 hours. GCV injections **(Figure 3)** were initiated at two days before melanoma induction (early depletion) or at day 8 after melanoma induction (late depletion).

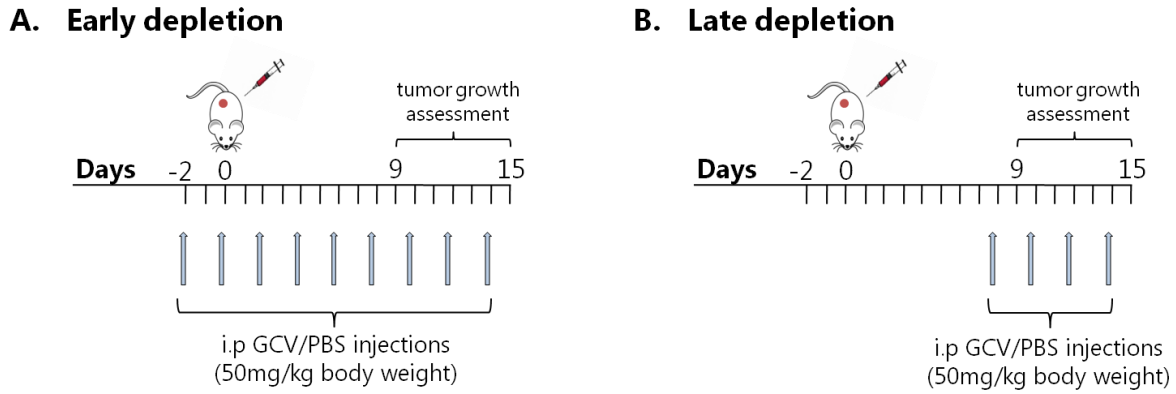


Figure 3 | Protocol for ablation (A: early vs. B: late) of α-SMA⁺ proliferating cells with GCV administration.

Mouse Genotyping

For αSMA-TK mice typing, genomic DNA was isolated from mouse tails and standard PCR was conducted. Four primers were used **(Table 2)** and the reaction yielded two possible products: a transgene of 200bp and an internal positive control of 521bp.

This mouse line was genotyped according to previously published protocols regarding the αSMA-TK mice from Jackson Laboratories (Bar Harbor, ME, USA).

Table 2 | Protocol primers for αSMA-TK PCR (jax.org)

	Sequence 5'→3'	Primer Type
Primer 1	GTG GTG GTT TTC CCC ATC C	Transgene Reverse
Primer 2	AGT GGC CTC TTC CAG AAA TG	Internal Positive Control Forward
Primer 3	TGC GAC TGT GTC TGA TTT CC	Internal Positive Control Reverse
Primer 4	ACC AAG AAC CCT GTC TGT GG	Transgene Forward

Brightfield Microscopy

Sorted $CD45^-CD31^-PDGFR\alpha^+$ cells from lungs (normal fibroblasts; NFs) and tumors (cancer-associated fibroblasts; CAFs) and $CD45^-CD31^-PD\text{-}L1^+$ cells from tumors were plated in 96-well plates at a density of 300,000 cells/well (total volume: 200μl). After culturing them for 6-9 days (37°C, 5% CO_2), their morphology was visualized with an Optical Microscope. Acquired images (magnification: 10x and 20x) were analyzed with the image processing software Fiji (SciJava).

Quantitative PCR (qPCR)

Total RNA from dLNs of αSMA-TK mice (treated with GCV or PBS) and FACS sorted $CD45^-CD31^-PDGFR\alpha^+PD\text{-}L1^-$, $CD45^-CD31^-PDGFR\alpha^-PD\text{-}L1^+$, $CD45^-CD31^-PDGFR\alpha^-PD\text{-}L1^-$, $CD45^+CD31^+$ cells from B16-F10 tumors was isolated with NucleoSpin® RNA kit (Macherey-Nagel). First-strand cDNA

synthesis was performed by reverse transcription with ThermoScript Reverse Transcriptase Kit (Invitrogen), according to manufacturer instructions.

qPCR was carried out in 20μl reactions using the iTaq Universal SYBR Green Supermix (BioRad Laboratories Inc) with a Step One Plus Real-Time PCR System (Applied Biosystems). Relative expression of target genes was calculated by comparing them to the expression of the house-keeping gene *HPRT*. All primers were purchased by Invitrogen **(Table 3)**:

Table 3 | Protocol primers for qPCR

	Forward Primer	Reverse Primer
HPRT	5′-GTGAAACTGGAAAAGCCAAA-3′	5′-GGACGCAGCAACTGACAT-3′
TYRP1	5′-CCGCCATTATCCCCACGATG-3′	5′-GCCCTGACAAAGTGGCTCT-3′
TYRP2/DCT	5′-TCCAGGACGCCCCTATAAGG-3′	5′-ACACTCGTTCTTCCCGGTTG-3′

Preparation of Tumor Explant Supernatants (TES)

Tumors from C57BL/6 mice and αSMA-TK mice were dissected at day 15 (see section: Tumor Cells Isolation) and the isolated single cell suspensions were plated in 6-well plates at a density of 10^7 cells/ml. The cells were incubated (37ºC, 5% CO_2) overnight (16 hours) and afterwards the supernatants were collected and stored at -80ºC.

T_{reg} Induction of $CD4^+FoxP3^-$ T cells in the presence of αSMA-TK TES

FACS sorted CellTrace labeled (ThermoFisher) $CD4^+GFP^-$ cells from total lymph nodes and spleen of Foxp3gfp.KI mice were plated in 96-well plates at a density of 150,000 cells/200μl/well. Cells were incubated (37ºC, 5% CO_2) for four days (96 hours) in the presence of plate bound aCD3 (10μg/ml; clone 145-2C11, BioLegend), aCD28 (1μg/ml; clone 37.51, BioLegend), IL-2 ($5x10^3$ units/ml), TGF-β (2ng/ml) and TES (20%) prepared from αSMA-TK mice treated with GCV or PBS. As control, $CD4^+GFP^-$ sorted cells were also cultured without TES.

After four days of culture the cells were collected and stained with anti-CD4 (GK1.5). The percentage of $CD4^+GFP^+$ (induced T_{regs}; iT_{regs}) cells and cell proliferation were assessed with flow cytometry.

Statistical Analysis

Statistical analyses were performed using unpaired two-tailed Student's t-test, with 95% confidence intervals. Data were presented as mean ± S.D. All data were analyzed using GraphPad Prism v5 software.

1. Isolation and characterization of CAFs subpopulations in mouse melanoma models

To examine the role of CAFs in regulation of the anti-tumor immunity, we set out to evaluate how the different CAFs subpopulations accumulate in the TME of our B16-F10 melanoma models with flow cytometry. Based on the existing bibliography for CAFs, until today there is no CAF-specific marker; moreover, it is established that CAFs share several markers with other inhabitants of the TME (e.g. myofibroblasts, muscle cells, myoepithelial cells, mesenchymal stem cells (MSCs), and endothelial cells). Followed by the aforementioned facts, we started this project by focusing on developing a strategy which would lead to their specific isolation and study.

After thorough research during which we surveyed several surface and intracellular markers, we decided to isolate CAFs based on their expression of a) the extracellular marker **PDGFRα (or CD140α)**, which is among the best characterized cell surface markers for robust expression in fibroblasts and is reportedly expressed by up to 90% of stromal fibroblasts in solid tumors[97,124] and b) the intracellular (cytosolic) marker **α-SMA**, which is the most common marker used up to date to characterize CAFs[97,124,78,5,6,86,87,90,91,92,93,94,95,96] and furthermore offers the advantage that it is specifically expressed in activated fibroblasts, thus allowing us to distinguish CAFs from NFs. Moreover, the purity of CAFs was further established by examining the expression of the cell surface molecules CD31, CD45 and EpCAM, which are specifically expressed on endothelial cells, cells of hematopoietic lineage and epithelial cells respectively. Finally, we also examined **PD-L1** expression on the cell surface of CAFs, as there is a number of reports denoting that CAFs express high levels of PD-L1[10,109,110,111].

Table 1 | Antibodies used for CAFs isolation and characterization in mouse melanomas

Marker	Selection	Description
α-SMA	positive	cytoskeletal protein associated with smooth muscle cells[78,125]
PDGFRα (CD140α)	positive	PDGF receptor; critical for tissue and organ development and maintenance[126]
PD-L1 (CD274)	positive	binds to the inhibitory checkpoint molecule PD-1, inhibiting immune responses[127,18]
CD45 (PTPRC)	negative	marker of all differentiated hematopoietic cells except for red blood cells[128,129]
CD31 (PECAM-1)	negative	endothelial cell marker; maintains and restores the vascular permeability barrier[130]

1.1 PDGFRα$^+$ CAFs characterization in the TME of mouse melanomas

To assess how CAFs accumulate in the TME, we induced melanomas in C57BL/6 mice. For tumor induction, we used a B16-F10 mouse melanoma cell line stably expressing ovalbumin with GFP (B16-OVA cells), which in other words allows us to immediately identify the GFP-expressing tumor

cells. $3x10^5$ B16-OVA cells were s.c. injected in the back of C57BL/6 mice and after 15 days the mice were sacrificed and fully grown melanomas were collected. Before the injections, GFP expression in B16-OVA cells was verified (B16-F10 cells were used as control) **(Figure 1A)**.

For CAFs characterization in the TME of fully grown mouse melanomas, we first used one of the best characterized cell surface markers for robust expression in fibroblasts, which is PDGFRα. More specifically **(Figure 1B)**, after first selecting all cells which did not express CD31/CD45 (therefore excluding endothelial cells and cells of hematopoietic lineage respectively), we analyzed the GFP^+ cells (tumor cells) and the $PDGFR\alpha^+$ cells (fibroblasts). Finally, we also assessed PD-L1 expression in the different subpopulations of our interest **(Figure 1C)**, finding that the tumor cells express this immune checkpoint molecule, whereas other populations do not.

Strikingly, we found that an important percentage of the $PDGFR\alpha^+$ cells were also GFP^+ positive, an observation which might have many possible explanations. Notably, i) due to multiple interactions between tumor cells and CAFs, the latter might simply take on albumin and thus be GFP^+, ii) $PDGFR\alpha^+$ CAFs might be autofluorescent and iii) although not supported by bibliography, PDGFRα might not be a specific marker for CAFs isolation in our mouse models, as it might be expressed by a small percentage of tumor cells as well.

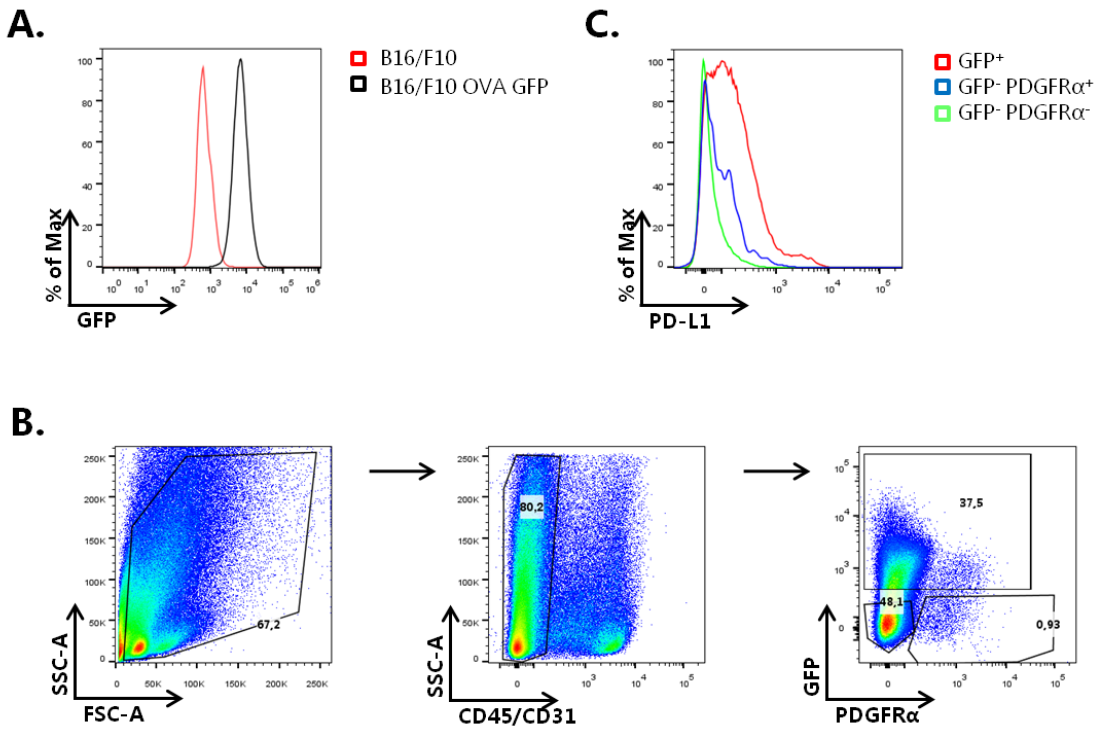


Figure 1 | Cell population analysis from the TME of melanoma-bearing mice.

