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Recent advances and trends

Fibroblast heterogeneity in Tumor Micro-environment: Role in Immunosuppression and New therapies

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Abstract

In tumors, Cancer-Associated Fibroblasts (CAFs) constitute the most prominent component of the tumor microenvironment (TME). CAFs are heterogeneous and composed of different CAF subsets exerting distinct functions in tumors. Specific CAF subpopulations actively influence various aspects of tumor growth, including cancer cell survival and proliferation, angiogenesis, extracellular matrix (ECM) remodeling, metastatic spread and chemoresistance. During the past decade, some CAF subsets have also been shown to modulate anti-tumor immune response. Indeed, they can increase the content in regulatory T lymphocytes and inhibit the activity of effector and cytotoxic immune cells. These functions are mainly controlled by their constitutive secretion of cytokines, chemokines, growth factors and ECM proteins, either directly in the surrounding extracellular space or through micro-vesicles. Some CAFs also express key regulators of immune checkpoints. The different roles played by CAFs, both as immunosuppressor or as physical support for tumor cell progression, set them as promising targets for anti-tumor therapies. In this review, we describe the main current knowledge on CAFs heterogeneity and immunosuppressive microenvironment, as well as their potential therapeutic implications.

Keywords: cancer associated fibroblasts, CAF, immunosuppression, cancer, tumor microenvironment, stroma, T lymphocytes, NK, macrophages

1. Generalities

It is now well-accepted that tumor progression is not strictly dependent on cancer cell genetic alterations or epigenetic modifications, but also controlled by components of the tumor microenvironment (TME). TME components are central to all stages of tumorigenesis and metastasis [1-4]. TME is composed of several cell types, such as fibroblasts, pericytes, immune and endothelial cells that are all able to interact with cancer cells in a dynamic way. Among cells present in the TME, cancer-associated fibroblasts (CAFs) represent one of the most abundant components that can contribute -at several levels- to the malignant phenotype [2-7]. In non-cancerous homeostatic conditions, fibroblasts are present at resident stage and represent an important sensor of tissue integrity. After a tissue damage signal, they differentiate into myofibroblasts and orchestrate tissue repair through synthesis of ECM components and crosstalk with immune cells [4]. Dysregulation of the physiological conditions during tumor development accompanied with a chronic inflammatory response drive fibroblast activation and their differentiation into myofibroblasts, i.e. fibroblasts with high constitutive expression of smooth-muscle α -actin (SMA). CAFs are described to significantly increase tumor development by regulating several processes, including cancer cell proliferation, tumor cell invasion, angiogenesis and ECM remodeling. The abundance of SMA⁺ CAFs in TME is associated with poor prognosis in multiple cancers [8-15]. Moreover, tumors with high stromal signatures are linked to therapy resistance and disease relapse [5, 16-19]. In addition to their role in controlling cancer cell behavior, CAFs emerge as central players in shaping the TME toward an immunosuppressive and growth-promoting phenotype, via increased production of immunosuppressive cytokines and enhanced expression of immune checkpoints [20-31]. Still, multiple findings showed that CAFs are not a homogenous cellular population. Indeed, several CAF subpopulations have been identified and have either tumor-promoting [25, 27, 31-36] or tumor-suppressive effects [37, 38]. Such heterogeneity might result from numerous causes, including the various cellular precursors of CAFs, the reciprocal interactions between CAFs and cancer cells or any other TME components, in addition to the cytokines, chemokines and growth factors secreted into the TME. Taking together, all these features highlight the importance of CAFs and define them as promising anti-cancer therapies, as a complement to existing treatments targeting cancer cells or boosting the immune system. In this review, we summarize recent advanced of CAFs heterogeneity in different cancer subtypes with a particular emphasis on the mechanisms involved in their immunosuppressive capacities. We will also highlight the potential therapeutic strategies for targeting CAFs in the field of cancer immunotherapy.

2. CAF heterogeneity in TME

CAFs are major components of stromal cells that surround cancer cells. They provide not only a mechanical support to cancer cells but also control their proliferation, survival, metastasis and resistance to therapies [3-7, 25]. Although CAF origins in tumors remain unclear and might be diverse, CAF heterogeneity has been unveiled in the last years thanks to intense researches. In that sense, CAF subsets rather represent distinct cellular states than different cell types. Many studies have reported different origins of CAFs including resident tissue fibroblasts, mesenchymal stem cells from bone marrow or adipose tissue, as well as endothelial cells, epithelial cells, pericytes and adipocytes undergoing trans-differentiation into mesenchymal cells [34, 39-52]. Fibroblast differentiation into myofibroblasts (SMA+ fibroblasts) is driven by different mechanisms highlighted in distinct cancer subtypes. In breast carcinoma, contact between cancer cells and stromal cells promotes myofibroblastic CAF differentiation. The release of reactive oxygen species (ROS) by cancer cells plays a key role in the increase in myofibroblast content through the activation of various signaling pathways, including CXCL12/CXCR4, TGF β and NOTCH signaling [2, 8, 52-54]. In mouse model of squamous cell carcinoma, the transition from normal fibroblasts to CAFs is mediated by two events, the suppression of p53 activity and down-modulation of the Notch effector *Csl/Rbpj* [55]. Interestingly, the induction of a pro-inflammatory gene signature in normal fibroblasts at early stage of squamous carcinogenesis promoted CAFs generation. Indeed, normal dermal fibroblasts can be educated by carcinoma cells to express pro-inflammatory genes, thereby promoting myofibroblastic differentiation [8, 56]. In addition, epigenetic modifications also contribute to fibroblasts reprogramming toward a pro-invasive phenotype, thereby promoting ECM remodeling and invasion of cancer cells [57-61]. Physical changes in ECM, including rigidity of collagen fibers and modulation of elasticity, govern fibroblast phenotypic modifications [62, 63 Arwert, 2020 #195, 64]. Mechanical stretching of normal fibroblasts in *in vitro* studies induces phenotypic changes similar to those of CAFs, increase their contractility and remodel TME to facilitate tumor invasion [65]. Furthermore, stretched fibroblasts produce ECM with a more organized and linearly aligned structure, with increased ability for redirecting co-cultured cancer cell migration [54, 65-75]. Tumor-stroma mechanics and ECM stiffening also coordinate non-essential amino-acid availability to sustain tumor malignancy [76]. In addition to the role of tumor cells in generating CAFs, signals from other cell types within the TME can also promote fibroblastic invasive functions. For instance, granulins secretion by tumor-associated macrophages activated hepatic stellate

cells, driven their differentiation into myofibroblasts that secreted periostin, resulting in a fibrotic microenvironment. Disruption of macrophages recruitment, or genetic depletion of granulins reduced CAFs activation and prevented liver metastasis [77, 78]. Furthermore, recent data show a reciprocal crosstalk between regulatory T lymphocytes and CAF subsets in breast cancers [25, 31].