A. Histogram denoting GFP expression in B16-OVA cells vs B16-F10 cells.

B. Gating strategy for analysis of tumor cell populations from B16-OVA injected C57BL/6 mice (Day15 following B16-OVA inoculation). Numbers on FACS plots denote frequency of gated population.

C. Histogram denoting PD-L1 expression of gated populations.

1.2 Characterization and morphology of PDGFRα$^+$ NFs and CAFs in mouse models

Following our first observations regarding the PDGFRα$^+$ CAFs in the TME, we proceeded to visualize their morphology, comparing them to PDGFRα$^+$ NFs (normal fibroblasts). For this reason, more specifically, we sorted with FACS all CD45$^-$CD31$^-$PDGFRα$^+$ cells from i) isolated lungs of naive mice **(Figure 2A)** and from ii) isolated tumors of melanoma-bearing mice on day 15 after tumor induction **(Figure 2B)**. Finally, we also sorted the CD45$^-$CD31$^-$PD-L1$^+$ cells from the isolated tumors **(Figure 2B)**, in order to investigate whether the cells that express this inhibitory checkpoint molecule have a fibroblastic morphology.

Following sorting, the obtained cells were plated in 96-well plates at a density of 300,000 cells/well (total volume: 200μl) and they were incubated for 6-9 days. After this time period, their morphology was visualized with brightfield microscopy **(Figure 2C)**. Although the PDGFRα$^+$ cells sorted from lungs were highly transparent, the vast majority of them exhibited the expected spindle-like (fibroblast-like) morphology. Furthermore, most of the PDGFRα$^+$ cells sorted from mouse melanomas were also characterized as spindle-shaped, having elongated ends, a characteristic of activated fibroblasts. Finally, regarding the PD-L1$^+$ cells, their morphology reminisced that of B16-F10 cells; from these data we concluded that this specific cell subpopulation is mostly comprised by tumor cells. Therefore, we didn't investigate further the properties of the latter sorted cell subpopulation.

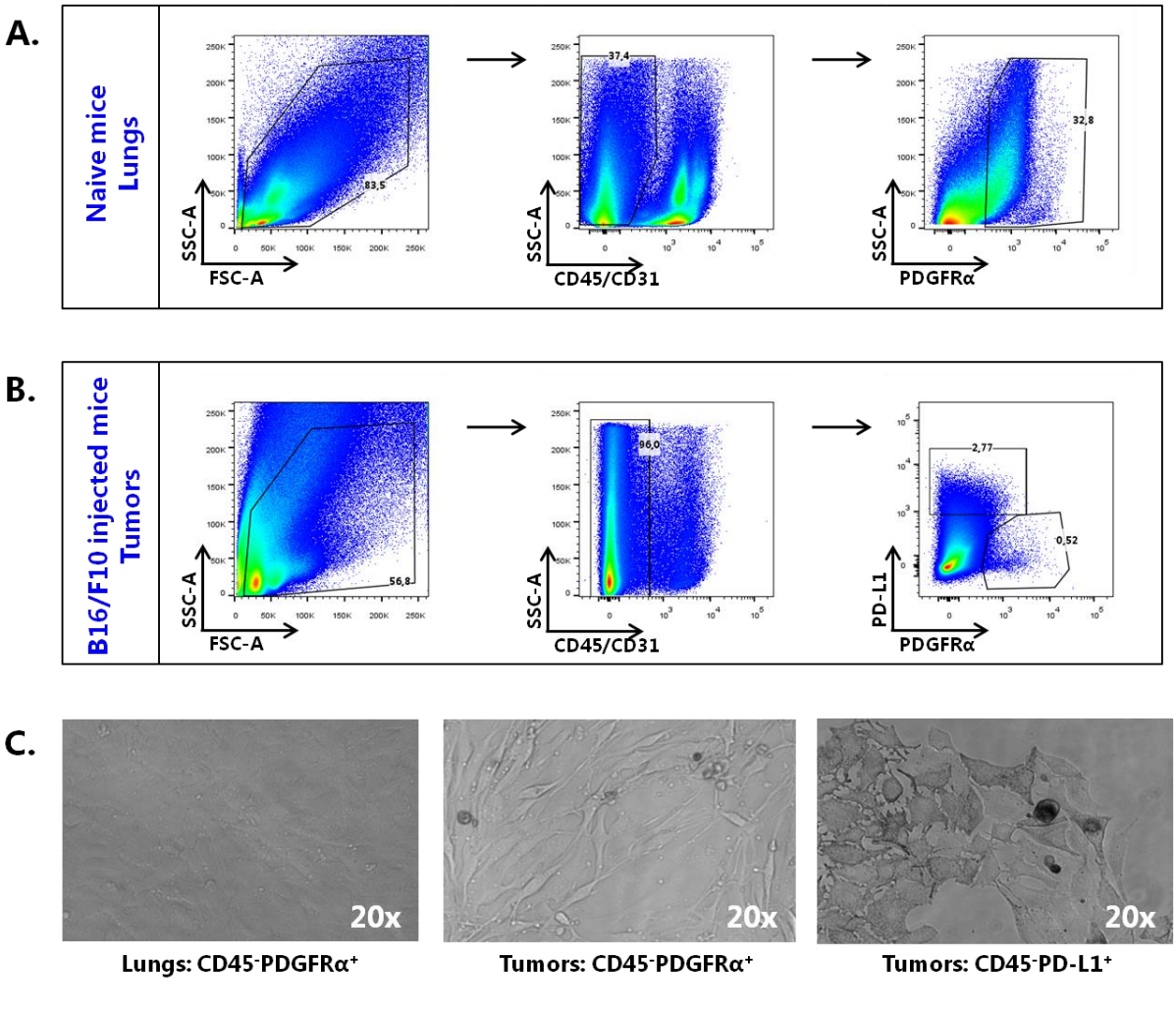


Figure 2 | FACS sorted PDGFRα$^+$ cells in the TME of melanoma-bearing mice exhibit a fibroblastic morphology.
A. Gating Strategy for NFs isolation from lungs of C57BL/6 mice. Numbers on FACS plots denote frequencies of gated population.
B. Gating Strategy for CAFs isolation from tumors of B16-F10 injected C57BL/6 mice (Day15 following B16-F10 inoculation). Numbers on FACS plots denote frequencies of gated population.
C. Brightfield images (20x) of sorted: CD45$^-$PDGFRα$^+$ NFs (from lungs), CD45$^-$PDGFRα$^+$ CAFs (from tumors), CD45$^-$PD-L1$^+$ B16-F10-like cells (from tumors).

1.3 TYRP1 mRNA expression in cell subpopulations of mouse melanomas

In another experiment conducted in order to further characterize all cell subpopulations of our interest in the TME and to gain a more comprehensive view of CAFs accumulation in mouse melanomas, we proceeded to sort the following cell subsets **(Figure 3B)**:

i) CD45$^+$ cells: coded as P8; shown with purple
ii) CD45$^-$PDGFRα$^-$PD-L1$^-$ cells: coded as P7; shown with blue
iii) CD45$^-$PDGFRα$^-$PD-L1$^+$ cells: coded as P5; shown with pink
iv) CD45$^-$PDGFRα$^+$PD-L1$^-$ cells: coded as P6; shown with green

Following FACS sort, in order to verify that the PDGFRα$^+$ cell population is not contaminated by tumor cells (therefore with this marker we can isolate a pure CAF population), we assessed *TYRP1* mRNA expression in all sorted cell populations with quantitative PCR (qPCR); *TYRP1* (**Ty**rosinase-**R**elated **P**rotein **1**) is a melanocyte-specific gene specifically involved in melanin synthesis[131] Relative expression of *TYRP1* was calculated by comparing it to the expression of the housekeeping gene *Hprt*. Strikingly, our results **(Figure 3C)** demonstrate a significant downregulation of *TYRP1* in all sorted cell populations besides P6, which are the PDL1 expressing cells. This observation is consistent with our previous results which depicted that the PDL1$^+$ cell population is morphologically similar to tumor cells. Finally, the P8 and P6 sorted cell populations exhibited the lowest relative *TYRP1* levels in a statistically significant manner, an observation which indicates that they are devoid of tumor cells.

So far, we have extensively characterized all cell populations in the TME, based on their expression for CD45, CD31, PD-L1, PDGFRα and α-SMA. Our results depict that the PDGFRα expressing cells of the TME have a fibroblast-like morphology and they do not upregulate the melanocyte-specific gene TYRP1. On the other hand, with FACS sorting of the PDGFRα$^+$ cells we are not entirely convinced that we can isolate a 100% pure population of CAFs, as this cell marker is also expressed on NFs (which are present in the TME), while our experiments showed that PDGFRα$^+$ cells expressed tumor antigens. Since our aim is to unravel the mechanisms in which only CAFs are implicated, we decided not to proceed on studying them based on their expression of PDGFRα and rather focus on α-SMA. Despite the fact that α-SMA is an intracellular marker which prohibits us from directly isolating them (the fixation step during the intracellular staining of α-SMA kills the isolated cells), by using this marker we can effectively differentiate CAFs from NFs;

moreover, there is a number of *in vivo* models targeting myofibroblasts based on α-SMA expression, providing us with more tools to study CAFs and their role in the anti-tumor immunity.

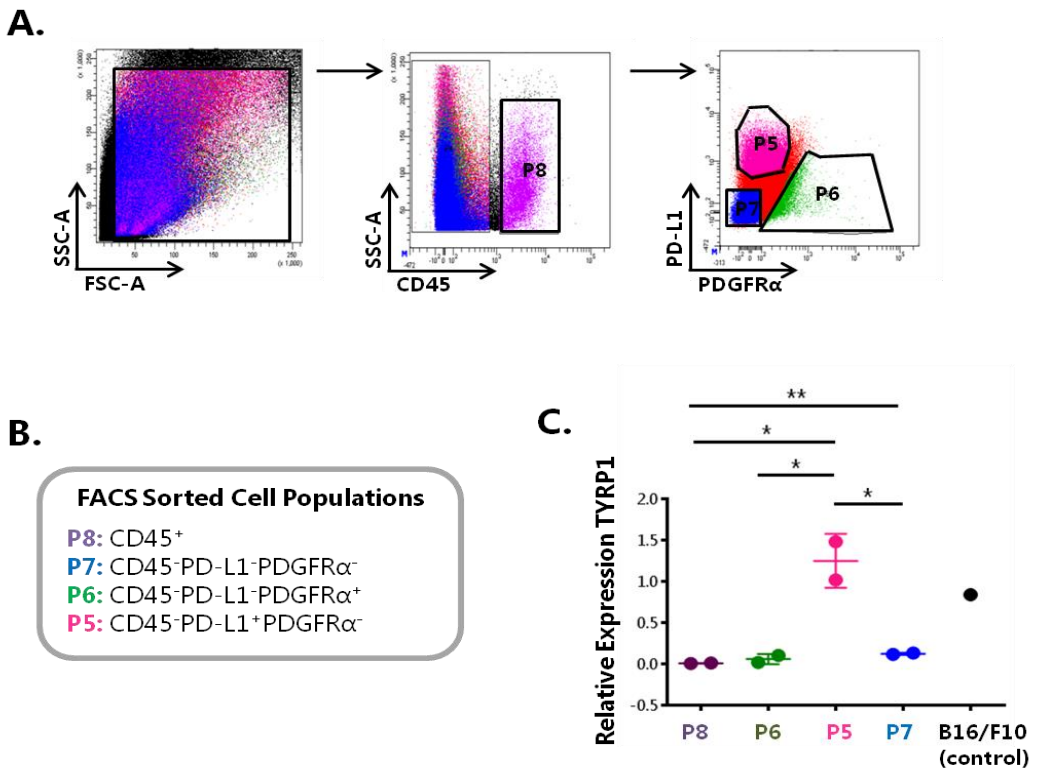


Figure 3 | PDGFRα⁺ cells in the TME of melanoma-bearing mice upregulate α-SMA and do not express TYRP1.
A. Gating Strategy for FACS sorted cell populations from tumors of B16-F10 injected C57BL/6 mice (Day15 following B16-F10 inoculation).
B. The four FACS sorted cell populations are P8: $CD45^+CD31^+$ (purple); P7: $CD45^-CD31^-PD\text{-}L1^-PDGFR\alpha^-$ (blue); P5: $CD45^-CD31^-PD\text{-}L1^+PDGFR\alpha^-$ (pink) and P6: $CD45^-CD31^-PD\text{-}L1^-PDGFR\alpha^+$ (green).
C. Quantitative PCR analysis of TYRP1 mRNA levels obtained from the four FACS sorted cell populations (P8/P6/P5/P7); B16-F10 TYRP1 mRNA levels were calculated as control; **P < 0.01, *P < 0.05; N=2 mice. Results are expressed as mean ± SD.

2. α-SMA$^+$ CAFs: Functional role in the anti-tumor immunity – Assessment in mouse melanoma models

2.1 Significant enrichment of α-SMA$^+$ CAFs in TME and blood of melanoma-bearing mice

To monitor the abundance of CAFs in the TME and peripheral blood, we induced melanomas in C57BL/6 mice. The presence of α-SMA$^+$ CAFs present was estimated with flow cytometry in specific time points following the immunizations **(Table 2)**.

Table 2 | Protocol for assessment of α-SMA$^+$ CAFs frequencies at different time points during tumor progression

Time point	Condition	Tissue Isolated
Day 0	no tumor/negative control	peripheral blood (PBMCs)
Day 6	pre-tumor appearance	peripheral blood (PBMCs)
Day 11	early tumor stage	peripheral blood (PBMCs); tumor
Day 15	late tumor stage	peripheral blood (PBMCs); tumor

As expected, we found an enriched presence of the α-SMA$^+$ CAFs in the TME of melanoma-bearing mice on days 11 and 15 after tumor induction **(Figure 4A & Figure 4C)**. This cell population is found among the CD45$^-$CD31$^-$ cells and is also PDGFRα$^-$. Interestingly though, we further detected a clear α-SMA$^+$ CAFs cell population in peripheral blood as well **(Figure 4B & Figure 4C)** as tumor progressed, providing us a solid indication that a portion of CAFs might leave the tumor and enter the circulation as tumor progresses. Finally, we observed expression of PD-L1 in α-SMA$^+$ CAFs of the TME during tumor progression, compared to α-SMA$^-$ cells **(Figure 4D)**.

2.2 Significant regression of melanoma growth upon early GCV administration in αSMA-TK mice

To understand the functional significance of αSMA$^+$ myofibroblasts in melanoma progression, we used the transgenic mice αSMA-TK, which express the viral thymidine kinase (TK) under control of the endogenous α-SMA promoter **(Figure 5A)**. As already described in Chapter 2 (Materials & Methods), upon administration of the prodrug Ganciclovir (GCV), TK phosphorylates GCV to GCV monophosphate, which is further converted to GCV-diphosphate and GCV-triphosphate in the cell by host kinases. GCV-triphosphate is an analog of deoxyguanosine (dGTP), therefore it is incorporated into the DNA by DNA polymerases during DNA replication. dGTP incorporation into the DNA leads to termination of DNA synthesis and, as a result, cell death occurs. The aforementioned process is initiated only when TK is expressed, when therefore the locus containing the α-SMA promoter is active, so non-myofibroblastic cells which have the transgenic construct are not affected by GCV administration. As a result, by using these mice we are able to specifically ablate the α-SMA$^+$ proliferating cells of the tumor, in other words the CAFs.

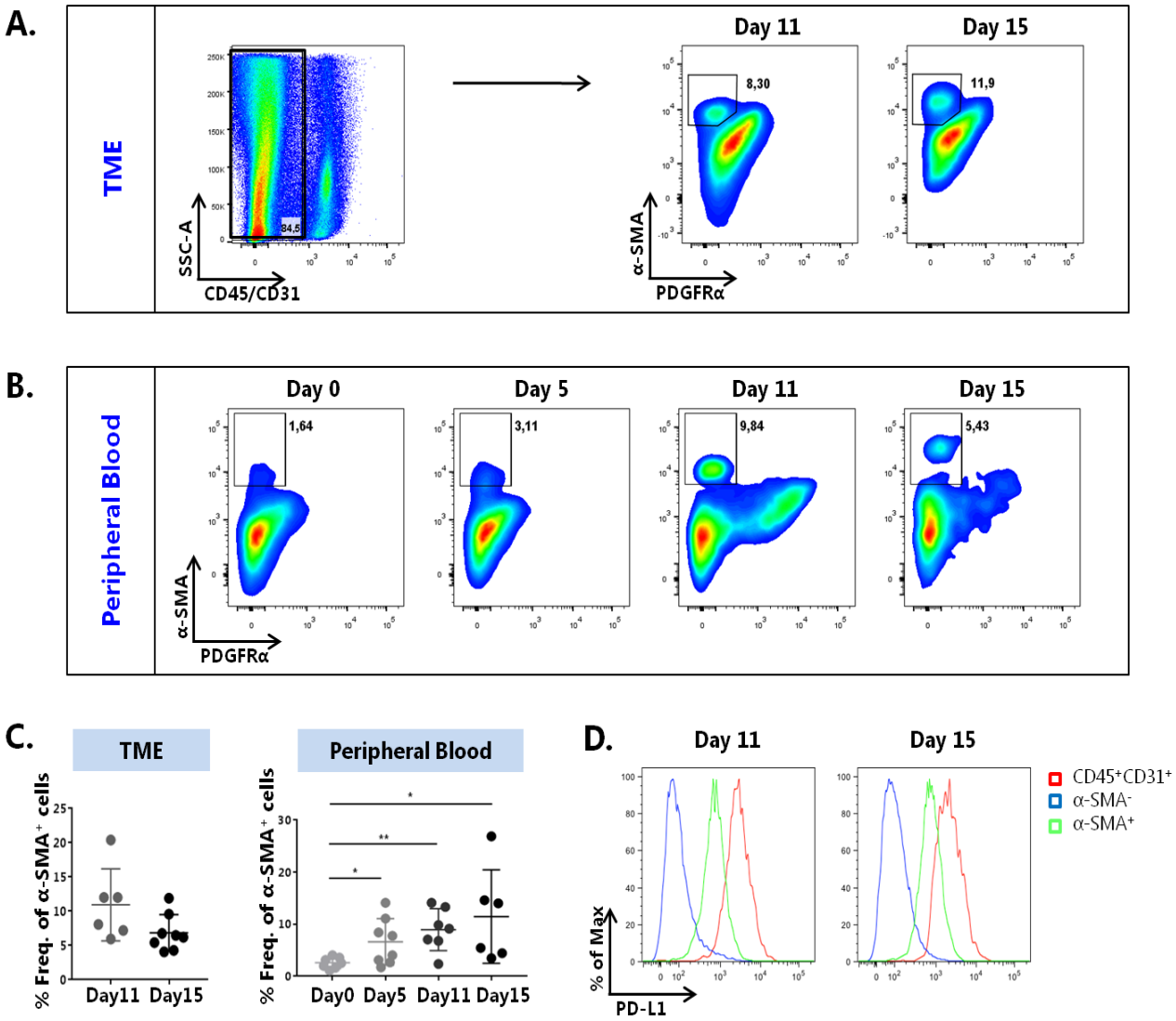


Figure 4 | α-SMA⁺ CAFs are enriched in the TME and blood of melanoma-bearing mice.

A. Gating strategy and frequencies of α-SMA⁺ CAFs in B16-F10 mouse melanomas of Day 11 and Day 15. Numbers on FACS plots denote frequencies of gated population.

B. Frequencies of α-SMA⁺ CAFs in peripheral blood of melanoma-bearing mice on Day 0, Day 6, Day 11 and Day 15 after B16/F10 inoculation (gated on $CD45^{-}CD31^{-}$). Numbers on FACS plots denote frequencies of gated population.

C. Mean and SD of α-SMA⁺ CAFs in TME and blood; N=6-8 mice per group. **P < 0.01, *P < 0.05.

D. Representative histograms depicting PD-L1 expression in $CD45^{+}CD31^{+}$ (red), $CD45^{-}CD31^{-}$α-SMA^{-} (blue) and $CD45^{-}CD31^{-}$α-SMA^{+} (green) cell populations in B16-F10 mouse melanomas of Day 11 and Day 15.

Heterozygous αSMA-TK mice were crossed with control C57BL/6 mice and the production of heterozygous littermates was verified with standard PCR **(Figure 5B)**. The mice were born at the expected Mendelian ratios with no obvious developmental defects. They were viable and fertile in adulthood and presented no obvious phenotype or altered life span. To verify that GCV does not produce any secondary effects in αSMA-TK mice besides ablation of α-SMA⁺ CAFs, we induced melanomas in C57BL/6 mice which were receiving GCV (or PBS as control). We found no differences between two groups (GCV-treated vs PBS-treated) regarding tumor growth and the percentages of tumor-infiltrating immune cells (data not shown).

We induced melanomas in αSMA-TK mice aged 8-10 weeks. To specifically ablate the α-SMA$^+$ proliferating cells, GCV injections were initiated at two days before melanoma induction (early depletion, see Materials & Methods). Finally, tumor growth was assessed starting from day 9 until day 17 after melanoma induction. As controls, αSMA-TK mice were used, which were treated with PBS.

As expected, flow cytometry analysis revealed a statistically significant reduction of αSMA$^+$ myofibroblasts in the TME of GCV-treated αSMA-TK$^+$ mice **(Figure 5C)**, verifying that our ablation protocol is successful. Moreover, our results also demonstrated a reduction in the α-SMA$^+$ cell population observed in the peripheral blood, an observation which is consistent with our previous results which depict that α-SMA$^+$ CAFs enter the periphery during tumor progression **(Figure 5D)**. α-SMA$^+$ myofibroblast depletion led to a robust regression of tumor growth **(Figure 5E)**; in GCV-treated mice, tumors were obviously smaller, weighed less, while they developed slowly in comparison with PBS-treated mice.

Having seen that α-SMA$^+$ myofibroblasts depletion is associated with a significant regression of melanoma growth, we proceeded subsequently to assess its direct impact on the anti-tumor immunity.

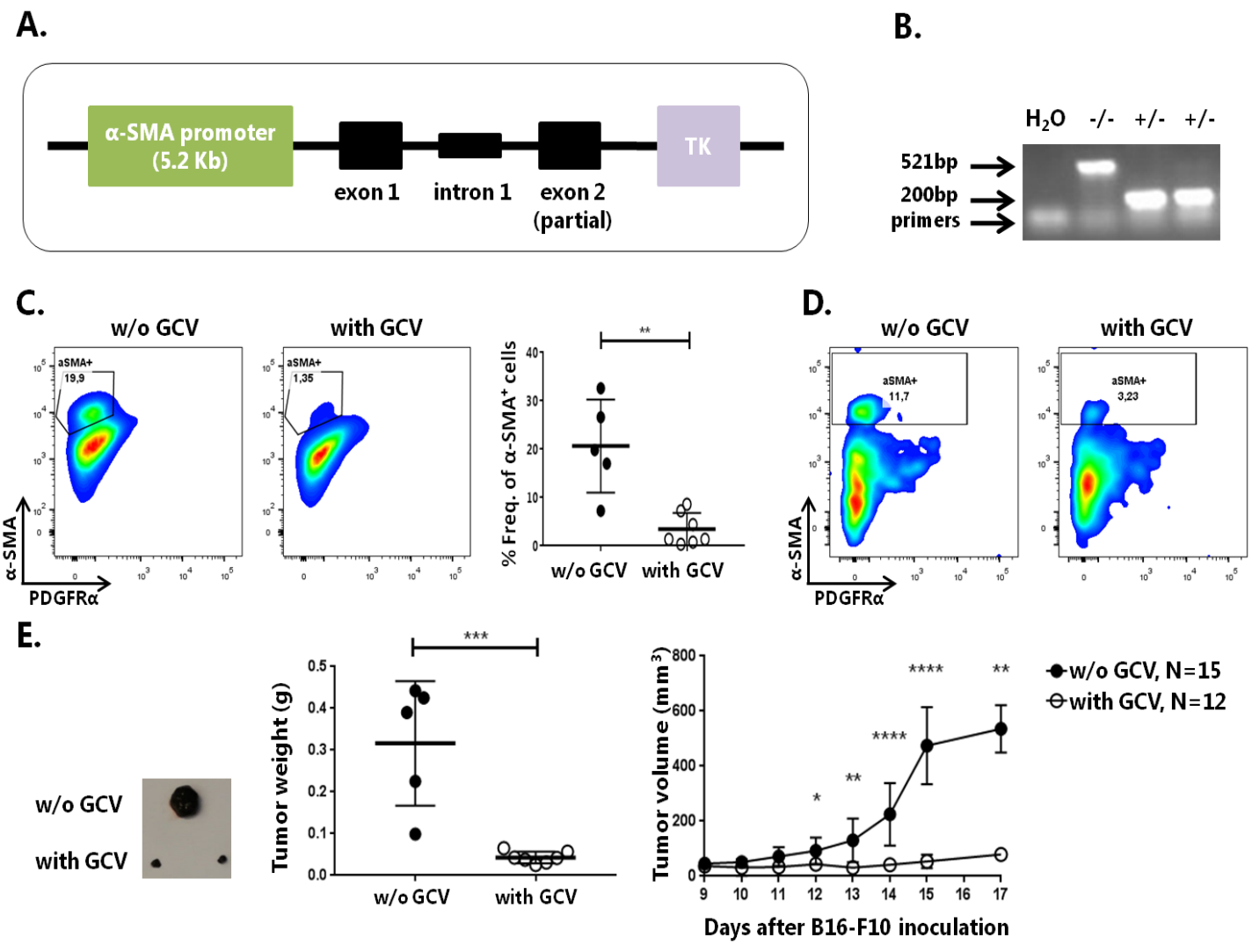


Figure 5 | α-SMA$^+$ CAFs are specifically ablated in the TME and blood of melanoma-bearing αSMA-TK mice upon GCV administration.