CAFs can be distinguished within the tumor by their morphological features and the lack of expression for epithelial, endothelial and immune cell markers. However, this is not sufficient to exclude other mesenchymal lineages, such as pericytes or adipocytes [4]. For these reasons, different markers, which are not or lowly expressed in normal fibroblasts compared to CAFs, were identified to better characterize activated CAFs, such as SMA, fibroblast activated protein (FAP), fibroblast specific protein-1 (FSP1, also known as S100A4), Integrin β 1 (CD29), platelet derived growth factor receptor α or β (PDGFR α / β) or podoplanin (PDPN) [79-84]. Still, these markers are not all regulated similarly or simultaneously in CAFs, thereby highlighting a strong degree of heterogeneity of these cells in TME. By combining the analysis of six CAF markers, four different CAF subpopulations (referred to as CAF-S1 to CAF-S4) were recently discovered in breast and ovarian cancers (Figure 1) [25, 27]. Both CAF-S1 (FAP^{High} CD29^{Med} SMA^{Med-High} FSP1^{Med} PDGFR β ^{Med-High} CAV1^{Low}) and CAF-S4 (FAP^{Neg-Low} CD29^{High} SMA^{High} FSP1^{Low-Med} PDGFR β ^{Low-Med} CAV1^{Low}) subsets are detected at high level in aggressive breast cancer subtypes (HER2 and TN) and in metastatic lymph nodes, confirming that stromal myofibroblasts are associated with poor prognosis [25, 54]. Both CAF-S1 and CAF-S4 promote metastases through complementary mechanisms [54]. Moreover, accumulation of FAP^{high} CAF-S1 subset in early luminal breast cancers is associated with distant relapse [85]. In contrast, the CAF-S2 subset (CD29^{Low} FAP^{Neg} FSP1^{Neg-Low} α -SMA^{Neg} PDGFR β ^{Neg} CAV1^{Neg}) is enriched in luminal breast cancer subtype and CAF-S3 fibroblasts (CD29^{Med} FAP^{Neg} FSP1^{Med-high} α -SMA^{Neg} PDGFR β ^{Med} CAV1^{Low}) accumulate in healthy tissues [25, 54, 85]. In all tumors and invaded lymph nodes, FAP^{high} CAF-S1 fibroblasts are defined by ECM, adhesion and wound-healing signatures, and FAP^{Neg} CD29^{High} CAF-S4 are characterized by a perivascular contractile signature [25, 27, 54, 85]. Several recent studies from bulk or single cell data on CAF from human cancers and mouse models confirmed the existence of ECM-rich (CAF-S1) and contractile (CAF-S4) sub-populations [36, 86-90]. By applying specific CAF-S1 and CAF-S4 molecular signatures on publicly available single cell data from lung cancer [91] and head and neck cancer [92], the existence of these two major CAF-S1/CAF-S4 myofibroblastic

subpopulations was validated in different cancer types [31]. Thus, these data indicate that both CAF-S1 (ECM/adhesion/wound) and CAF-S4 (perivascular/contractile) myofibroblastic cells can be detected in distinct cancer types and across species.

Among the FAP^{High} CAF (CAF-S1) subpopulation, two distinct subsets were recently identified in pancreatic adenocarcinoma, exhibiting either a matrix-producing myofibroblastic phenotype termed myCAF or an immunomodulatory secretome, inflammatory CAFs named iCAF [33]. More precisely, CAFs most proximal to the cancer cells exhibit a myofibroblastic myCAF phenotype with high expression of SMA. More distal CAFs express high levels of IL-6 and are defined as iCAFs [33, 87]. Furthermore, two FAP⁺ mesenchymal subsets were also identified on the basis of PDPN expression [26]. Although both subsets express ECM molecules, FAP⁺ PDPN⁺ fibroblasts exhibit higher expression of genes associated with TGF β signaling and fibrosis than FAP⁺ PDPN⁻ cells. These observations demonstrated that the FAP^{High} CAF-S1 subpopulation could be -by itself- heterogenous. Several laboratories have addressed this question by performing single cell analysis from FAP^{High} CAF cells [31, 87-90]. All these studies demonstrated the existence of the iCAF/myCAF subpopulations in the different cancer types analyzed. Interestingly, the large number of FAP^{High} CAF (CAF-S1) fibroblasts recently sequenced at single cell levels in [31] reached -to our knowledge- an unprecedented resolution of this subpopulation. This study identified 8 different FAP^{High} CAF clusters with 3 clusters belonging to the iCAF subgroup and 5 clusters to the myCAF subgroup (Figure 1). By dissecting the most prominent pathways and gene signature specific of each cluster, these 8 clusters can be characterized as followed: ECM proteins (ecm-myCAF), detoxification pathway (detox-iCAF), interleukin-signaling (IL-iCAF), TGF β -dependent pathway (TGF β -myCAF), wound-healing signaling (wound-myCAF), IFN γ - and IFN $\alpha\beta$ -related pathway (IFN γ -iCAF, IFN $\alpha\beta$ -myCAF) and acto-myosin-signaling (acto-myCAF) [31]. Interestingly, the ecm-myCAF specific signature contains the LRRC15 gene that has been recently identified in pancreatic cancer [90]. Moreover, the IFN γ -iCAF cluster express high levels of *CD74*, encoding *Major Histocompatibility Class (MHC) II invariant chain*, which characterizes the antigen-presenting CAF (“apCAF”) recently identified in pancreatic cancer [89]. Finally, the existence of the most abundant FAP^{High} CAF clusters has been validated in head and neck squamous cell carcinoma and in non-small cell lung cancer [31].