A. Schematic illustration of the transgenic construct inserted in αSMA-TK mice, allowing for selective depletion of proliferating α-SMA$^+$ myofibroblasts upon Ganciclovir (GCV) administration. TK, thymidine kinase.

B. PCR for genotyping of αSMA-TK mice. A set of four primers is chosen which amplify the TK transgene (200bp) and the internal positive control (521bp). A wild type animal (-/-) produces only the higher (521bp) amplicon; an animal heterozygous for the transgene (+/-) produces the higher (521bp) and the lower (200bp) amplicons. For size verification of PCR amplicons, 100 kb DNA ladder is used (not shown).

C. Frequencies; mean and SD of α-SMA$^+$ CAFs in the TME of day 15 melanomas of control αSMA-TK mice (w/o GCV; treated with PBS) and mice with depleted α-SMA$^+$ CAFs (with GCV). N=5 mice for controls, N=7 mice for GCV-treated mice. Results of one representative experiment are shown. **P < 0.01.

D. Frequencies of α-SMA$^+$ CAFs in the peripheral blood of control αSMA-TK mice (w/o GCV; treated with PBS) and mice with depleted α-SMA$^+$ CAFs (with GCV), on day 15 after B16-F10 inoculation.

E. Representative image, tumor weight and tumor volume of excised tumors of B16-F10 inoculated αSMA-TK mice. For tumor weight assessment, results from two independent experiments are combined; for tumor volume assessment, results from three independent experiments are combined. ****P < 0.00001; ***P < 0.0001; **P < 0.01, *P < 0.05. Results are expressed as mean ± SD.

2.3 α-SMA$^+$ CAFs ablation is associated with major changes of immune cell populations infiltrating the TME

To dissect their potential role as regulators of the anti-tumor immunity, we monitored the presence of immune cell populations infiltrating the tumor site after specific CAFs elimination. Specifically, we isolated and studied with flow cytometry the CD45$^+$ cells, the myeloid derived suppressor cells (MDSCs), the dendritic cells (DCs), the CD4$^+$ T cells, the CD8$^+$ T cells and the natural killer (NK) cells **(Figure 6A and Figure 6B)**. We found that upon ablation of CAFs, the frequencies and absolute cell numbers of the hematopoietic CD45$^+$ cells are drastically augmented **(Figure 6C; left and Figure 6D; left)**, clearly demonstrating that tumors are characterized by a greater immune infiltration.

The increase in the CD45$^+$ cells was accompanied by a decrease in the relative percentages of MDSCs, a major suppressive cell population of the TME, indicating that CAFs may boost their presence and survival **(Figure 6C)**. Furthermore, we observed that the frequencies of the DCs, the CD4$^+$ T cells and the CD8$^+$ T cells were decreased as well in a statistically significant manner **(Figure 6C)**, despite that these differences are not reflected on their absolute numbers **(Figure 6D)**. Finally, we found that the frequencies of the NK cells were higher upon ablation of CAFs in a marginally significant fashion **(Figure 6C)**.

Overall, our results provide evidence that α-SMA$^+$ CAFs in B16/F10 melanoma solid tumors promote tumor growth and dampen antitumor immune responses, while facilitating the accumulation of immunosuppressive cell populations, such as the MDSCs.

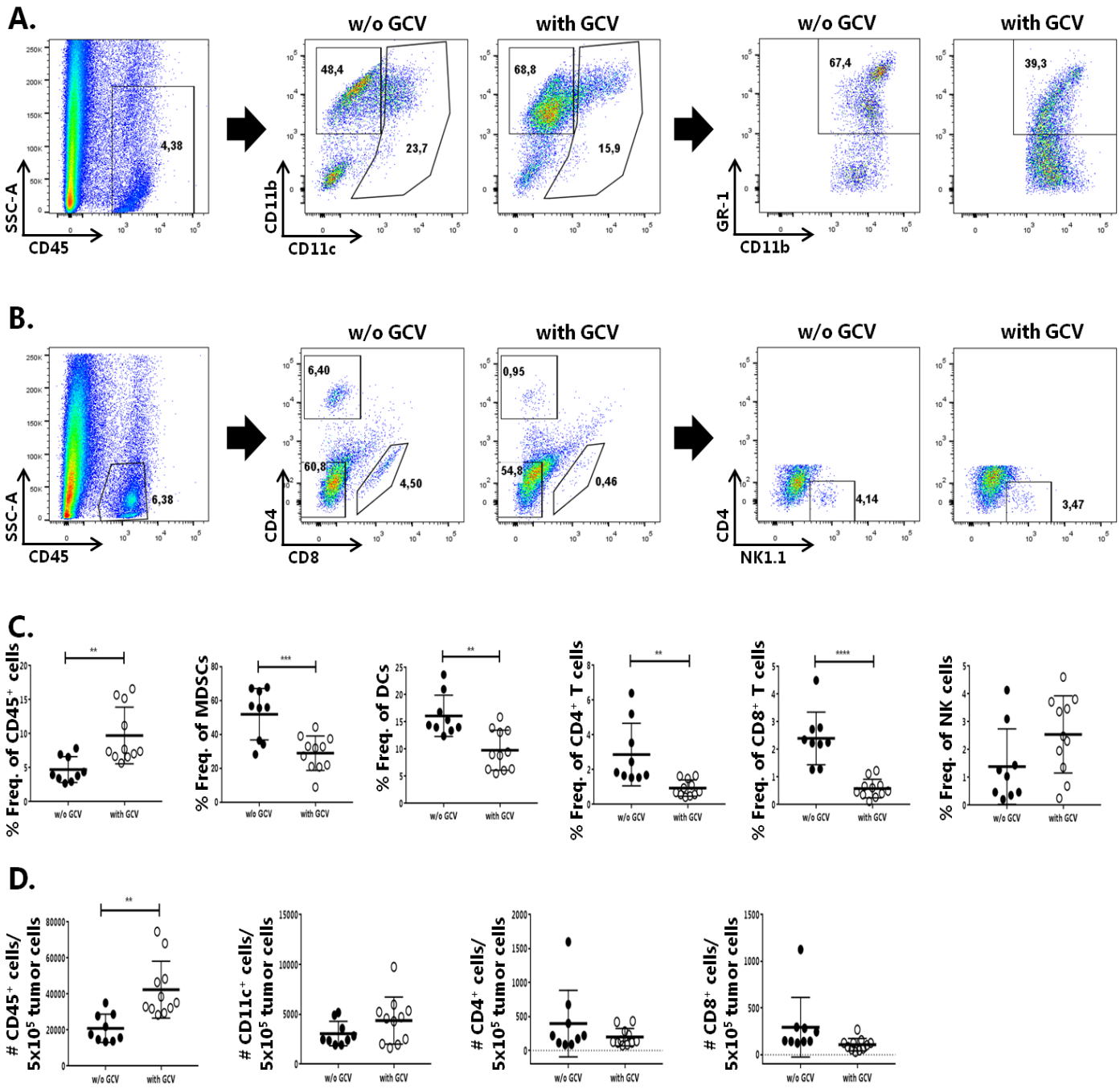


Figure 6 | Tumors from aSMA-TK mice are associated with a greater immune infiltration.

A. Gating strategy for analysis of tumor-infiltrating DCs ($CD45^+CD11c^+$) and MDSCs ($CD45^+CD11b^+GR\text{-}1^+$); representative dot plots from each group are shown. Numbers on FACS plots denote frequencies of gated population.
B. Gating strategy for analysis of tumor-infiltrating $CD4^+$ T cells ($CD45^+CD4^+$), $CD8^+$ T cells ($CD45^+CD8^+$) and NK cells ($CD45^+CD4^-CD8^-NK1.1^+$); representative dotplots from each group are shown. Numbers on FACS plots denote frequencies of gated population.
C. Frequencies of tumor infiltrating immune cell populations (left to right: $CD45^+$ cells, MDSCs, DCs, $CD4^+$ T cells, $CD8^+$ T cells, NK cells). Results from three independent experiments are combined. ****P < 0.00001; ***P < 0.0001; **P < 0.01. Results are expressed as mean ± SD.
D. Numbers per 5×10^5 total tumor cells of tumor infiltrating immune cell populations (left to right: $CD45^+$ cells, DCs, $CD4^+$ T cells, $CD8^+$ T cells). Results from three independent experiments are combined. **P < 0.01. Results are expressed as mean ± SD.

2.4 Intratumoral CD45$^+$ cells express more PD-L1 upon α-SMA$^+$ CAFs ablation

To investigate how CAFs affect the expression of inhibitory immune checkpoint molecules, we assessed PL-L1 expression upon their absence. Our results demonstrate that α-SMA$^+$ cells express more PD-L1 than α-SMA$^-$ cells, either when mice were treated with GCV or PBS **(Figure 7A)**, a result which is consistent with our previous observations. Strikingly though, we observed that upon CAFs elimination, CD45$^+$ cells express more PD-L1 in a statistically significant manner **(Figure 7B)**.

Our observations depict that in the absence of α-SMA$^+$ myofibroblasts, immune cells in the TME have an enhanced expression of PD-L1. Undoubtedly, more assays are required in order to find out why tumor-infiltrating CD45$^+$ cells downregulate PD-L1 in the presence of CAFs and the functional significance of this fact.

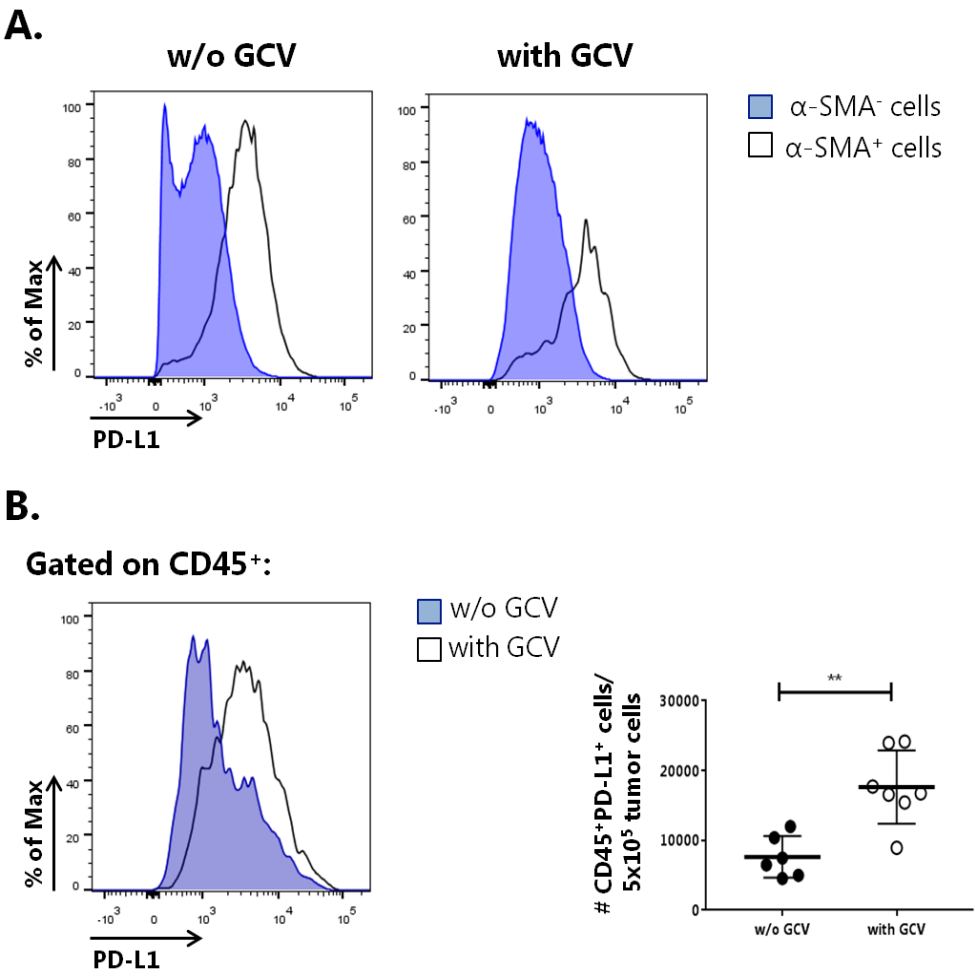


Figure 7 | PD-L1 expression in α-SMA$^+$ cells and in CD45$^+$ cells.

A. Representative histograms depicting PD-L1 expression in α-SMA$^+$ and α-SMA$^-$ cells in αSMA-TK melanomas.
B. Representative histogram; numbers per 5 × 10^5 total tumor cells of CD45$^+$PD-L1$^+$ cells. Results from two independent experiments are combined. **P < 0.01. Results are expressed as mean ± SD.

2.5 Immune profiling of dLNs & spleens did not reveal any significant differences in melanoma-bearing αSMA-TK mice

To assess whether the absence of CAFs impacts on immune cell populations of the peripheral organs, we measured the levels of MDSCs and DCs in spleens and the levels of T cells and NK cells in draining lymph nodes (dLNs) of αSMA-TK mice treated with GCV (having as controls mice treated with PBS). Our results depict that frequencies of DCs in spleens are elevated upon CAFs absence **(Figure 8A)**. Yet, no other significant differences were observed regarding the other immune cell populations of our interest **(Figure 8A and Figure 8B)**.

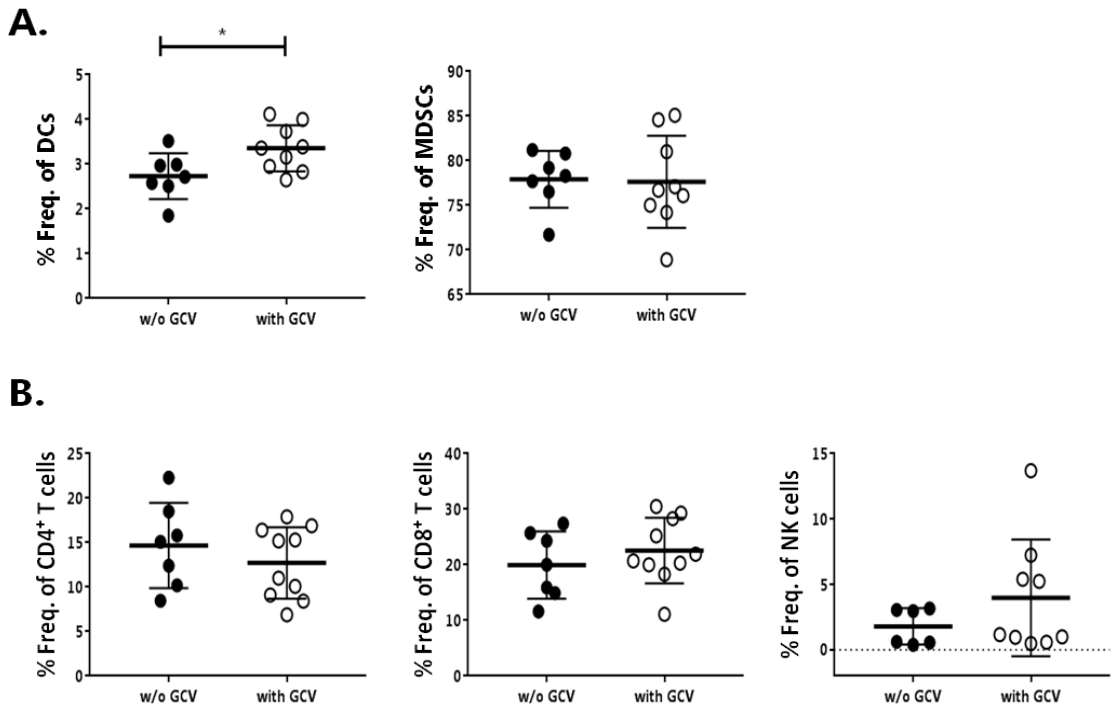


Figure 8 | Immune profiling of dLNs and spleens.

A. Frequencies of DCs and MDSCs in the spleens of melanoma bearing αSMA-TK mice. *P < 0.05. Results are expressed as mean ± SD.

B. Frequencies of $CD4^+$ T cells, $CD8^+$ T cells and NK cells in the draining lymph nodes of melanoma bearing αSMA-TK mice. Results are expressed as mean ± SD.

2.6 Enhanced infiltration of T_{regs} in the TME of melanoma-bearing αSMA-TK mice

To investigate whether CAFs affect the infiltration of the immune suppressive T regulatory cells, we assessed the frequencies and numbers of T_{regs} upon the absence of α-SMA^+ CAFs. We found that GCV-treated αSMA-TK mice were characterized by a significantly enriched infiltration of T_{regs} in the TME **(Figure 9A)**. Strikingly though, when we measured their frequencies and numbers in

draining lymph nodes (dLNs), we found a statistically significant regression of their presence **(Figure 9B)**.

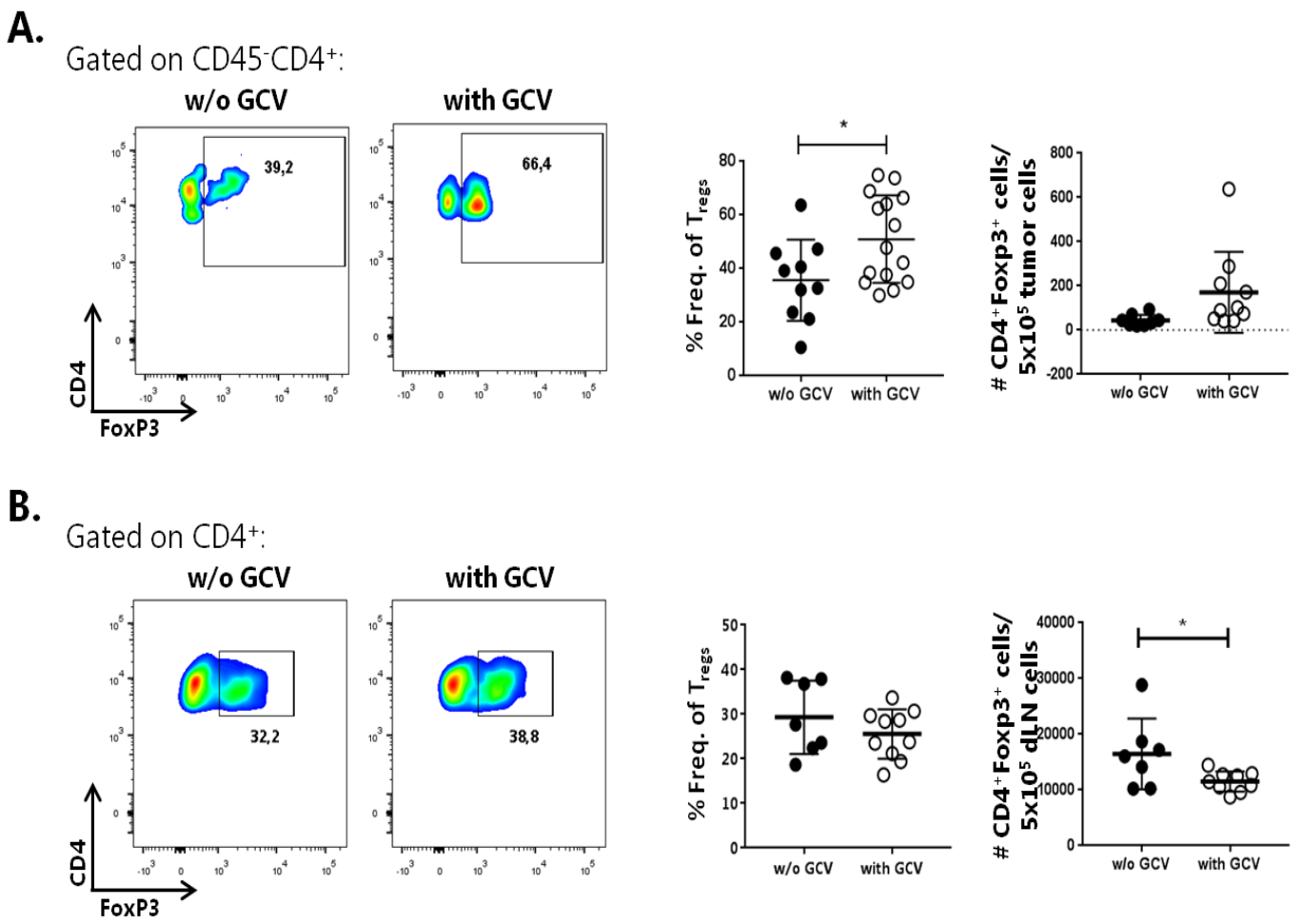


Figure 9 | Infiltration of T_{regs} in the TME and dLNs of melanoma-bearing mice.

A. Representative flow cytometric analysis, frequencies and total numbers of tumor-infiltrating T_{regs} in the TME of day 15 melanomas of control αSMA-TK mice (w/o GCV; treated with PBS) and mice with depleted α-SMA⁺ CAFs (with GCV). N=8 mice for controls, N=10 mice for GCV-treated mice. Results of one representative experiment are shown; results are expressed as mean ± SD. *P < 0.05. Numbers on FACS plots denote frequency of gated population.

B. Representative flow cytometric analysis, frequencies and total numbers of T_{regs} in the dLNs of day 15 melanomas of control αSMA-TK mice (w/o GCV; treated with PBS) and mice with depleted α-SMA⁺ CAFs (with GCV). N=8 mice for controls, N=10 mice for GCV-treated mice. Results of one representative experiment are shown; results are expressed as mean ± SD. *P < 0.05. Numbers on FACS plots denote frequency of gated population.

3. Effect of CAFs ablation on T_{reg} induction

We have found that in the absence of CAFs, the numbers of tumor-infiltrating T_{regs} are augmented. To this end, we set out to investigate whether soluble factors secreted by CAFs are potent of directly influencing the induction of T_{regs}, either acting as enhancers or inhibitors. Using an *in vitro* established protocol **(Figure 10)**, we sorted with FACS all $CD4^+GFP^-$ cells from total lymph nodes and spleen of Foxp3gfp.KI mice. Sorted cells were labeled with CellTrace and cultured in T_{reg} induction conditions, in the presence of tumor explant supernatants from PBS-treated (TES, control) or GCV-treated (TES, $CAFs^{\Delta}$) melanoma-bearing mice. As an additional control, we also cultured CellTrace labeled cells in T_{reg} induction conditions without TES.

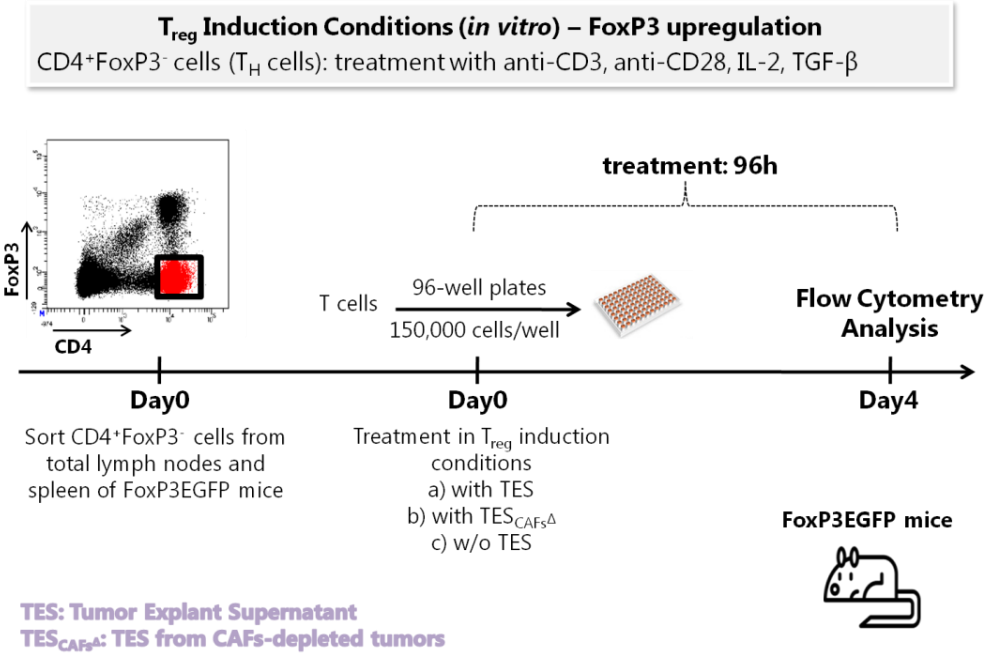


Figure 10 | Experimental protocol for T_{reg} induction of T effector cells in the presence of TES.

3.1 $CAFs^{\Delta}$-TES treatment without TGF-β does not impact on T_{reg} induction

Because CAFs are a major source of immunosuppressive cytokines in the TME, we first formed the hypothesis that TGF-β produced by this cell subpopulation can be potent of inducing Foxp3 expression in conventional T cells. Therefore, we cultured $CD4^+Foxp3^-$ cells without TGF-β in the presence of i) no TES (condition A), ii) TES, control (condition B) and iii) TES, $CAFs^{\Delta}$ (condition C); according to our hypothesis, we expected that Foxp3 would be upregulated only in condition B.