In conclusion, these recent data demonstrate that CAF heterogeneity in cancer has been until now largely under-estimated. Still, a common spectrum of CAF subpopulations is

emerging, with both CAF-S1 (FAP^{High} CD29^{Med} SMA^{Med-High}; ECM/adhesion/wound signatures) and CAF-S4 (FAP^{Neg-Low} CD29^{High} SMA^{High}; perivascular/contractile signatures) myofibroblastic CAF detected in distinct cancer types and across species. Moreover, among FAP^{High} CAF-S1 subpopulation, the iCAF/myCAF subsets are validated in all cancers analyzed until now. Finally, these iCAF and myCAF subsets can be -by themselves- subdivided into different clusters identified as detox-iCAF, IL-iCAF, IFN γ -iCAF and ecm-myCAF, TGF β -myCAF, wound-myCAF and IFN $\alpha\beta$ -myCAF, respectively. These FAP^{High} CAF clusters have been detected in various cancer types, thereby demonstrating their validity and their potential important roles in tumors.

3. Role of CAFs in anti-tumor immune response

As discussed above, it is now well established that CAFs exhibit a phenotypic diversity and functional heterogeneity that define their role within the tumor [7, 8, 20, 25, 26, 31, 37, 38, 54, 87, 90, 93-95]. The pro-tumorigenic functions of CAFs could be attributed either to their ability to produce pro-survival factors, which directly and positively impact tumor progression by enhancing cancer cell proliferation, survival and metastasis, or to their role in regulating antitumor immune response by inducing an immunosuppressive microenvironment [8, 21-25, 27, 31, 87, 96-102]. The immunomodulatory functions of these stromal cells can affect both innate and adaptive antitumor immune response (Figure 2). This can be either direct via the secretion of cytokines and chemokines such as IL-6, CXCL1, CXCL12, CCL2, which can retain suppressive immune subsets and counteract effector functions of activated immune cells, or indirect via the remodeling of the ECM protein network, forming a physical barrier for immune cell entry [29, 30, 62, 65, 70, 103-108].

3.1. CAF-dependent regulation of the adaptive anti-tumor immune response

3.1a- Role of CAF subsets in adaptive anti-tumor response

The pleiotropic immunomodulatory functions of CAFs can interfere with the adaptive anti-tumor immune responses at different stages of cancer progression (Figure 2). In this context, multiple studies have confirmed the presence of functionally heterogeneous CAFs subpopulations within the same tumors. For example, in breast cancer, among FAP^{High} CAF, it was shown that PDPN⁺ fibroblasts suppress T cell proliferation in a nitric-oxide-dependent manner, whereas PDPN⁻ cells are unable to do so [26]. Importantly, a multistep mechanism unraveling FAP^{High} CAF-mediated immunosuppressive activity has been recently highlighted in breast and ovarian cancer [25, 27]. By secreting CXCL12 -through regulation by mir-200