Our results did not reveal an upregulation of Foxp3 expression among the three groups **(Figure 11)**, signifying that our hypothesis was not correct. These observations might either mean that CAFs do not play a role in T_{reg} induction through their secretome, or that their secreted factors are not potent of replenishing the effect that a specific amount of TGF-β exerts on this phenomenon.

To assess the second possibility, we decided to add TGF-β in the culture medium and investigate again how treatment of T effector cells with TES impacts on T_{reg} induction.

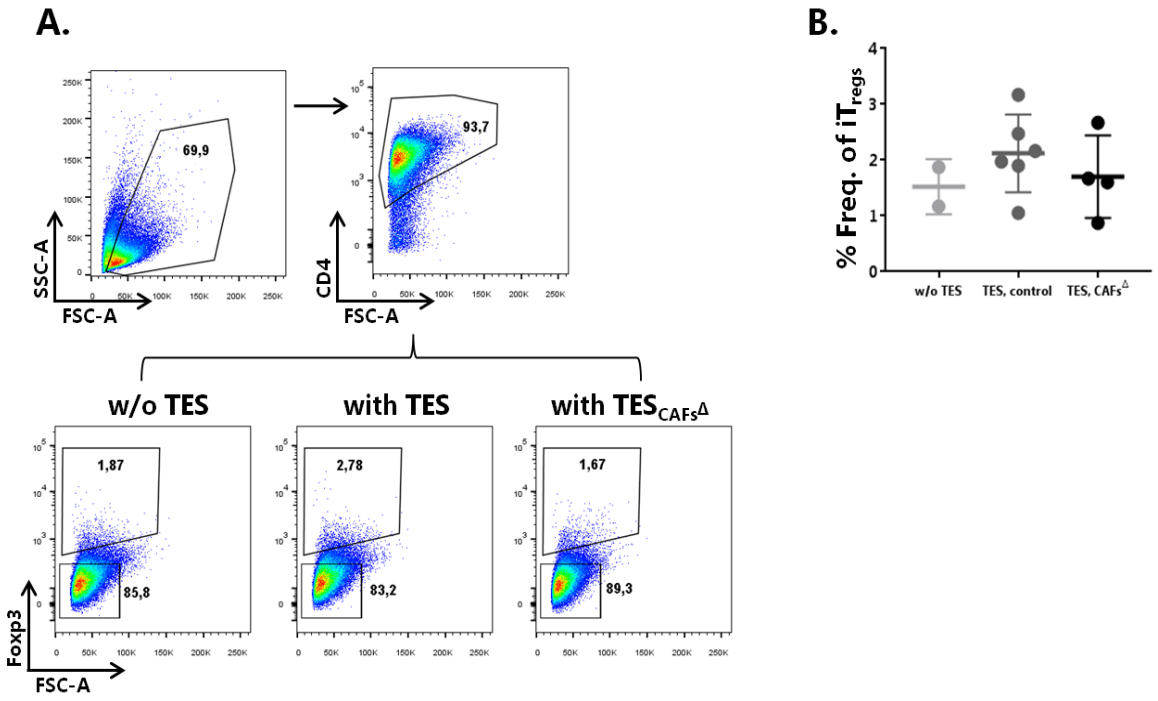


Figure 11 | Treatment of T effector cells with TES in the absence of TGF-β does not lead to Foxp3 upregulation.

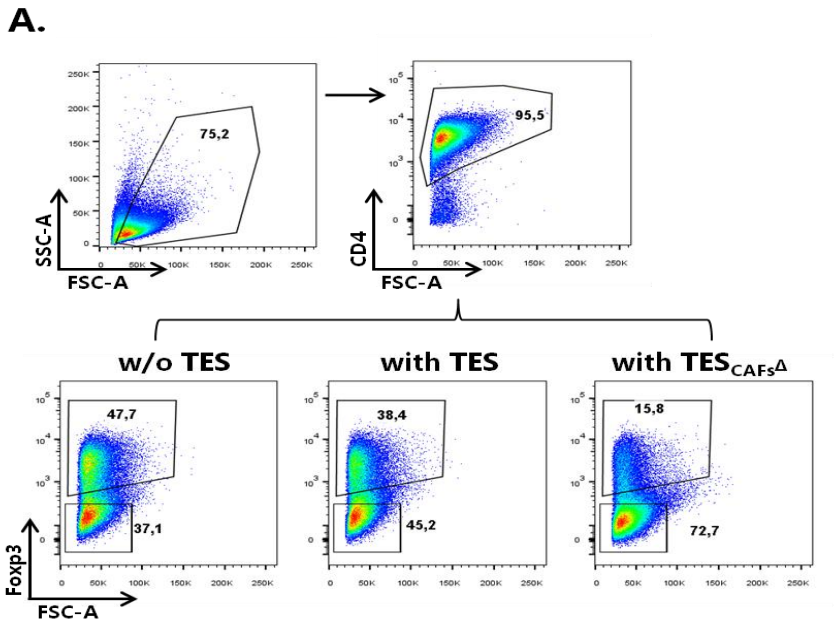
A. Gating strategy for analysis of Foxp3 expressing cells upon culture of $CD4^+Foxp3^-$ cells in T_{reg} induction conditions. Cells were treated i) without TES, ii) with TES, control and iii) with TES, $CAFs^{\Delta}$; representative dot plots from each group are shown. Numbers on FACS plots denote frequencies of gated population.
B. Frequencies of $CD4^+Foxp3^+$ cells (iT_{regs}). Results from two independent experiments are combined; results are expressed as mean ± SD.

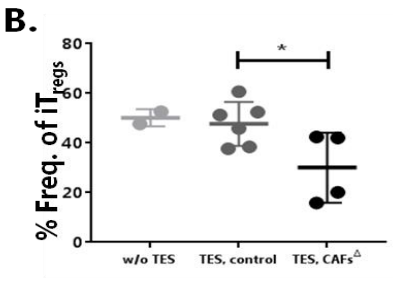
3.2 $CAFs^{\Delta}$-TES treatment with TGF-β hampers T_{reg} induction and hinders iT_{reg} proliferation

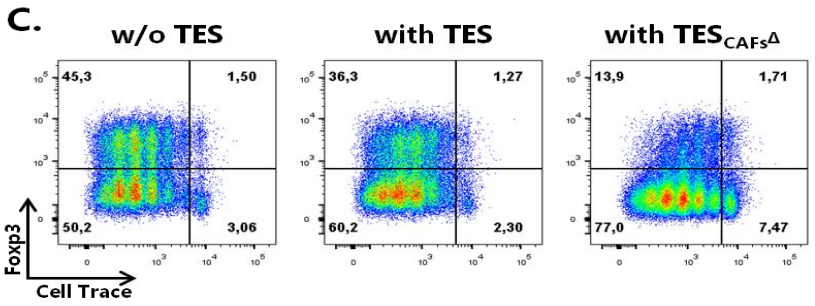
In the presence of TGF-β, we did not observe any significant differences regarding the $Foxp3^+$ induced T cells between T effector cells treated with control TES and untreated T effector cells **(Figure 12B)**. This depicts that once $CD4^+Foxp3^-$ cells are exposed in the secretome from all cells present in the tumor T_{reg} induction is not blocked, but facilitated. Interestingly though, we found a significant decrease of $Foxp3^+$ T cells when T effectors were treated with $CAFs^{\Delta}$-TES **(Figure 12B)**, an observation which confirms that CAFs are required for T_{reg} induction not to be obstructed.

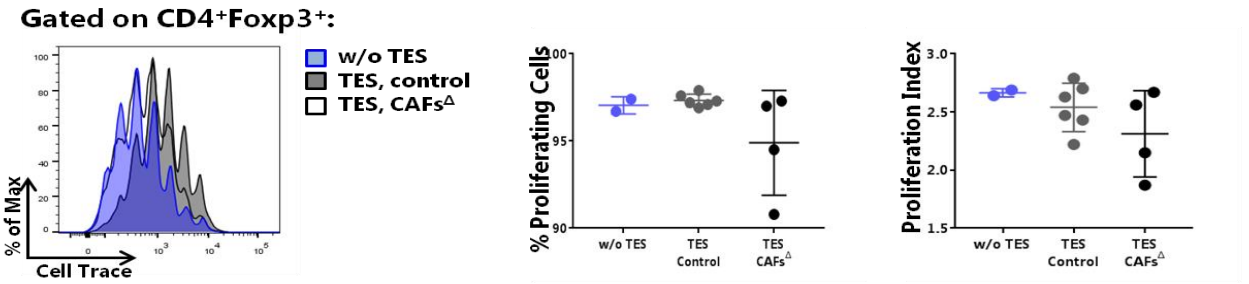
To examine how the absence of CAFs impacts on iT_{reg} proliferation, we labeled all sorted cells with CellTrace Violet. In this way, we were able to trace all cells generations due to dye dilution **(Figure 12C)**. We examined the rate of proliferation among our three groups. We found that without TES and in the presence of control TES, $Foxp3^+$ and $Foxp3^-$ cells proliferated in the same means; multiple generations of cells were observed **(Figure 12D)**. However, when cells were treated with $CAFs^{\Delta}$ TES, proliferation seemed to be slightly hindered, albeit not in a statistically

important fashion when compared with the other two groups **(Figure 12D)**. The same conclusion was reached when we compared the percentages of proliferating cells (% of cells not belonging in the initial generation) and when we compared the proliferating index (which reflects how many cell divisions a cell is most likely to make). Collectively, our observations demonstrate that CAFs might facilitate T_{reg} induction, while having an additional enhancing effect on iT_{reg} proliferation.









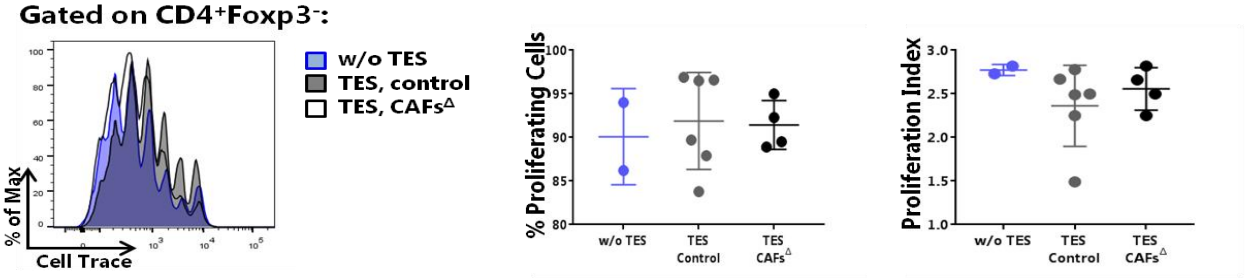


Figure 12 | Treatment of T effector cells with CAFs$^{\Delta}$-TES in the presence of TGF-β hampers Foxp3 upregulation and hinders iT$_{reg}$ proliferation

A. Gating strategy for analysis of Foxp3 expressing cells upon culture of CD4^{+}Foxp3^{-} cells in T$_{reg}$ induction conditions. Cells were treated i) without TES, ii) with TES, control and iii) with TES, CAFs$^{\Delta}$; representative dot plots from each group are shown. Numbers on FACS plots denote frequencies of gated population.
B. Frequencies of CD4^{+}Foxp3^{+} cells (iT$_{regs}$). Results from two independent experiments are combined; results are expressed as mean ± SD.
C. Representative flow cytometric analysis of CellTrace-labeled T effector cells which were cultured in T$_{reg}$ induction conditions in the presence of TES. Numbers on FACS plots denote frequencies of gated population. Results from two independent experiments are combined.
D. Representative histograms for cell proliferation of iT$_{regs}$ and non-induced T$_{effs}$; mean and SD of proliferating cells and proliferating index of iT$_{regs}$ and non-induced T$_{effs}$, N=2-7 mice per group. Results from two independent experiments are combined.

4. Effect of late CAFs depletion on tumor growth and the anti-tumor immunity

The robust effect of early CAFs depletion on tumor growth and the anti-tumor immunity prompted us to investigate their involvement once tumors would manifest. Therefore, we developed an experimental protocol in which we depleted α-SMA$^+$ CAFs after melanomas started to be visible (usually on days 8-9 following B16-F10 inoculation). As implied, with this specific strategy we aimed to see whether by simulating a condition in which tumors arise we can utilize specific depletion of CAFs as a potential therapeutic strategy.

4.1 Late administration of GCV in melanoma-bearing αSMA-TK mice i) does not affect tumor development and ii) does not lead to CAFs depletion

To assess the functional role of CAFs after tumor appearance, we administered GCV (and PBS as control) on day 8-9 following B16-F10 inoculation (late depletion, see Materials & Methods). Two experiments have been conducted so far using this setting; tumor weight has been assessed once and tumor volume was assessed both times. Although we expected that with GCV administration (thus proliferating myofibroblast depletion) tumor development would be significantly halted, we did not observe any differences between two groups **(Figure 13A)**. This implied either that α-SMA$^+$ CAFs were not depleted along the process, or that their depletion after tumor appearance did not have a significant impact on tumor growth.