family members in ovarian cancer [27]-, FAP^{High} CAF attract CD4⁺ CD25⁺ T lymphocytes and retain them at their surface by high expression of OX40L/TNFSF4, PD-L2/PDCD1LG2 and JAM2 in several cancer types[25, 27]. Moreover, FAP^{High} CAF increase CD4⁺ T cell survival and their differentiation into CD25^{High} FOXP3^{High} functional Tregs through B7H3/CD276, CD73/NT5E, and DPP4 [25, 27]. As detailed above, among FAP^{High} CAF, recent single cell analyses reveal the heterogeneity within this population [31, 33, 87-90]. On line with the identification of different clusters among FAP^{High} CAF, recent data demonstrated that only specific clusters, in particular those characterized by ECM accumulation, wound-healing signature and TGF β -signaling, are associated with an immunosuppressive environment [31, 89, 90]. Consistent with this correlation between the abundance of FAP^{High} ecm-myCAF and TGF β -myCAF cellular clusters with the content in FOXP3⁺ T lymphocytes in breast cancer, *in vitro* functional assays showed that ecm-myCAF are able to recruit FOXP3^{high} Tregs and to increase PD-1 and CTLA-4 protein levels at their surface [31]. Interestingly, accumulation of ecm-myCAF, wound-myCAF and TGF β -myCAF is correlated with resistance to immunotherapy in melanoma and non-small cell lung cancer patients [31].

3.1b- Role of CAF subsets on immune checkpoint expression

Regulating immune checkpoint expression emerged as an important process by which CAFs directly modulate T cell function. An interesting point was recently raised showing that CAFs from breast, ovarian, lung, pancreas and colon cancer express programmed death ligand-1/2 PD-L1 and/or PD-L2 [25, 27, 109-112], in particular in the FAP^{High} CAF subset [25, 27]. These ligands bind to PD-1 receptor expressed by T cells and inhibit T cell activity [25, 110-112]. PD-L1 and PD-L2 ligands can be upregulated by IFN- γ highly secreted by activated T cells, thereby impairing their function [109]. Moreover, fibroblasts isolated from melanoma patients' biopsies secrete IL-1 α/β that could directly suppress CD8⁺ T cell proliferation and function via upregulation of PD-L1 expression at their surface [113], suggesting that IL-1 α/β clinical blockade might synergize with immunotherapeutic interventions and benefit melanoma patients. Similarly, CAFs isolated from pancreatic cancer also promote expression of immune checkpoint inhibitors TIM-3, PD-1, CTLA-4 and LAG-3 at the surface of proliferating T cells, via the activity of CAF-derived PGE2 [112]. Finally, α -SMA⁺ CAF are positively correlated with PD-L1 expression by tumor cells in melanoma and colorectal carcinoma [114]. Mechanistically, CXCL5 secreted by CAFs promotes PD-L1

expression at the surface of tumor cells which in turn abolished T cell anti-tumor immune response [114].

3.1c- Role of metabolism in CAF-mediated immunosuppressive action

Another suggested mechanism by which CAFs impair T cell proliferation and function is depending on the production of metabolic reprogramming factors [115-120]. First, in contrast to normal fibroblasts, CAFs can use aerobic glycolysis as a source of energy, thus producing high levels of pyruvate and lactate that modify T cell polarization, reducing the percentage of antitumoral helper T cell (T_H1) subset and increasing Treg proliferation and function [119-123]. Another example of immunosuppressive metabolites secreted by CAFs is mediated by the immunomodulatory indoleamine 2,3-dioxygenase (IDO) enzyme that acts as a T-cell inhibitory effector [124, 125]. Indeed, IDO facilitates the conversion of tryptophan to kynurenine and production of downstream metabolites that inhibit T-cell proliferation and function [124, 125]. Similarly, CAF-dependent expression of Arginase 2, which hydrolyzes arginine to ornithine and urea, reduces Arginine content in TME, which is indispensable for T cell functions [126, 127]. Consistent with this key function, the abundance of Arginase 2-expressing stromal cells is associated with poor clinical outcome in pancreatic cancer [126, 127]. Furthermore, CAFs are a major source of PGE₂, which plays an important role in promoting tumor growth, angiogenesis and resistance to established cancer therapies [128-133]. In addition, it was shown that PGE₂ drives immunosuppression by multiple mechanisms, either by shifting the balance from anti-tumor T_H1 immune response toward immunosuppressive T_H2 response, or by suppressing T cell cytotoxic activity and promoting Treg function [134-139]. Moreover, COX2, which catalyzes PGE₂ production, is highly expressed in CAF and results in an exacerbated Treg recruitment in tumors [139-142]. CAFs also produce galectins, known as S-type lectins, carbohydrate-binding proteins that mediate cell-cell or cell-ECM communications [143, 144]. Galectin-1 induces T cell apoptosis by binding on CD7 and CD45 on T cell surface. In addition, galectin-1 is overrepresented in Tregs and contributes to their immunosuppressive activity [145-147]. Furthermore, mesenchymal stromal cells isolated from cervical tumors showed elevated expression levels of CD39 and CD73 as compared to normal fibroblasts [148]. This expression reduces T cell activity and proliferation, through the generation of a large amount of adenosine, a well-known immunosuppressive molecule produced by the hydrolysis of ATP, ADP and AMP [148]. On line with this observation, FAP^{High} CAF express high levels of CD73, which promotes immunosuppression by increasing Tregs in breast and ovarian cancer [25, 27].

3.1d- TGFβ-dependent signaling pathway in CAF-mediated immunosuppression

One cellular cluster recently identified among FAP^{High} CAF by single cell analysis in breast cancer is characterized by TGFβ signaling and is directly involved in immunosuppression and resistance to immunotherapies [31]. Indeed, some CAFs, in particular some FAP^{High} CAFs, secrete high levels of TGFβ ligands that act on both CD8⁺ and CD4⁺ T cells [25, 31, 149-156]. TGFβ was shown to promote cell death of effector CD8⁺ T lymphocytes by inhibiting the expression of the pro-survival protein BCL2 [111]. It attenuates the acquisition of effector function by memory CD8⁺ T cells, preventing their access to cancer cells and providing a barrier for the therapeutic cancer vaccines [104, 157]. TGFβ also alters CD8⁺ T cell function by inhibiting the expression of key genes involved in their cytotoxic activity (perforin, granzymes A and B, FAS ligand and IFNγ). For instance, repression of granzyme B and IFNγ transcription is mediated by the binding of TGFβ-activated SMAD and ATF1 transcription factors on their promoters. Neutralization of systemic TGFβ in mice enables tumor clearance with restoration of cytotoxic gene expression in activated CD8⁺ T cells [158]. In head and neck cancer, α-SMA⁺ FAP⁺ CAFs inhibit the proliferation of CD8⁺ T cells and promote the recruitment of CD4⁺ CD25⁺ T cells by secreting TGFβ and IL6 [22]. TGFβ stimulates also class switch recombination, converting IgM expressing-B cells to IgA expressing-cells which present regulatory activity. In hepatocellular carcinoma, these IgA⁺ cells interfere with activation of cytotoxic CD8⁺ T lymphocytes inhibiting thus, tumor-directed immune response [159, 160]. In addition to TGFβ, it was shown in a mouse model of colon cancer that CAFs release high levels of IL6 which in turn, decrease CD8⁺ T cells infiltration within the tumor and increased Foxp3⁺ Treg activation, effect attenuated by treatment with IL6 blocking antibodies [161].

Consistent with their immunosuppressive functions, TGFβ signature in CAFs is associated with poor response to immunotherapies in metastatic urothelial and colon cancer [28, 162], as well as in non-small cell lung cancer and in melanoma patients [31]. This can occur particularly in patients with tumors presenting an exclusion of CD8⁺ T cells from the tumor [28, 162] and an accumulation in the collagen-rich peritumoral stroma, in line with ECM-enriched FAP^{High} CAFs [31]. Interestingly, co-administration of TGFβ and anti-PD-L1 blocking antibodies reduces TGFβ signaling in stromal cells, facilitates T cell penetration, reduces tumor progression and improves patient prognosis [28, 163-166].