On day 16-18 following B16-F10 inoculation, tumors were dissected and α-SMA expression was assessed with flow cytometry in the TME and blood. Strikingly, we observed that an enhanced population of α-SMA$^+$ CAFs remained in the TME **(Figure 13B)**, while no statistical differences were detected when we compared the MFI for α-SMA between PBS and GCV-treated melanomas. In addition, when we assessed the presence of α-SMA$^+$ CAFs in the peripheral blood, a clear cell population was detected, further showing that despite GCV administration CAFs were not depleted **(Figure 13C)**.

All taken together, these data require further investigation, since our GCV administration protocol has been proven successful on early depletion settings. The fact that we are not observing a specific ablation when we start injections from the 8th day and on must be investigated, as it might prove new insights regarding the biology of CAFs. Undoubtedly, more experiments are needed which will allow us to interpret these results.

4.2 Late administration of GCV in melanoma-bearing αSMA-TK mice does not affect cell populations of the immune system

To dissect whether ablation of proliferating myofibroblasts after tumor appearance affects the immune system, we monitored with flow cytometry the presence of immune cell populations infiltrating the tumor site, specifically the CD45$^+$ cells, the MDSCs, the DCs, the CD4$^+$ T cells, the

CD8$^+$ T cells and the natural killer (NK) cells. After isolating tumors (day 18 after B16-F10 inoculation), we found that the frequencies **(Figure 14A)** and absolute cell numbers **(Figure 14B)** of the cell populations of our interest did not differ between our two groups (GCV-treated vs PBS-treated). Overall, these results are in accordance with the aforementioned analyses of tumor growth and α-SMA$^+$ cell presence upon late GCV administration, collectively indicating that no effects were exerted in the immune system.

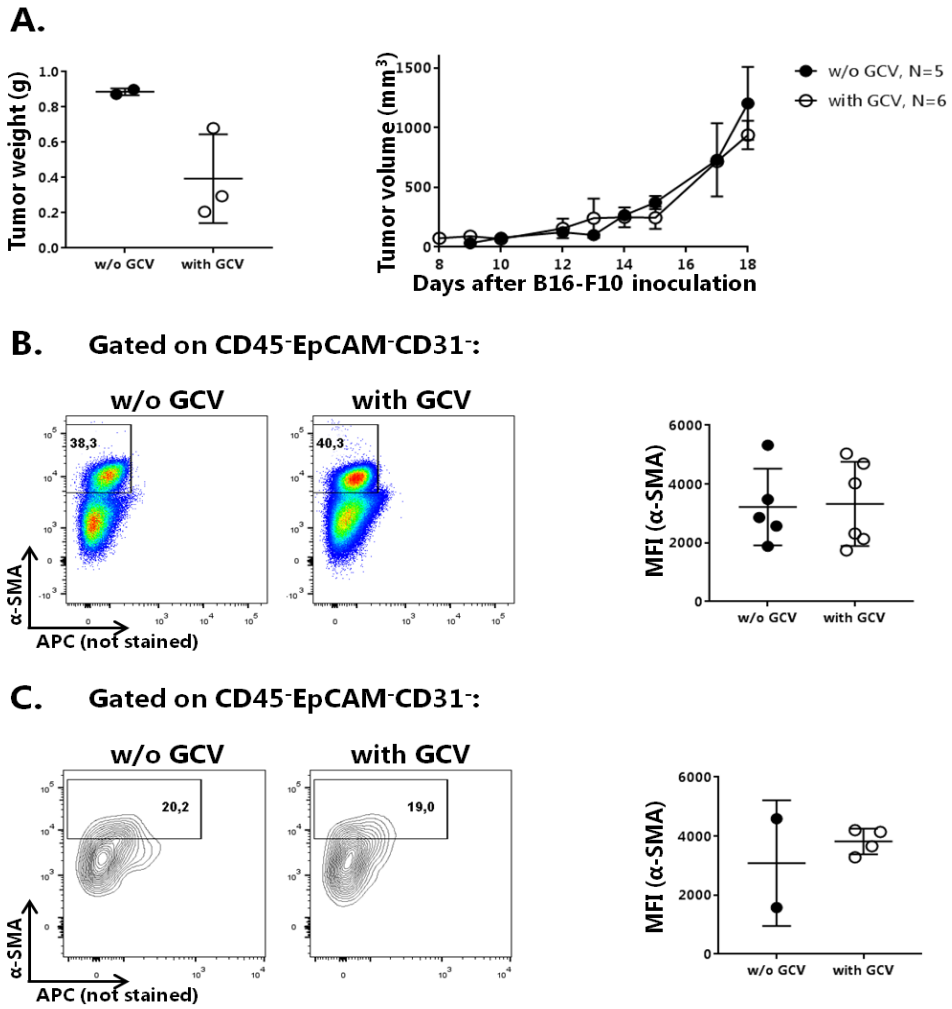


Figure 13 | Late GCV administration does not impact on tumor development and α-SMA$^+$ populations in the TME and peripheral blood.

A. Tumor weight and tumor volume of excised tumors of B16-F10 inoculated αSMA-TK mice. For tumor weight assessment, results from one experiment are depicted; for tumor volume assessment, results from two independent experiments are combined. Results are expressed as mean ± SD.

B. Frequencies in the TME of day 18 melanomas of control αSMA-TK mice (w/o GCV; treated with PBS) and mice with depleted α-SMA$^+$ CAFs (with GCV); MFI of α-SMA in CD45$^-$EpCAM$^-$CD31$^-$ cells of the TME. N=5 mice for controls, N=6 mice for GCV-treated mice; results from two independent experiments are combined; results are expressed as mean ± SD.

C. Frequencies in peripheral blood of αSMA-TK mice (w/o GCV; treated with PBS) and mice with depleted α-SMA$^+$ CAFs (with GCV) on day 18 after B16-F10 inoculation; MFI of α-SMA in CD45$^-$EpCAM$^-$CD31$^-$ cells of the peripheral blood. N=2 mice for controls, N=4 mice for GCV-treated mice; results from one experiment are depicted; results are expressed as mean ± SD.

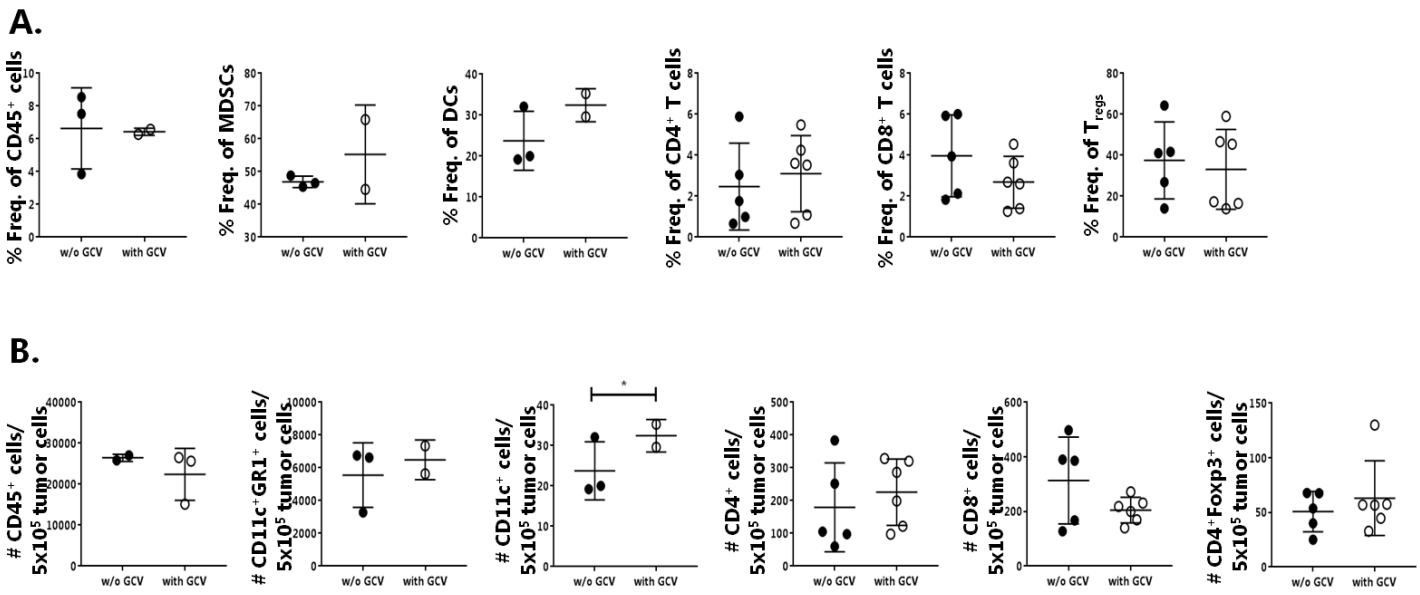


Figure 14 | Late GCV administration does not impact on immune cell populations of the TME.

A. Frequencies of tumor infiltrating immune cell populations (left to right: $CD45^+$ cells, MDSCs, DCs, $CD4^+$ T cells, $CD8^+$ T cells, T_{regs}). For $CD45^+$ cells, MDSCs and DCs results from one independent experiment are depicted; for $CD4^+$ T cells, $CD8^+$ T cells and T_{regs} results from two independent experiments are combined. Results are expressed as mean ± SD.
B. Numbers per 5×10^5 total tumor cells of tumor infiltrating immune cell populations (left to right: $CD45^+$ cells, MDSCs, DCs, $CD4^+$ T cells, $CD8^+$ T cells, T_{regs}). For $CD45^+$ cells, MDSCs and DCs results from one independent experiment are depicted; for $CD4^+$ T cells, $CD8^+$ T cells and T_{regs} results from two independent experiments are combined. *$P < 0.05$. Results are expressed as mean ± SD.

Discussion & Future Directions

The recognition of the determining role that the TME plays regarding tumor progression and metastasis has furthered the complexity of cancer biology, but has also brought an opportunity for development of novel anti-cancer therapies[1,3,82,94,124,132,133]. Undoubtedly, its soluble and cellular components are considered important targets in emerging anti-cancer therapies[1,3] and unraveling how they support cancer cells and facilitate malignant progression is essential to predict response to immunotherapy and to establish more efficient treatments for cancer patients.

One of the most abundant yet quite unexplored subsets of the TME is CAFs[3,4,5,10,78-83]. This cell population is characterized by high heterogeneity, yet they are overall considered as strong tumor promoters. Indeed, they are believed to play a role in almost all processes dictating tumor progression, while it has been proven that they mediate therapeutic resistance. However, their multiple origins[5,6,79,80,87,90-92] and the lack of CAF-specific markers[5,6,86,87,90-98] have limited extended study of their many proposed functions. As a result, despite their abundance in the TME, their tumor-promoting capabilities haven't been fully explored yet.

Recently, the interactions between CAFs and infiltrating immune cells in the TME have started to be explored. So far, accumulating evidence depict that CAFs are characterized by strong immunosuppressive properties, affecting almost all immune cells of the innate and adaptive immunity; through their secretome they are capable of modulating the characteristics of immune cells, therefore potentiating immune evasion of cancer cells[33,10,80,91,96,101,102,134]. Undoubtedly, their role in tumor-enhancing inflammation must be fully characterized, since they can be key players in the creation of the highly immunosuppressive TME, and therefore they could be among the main reasons behind failed immunotherapeutic approaches.

Despite the field is still at its infancy, a great interest has risen over the past years to study the mechanisms which facilitate the recruitment and expansion of immunosuppressive cell populations in the TME. Importantly, through their secreting molecules (eg COX-2, PGE-2, TGF-β) CAFs have are completely potent in recruiting and inducing T_{regs}, which are indeed the major immunosuppressive immune cell subpopulation during tumor progression[116-119]. However, the correlation between T_{regs} and CAFs has not been thoroughly investigated, despite data from the literature depicting a direct association between these two subpopulations, which as a result favors T_{reg} recruitment and differentiation. Delineation of the precise mechanisms and molecules which characterize the interplay between CAFs and T_{regs} in the TME will undoubtedly provide a better understanding of the global regulation of the anti-tumor immune response that shall ultimately lead to development of more efficacious and enduring cancer therapies.

To this end, for the present Master Thesis we set out to explore whether CAFs are required for the induction and expansion of tumor-specific FoxP3$^+$ T_{regs}, leading to tumor development and hampering the effectiveness of immunotherapies. We propose that through these functions of

theirs, CAFs are key players in the formation of the highly immunosuppressive TME, and that future research on antitumor treatments should include a viable strategy that disrupts the relationship between CAFs and T_{regs}.