In addition to CD8⁺ T cell and Tregs, CAFs have the potential ability to influence CD4⁺ helper T cell phenotype, switching them from anti-tumoral to pro-tumoral cells. Indeed, CD4⁺ T cells can be classified into multiple sub-lineages, based on their functions and the profile of cytokine production. In general, T_H2-mediated immunity is considered tumor promoting, as it activates M2-like macrophages function and inhibits cell-mediated tumor killing. Many findings emerged from murine models of cancer, showed that targeting of specific signaling molecules in CAFs resulted in reduction of tumor growth, accompanied by a shift in the T cell response[20, 167-174]. For example, in a mouse model of breast cancer, elimination of FAP⁺ CAFs by a DNA vaccine targeting FAP results in a shift of the immune population from a T_H2 to a T_H1 phenotype [167]. This shift was associated with an increased expression of IL-2, an increase of CD8⁺ T cell population and a diminished recruitment of tumor-associated macrophages (TAM) and Tregs. Furthermore, targeting of CAF-derived Chitinase-3-like 1 (Chi3L1) has a similar effect in another transplantable model of breast cancer [175]. Indeed, it was demonstrated that Chi3L1 is highly upregulated in CAFs from mammary tumors and pulmonary metastases of transgenic mice. Genetic ablation of Chi3L1 in fibroblasts attenuated tumor growth, macrophage recruitment and reprogramming to an M2 phenotype and enhanced CD8⁺ and CD4⁺ T cells accumulation within the tumor [175]. Consistent with these findings, CHI3L1 has been shown to promote tumor progression through TGFβ pathway and pro-inflammatory signals and to be associated with poor prognosis in many cancers [176-182].

In conclusion, through distinct molecular mechanisms, CAFs modulate T cell-dependent antitumor immune response at multiple levels, i.e. by recruiting CD4⁺ CD25⁺ Tregs, by switching a T_H1 to a T_H2 phenotype, and by inhibiting CD8⁺ cytotoxic activity.

3.2. CAF regulation of the innate anti-tumor immune response

Besides the adaptive immunomodulatory functions, myofibroblastic CAFs also interfere with the innate immune response at different levels (Figure 2). First, it has been shown that CAFs affect the differentiation of tumor associated macrophages (TAMs) [183-185]. TAMs derive from infiltrating monocytes or resident macrophages and represent a major component within tumors. They are involved in various tumor-promoting tasks including pro-inflammatory signaling, enhancement of angiogenesis, metastasis and therapy resistance. CAFs actively polarize resident macrophages toward a pro-tumoral phenotype via the secretion of IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF) [186-188]. In addition, the glycoprotein CHI3L1, highly expressed by CAFs, enhances TAM recruitment and their

differentiation into pro-tumoral M2 [175]. CAFs also increase the recruitment of monocyte in tumors through CXCL12 delivery and promote their differentiation toward the M2 phenotype [185, 186, 189-194]. This interaction between CAFs and M2 enhances tumor progression and metastasis formation. CAFs abundance is correlated with a higher number of TAMs in human breast cancer, correlation associated with a high Ki-67 proliferation index and a high tumor volume [193].

Similarly as the phenotypic macrophages (M1/M2), the concept of immune cell polarization has also been extended to neutrophils, named Tumors-associated neutrophils (TANs). N1 neutrophils show an anti-tumor phenotype with their capability of efficiently killing microorganisms and tumor cells, while N2 neutrophils exhibit a pro-tumor phenotype [195, 196]. CAF-mediated TGF β signaling redirects TAN differentiation toward the N2 pro-tumorigenic phenotype. Conversely, TGF β blockade attenuates tumor growth via TAN polarization to an anti-tumor N1 phenotype, thereby providing additive strategies for cancer therapies [197]. Moreover, CAF-derived IL6 increases survival, proliferation and activation of TAN in a STAT3-dependent manner and induces PD-L1 expression in TAN [198], indicating that IL6-STAT3-PD-L1 signaling cascade by CAF could provide novel targets for therapies [198-204].