To investigate their role in modulation of anti-tumor immunity, we first aimed to develop a strategy allowing for specific CAFs isolation and study. Based on the existing bibliography, we used PDGFRα and α-SMA as specific markers expressed in CAFs. We found that PDGFRα$^+$ cells have a spindle-shaped morphology (characteristic of fibroblasts) and they do not upregulate the melanocyte-specific gene TYRP1. However, when we analyzed the accumulation of these cells in the TME of B16-OVA melanomas (B16 melanocytes stably express ovalbumin with GFP), we found that a significant percentage of the PDGFRα expressing cells were also GFP$^+$, an observation which definitely requires further investigation. Importantly, CAFs, through their interaction with cancer cells, could uptake cancer-cell derived molecules and thus be GFP$^+$; alternative explanations include the possibilities are that CAFs are auto-fluorescent and, finally, that the marker PDGFRα is not CAF-specific, unlike what is suggested by bibliography[97,135].

The aforementioned unsolved issues imply that we cannot isolate a 100% pure population of CAFs; along with the fact that this cell marker is also expressed on NFs (which are present in the TME), we decided to abandon PDGFRα as a marker for CAFs study and rather focus on α-SMA, which is expressed abundantly in the cytosol of activated myofibroblasts, thus allowing us to distinguish CAFs from NFs. Consistently with other studies, we observed an enriched presence of α-SMA$^+$ CAFs in the TME of melanoma-bearing mice during tumor progression. These cells were positive for expression of the immune checkpoint molecule PD-L1, a fact that strongly implies that CAFs might be targeted with current immunotherapy regimens. Interestingly though, we further detected a clear α-SMA$^+$ CAFs cell population in peripheral blood as tumor progressed, providing us a solid indication that a portion of CAFs might leave the tumor and enter the circulation as tumor progresses. This observation is consistent with the theory that CAFs facilitate metastasis, by aiding circulating tumor cells (CTCs) survival and creation of the new tumor niche[113,114,115,136]. More information is required to assess whether circulating CAFs may complement CTCs as a clinically relevant biomarker for metastasis initiation.

Unfortunately, the fact that α-SMA is located intracellularly prohibits us from directly isolating CAFs with this marker; however, there are a number of *in vivo* models targeting myofibroblasts based on α-SMA expression, providing us with significant tools to study their functional importance regarding the anti-tumor immunity. In this line, we utilized the αSMA-TK transgenic mouse model, which, as extensively described above, allows for selective depletion of proliferating α-SMA expressing cells upon GCV administration. In our melanoma model, we expected that cells with these characteristics that are present in the TME and the peripheral blood are only the α-SMA$^+$ CAFs.

We showed a depletion of the majority of infiltrating myofibroblasts upon early GCV administration in melanomas (GCV injections were initiated at two days before melanoma induction). Moreover, we also observed a reduction in the α-SMA$^+$ cells in the periphery, an

observation which is consistent with our previous results which depict that α-SMA$^+$ CAFs enter the periphery during tumor progression. Importantly, we found that upon early elimination of CAFs, a robust melanoma regression was observed. Despite the fact that our results collectively confirm the major role that α-SMA$^+$ cells play for cancer initiation, and potentially cancer progression, how the drastic changes in tumor growth upon α-SMA$^+$ CAFs depletion impact on the vasculature and composition of the TME still remains unanswered. To answer this question, in the future we aim to dissect α-SMA$^+$ CAFs depleted tumors visualize them with immunohistochemistry.

We demonstrated that depletion of aSMA$^+$ myofibroblasts resulted in drastic changes regarding composition of the immune infiltrates in the TME. Strikingly, shrinkage of tumor growth was accompanied by a greater infiltration of the hematopoietic CD45$^+$ cells compared with control tumors, contradicting the results published in 2014 by Ozdemir et al., who showed that upon early CAFs depletion in pancreatic ductal adenocarcinome (PDAC), infiltrating CD45$^+$ cells were decreased[119]. Furthermore, we found a significant reduction regarding the cell populations of infiltrating DCs, CD4$^+$ T cells and CD8$^+$ T cells in the TME. Finally, NK cell infiltration upon myofibroblasts depletion remained unchanged. These changes regarding the profile of tumor infiltrating immune cells were not reflected on peripheral organs (draining lymph nodes and spleens).

Undoubtedly, these observations show an important shrinkage of immune cell populations which belong in the lymphoid lineage (notably T cells and NK cells) and in the myeloid lineage (DCs), strongly suggesting an interplay between CAFs and infiltrating immune cells. On the other side, we found that the total number of CD45$^+$ cells in the TME is augmented. These results are highly ambiguous; while on the one hand, the fact that we observe that more immune cells are present following CAFs ablation outlines an inhibitory role of CAFs regarding the anti-tumor immunity, on the other hand, by observing the diminished levels of effector immune cells in the TME, one could suggest an opposing enhancing role. Alternatively, one could hypothesize that the immune effector cells that remain in the TME upon myofibroblast depletion are more activated, producing more cytokines and soluble factors (eg perforin, granzyme B, IFN-γ) which inhibit tumor cell expansion and immunosuppressive cell populations accumulation. Unfortunately, limited bibliography on how ablation of α-SMA$^+$ CAFs impacts on the immune system prohibits us from directly interpreting these conflicting observations. However, despite bibliography outlining an oncogenic supportive role for α-SMA$^+$ CAFs, increased myofibroblast presence in the TME has been associated with protective responses from the host in the past[119,137,138], while high α-SMA expression has been correlated with improved survival[139].

These results become more complex to interpret when we take into account the infiltration of immunosuppressive cell populations in the TME upon elimination of CAFs. Importantly, in GCV-treated αSMA-TK mice, we observed a significant decrease in the MDSC cell population, while no differences were noted when we examined their frequencies and numbers in spleens. However, consistently with the results by Ozdemir et al[119], we also observed a significant increase in the frequencies and numbers of T regulatory cells. Furthermore, when we examined the levels of T_{regs}

in draining lymph nodes, we found that they are significantly hindered when CAFs are absent. These data indicate that CAFs might hinder T_{regs} accumulation in the TME; however, despite their enhanced presence upon CAFs absence, with current experiments we cannot be sure of how functional these intra-tumoral T_{regs} are. For future experiments, we would like to expand our knowledge on how ablation of α-SMA$^+$ myofibroblasts shapes the characteristics of the T_{reg} population (whether CAFs affect differentially the subpopulation of nT_{regs} vs iT_{regs}, whether the TME formed upon CAFs elimination results in "T_{reg} fragility" and thus loss of their suppressive identiy, etc).

With all information acquired from our experiments taken together, we cannot be entirely sure whether CAFs exert different functions in immune cell populations which collectively facilitate melanoma progression. Until today our results seem rather contradictory; our data indicate that this cell population of the TME acts as both an immune suppressor and an immune enhancer in melanomas. Further characterization of all immune cell populations infiltrating the tumor (eg. B cells, macrophages, NK T cells etc), as well as measurement of the levels of cytotoxic mediators secreted by immune effector cells are required to determine exactly how CAFs shape the anti-tumor immune response. Additionally, to understand whether CAFs in melanomas impede or enhance the anti-tumor immunity, we aim to directly isolate melanoma α-SMA$^+$ CAFs in order to perform transcriptomic analysis: RNA-Sequencing (RNA-Seq) of sorted CAFs is expected to identify differentially expressed genes associated with the anti-tumor immunity and shall ultimately provide a specific direction for this potential role of theirs. To easily sort CAFs based on their expression for α-SMA, despite its intracellular location, we aim to use the αSMA-RFP transgenic mice **(Figure 1)**, which have a DsRed fluorescent reporter for α-SMA expressing myofibroblasts.

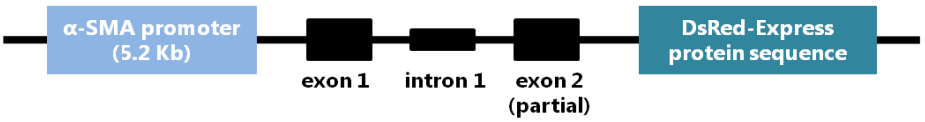


Figure 1 | Schematic illustration of the transgenic construct inserted in αSMA-RFP mice, which have a DsRed fluorescent reporter for α-SMA expressing myofibroblasts.

Followed by the contradicting results of α-SMA myofibroblasts ablation in the immune profiling of melanomas, we decided to expand our knowledge on the relationship between CAFs and T_{regs}. Data acquired from the immune phenotyping of GCV-treated αSMA-TK mice dictate that CAFs hinder T_{reg} accumulation in the TME, but what about their capability to induce T_{reg} induction? As already described, the phenomenon of T_{reg} induction is quite extended in the TME, yet the exact mechanisms and cell populations that determine this process remain unidentified. We hypothesized that secreted molecules (eg immunosuppressive cytokines such as TGF-β) by CAFs may have an important role in T_{reg} induction in the TME, therefore we examined whether T_{regs} could be induced by TES made from CAFs-depleted tumors. Compared with T_{reg} induction upon exposure of naive T_H cells to control TES, we found a significant decrease in the number of induced T_{regs} when naive T_H cells were exposed to TES made from CAFs-depleted tumors. This observation

confirms that the presence of CAFs is required for T_{reg} induction not to be obstructed. Moreover, we saw that when cells were treated with $CAFs^{\Delta}$ TES, proliferation seemed to be slightly hindered, albeit not in a statistically important fashion.

Our results strongly suggest that CAFs, facilitate T_{reg} induction and iT_{reg} proliferation. There are a number of potential mechanisms behind the hampered Treg induction observed following CAFs specific ablation. Indeed, a reduction in the Foxp3 expressing cells might be explained due to hampered IL-2 signaling or negative regulation of Foxp3 gene expression through TCR/TGF-β signaling. Furthermore, the reason behind the smaller observed percentages of iT_{regs} could merely be the result of their cell death: absence of the secretome of CAFs might not allow survival of naive T cells after they upregulate Foxp3 transcription factor. Finally, elimination of CAFs (and thus of their secreted molecules) might facilitate differentiation of naive T cells towards a T_{H17} lineage, which might be the reason why we observe decreased iT_{reg} numbers.

For future experiments, we aim to dissect the role of CAFs on the process of T_{reg} induction, by specifically assessing on induced T_{regs} the protein levels of key molecules involved in signaling pathways that might influence T_{reg} induction and proliferation **(Table 1)**. Further on, we intend to perform proteomics analysis of TES made from CAFs-depleted tumors in order to identify changes regarding the proteome upon CAFs ablation. Currently, we are on the process of developing a protocol which will enable for proteomics analysis of TES.

Table 1 | Molecules belonging in signaling pathways which might impact on T_{reg} induction.

Molecule	Signaling Pathway
CD25, STAT-3	IL-2 Signaling
pSMAD-3	TGF-β Signaling
ZAP70	TCR Signaling

Last but not least, after witnessing the dramatic effect that early CAFs depletion had on tumor growth, we developed an experimental protocol, aiming to deplete α-SMA^{+} CAFs after melanomas were established (usually on days 8-9 following B16-F10 inoculation). As implied, with this specific strategy we aimed to see whether by simulating a condition in which tumors arise we can utilize specific depletion of CAFs as a potential therapeutic strategy. Interestingly, following late GCV administration, α-SMA^{+} myofibroblasts were not eliminated, while tumor growth was not halted and tumor-infiltrating immune cell populations did not show any differences compared to controls. These observations conflict the published results by Ozdemir et al., who also utilized a similar late depletion protocol in murine PDACs and found a 76% depletion of α-SMA^{+} CAFs[119], associated with increased T_{regs} and MDSCs infiltration.

In melanomas, we concluded that after tumors arise, for an unknown reason α-SMA^{+} myofibroblasts cannot be ablated, however we haven't been able to explain this until today. The simplest explanation that might be given for these observations is that after a certain time-point CAFs, which have developed a solid cell population, exert all of their functions in a non-

proliferative state. More experiments are required in order to investigate i) whether CAFs do not proliferate after tumor appearance and ii) why they acquire this characteristic. The reason behind failed ablation of CAFs on a late GCV administration protocol might lead to new insights regarding the biology of CAFs.

All conclusions taken into consideration, this project brings into focus CAFs as an important contributor of the TME-induced immune suppression. Our data so far suggest that activated myofibroblasts accumulate in the TME, where they affect almost all immune cell populations we examined. Importantly, absence of CAFs is associated with a significant rise of the tumor-infiltrating T_{regs}, while their secretome positively affects T_{reg} induction and iT_{reg} proliferation. Despite that the interplay between cancer-associated fibroblasts and immune cells has long been recognized as a major contributor of cancer development, our research expands our knowledge over how they suppress immune surveillance, by facilitating the survival and accumulation of suppressor T cells in the TME.

Concluding, with our study, new insights on melanoma anti-tumor immunity were offered. Undoubtedly, fully understanding how CAFs induce immunosuppression in the TME will result in a better mechanistic insight of how they influence anti-tumor immune responses, while simultaneously providing tools for prediction of immunotherapy responses. Continuation of this project is expected to provide a more comprehensive view of cancer immunity regulation, which will undoubtedly lead to development of more efficacious immunotherapeutic approaches involving cancer patients.

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