Another mechanism by which CAFs trigger anti-tumor immune responses is by affecting the function of dendritic cells (DC), the most important population of antigen presenting cells, needed to activate T lymphocytes. As a major source of TGF β , CAF mediate downregulation of MHC class II molecules and of CD40, CD80 and CD86 co-stimulatory molecules at the surface of DC, thus inhibiting their antigen presenting function and their ability to activate cytotoxic T cell responses [205]. Moreover, CAF-produced IDO facilitates the generation of regulatory DCs, characterized by low expression of costimulatory molecules, high levels of suppressive cytokines, impaired T-cell proliferation and enhanced Treg expansion [206-208]. Furthermore, proinflammatory cytokines (such as TNF α and IL-1 β) released by pancreatic tumor cells activate CAFs and facilitate secretion of thymic stromal lymphopoietin (TSLP) by CAFs [209]. TSLP induces activation/maturation of tumor-resident DCs, that migrate to the draining LNs where they activate CD4⁺ T_H2 cells and exert tumor-promoting functions [209].

Another immune cell population affected by CAFs immunosurveillance is mast cells. It is a heterogeneous population of immune cells, widely distributed throughout all tissues. Upon activation, mast cells release a large spectrum of cytokines and inflammatory molecules

that modulate tumor initiation and progression. Interestingly, in pancreatic tumors, stellate cells (known as precursors of CAFs) activate mast cells and facilitate their degranulation and cytokine release. As a result, activated mast cells secrete IL-13 and tryptase, in the tumor microenvironment, leading to stromal cell proliferation, and favoring tumor growth [210]. Of note, activated mast cells not only increase tumor progression but also alter anti-tumor immune response. Indeed, mast cells mobilize myeloid-derived stem cells (MDSCs) infiltration into tumors and induce production of IL-17 by MDSCs; MDSCs-derived IL-17 indirectly attract Tregs and enhance their suppressive function [211]. MDSCs are a group of heterogeneous cells that are derived from bone marrow and have a remarkable ability to suppress immune cell responses. Multiple studies have shown a direct interaction between CAFs and MDSCs. Indeed, CAFs, isolated from hepatocellular carcinoma attract monocytes through the CXCL12/CXCR4 pathway and induce their differentiation into MDSCs through IL-6 mediated STAT3 activation [198, 212]. This interaction subsequently impairs T cell proliferation and alters their cytotoxic function. Moreover, FAP⁺ CAF, as a major source of CCL2, promote tumor growth via the recruitment of MDSCs in a mouse model of hepatic cancer and in lung squamous cell carcinoma [24, 213].

Finally, CAFs can also affect the activity of major innate effector cells, Natural Killer (NK) cells, which orchestrate early immune response through their cytotoxic activity [214]. CAF-produced TGF β ligands are important regulators of NK immune function. Indeed, TGF β is well known to downregulate the expression of NKp30 and NKG2D activating receptors, thereby decreasing NK cell cytotoxic activity. In addition, TGF β induces the expression of the miR-183, which bind and repress DNAX activating protein 12 (DAP12), a signal adaptor for lytic function in NK cells [215-217]. TGF β also inhibits CD16-mediated IFN- γ production and antibody-dependent cellular cytotoxicity, an effect that reduces T_H1 immune cell differentiation [218, 219]. Moreover, melanoma and colorectal carcinoma-derived CAFs sharply interfere with NK cell functions including cytotoxicity and cytokine production by repressing the expression of several NK activating receptors including NKp44, NKp30 and DNAX accessory molecule 1 (DNAM-1), as well as the production of cytolytic granules containing perforin and Granzyme B [220, 221]. This modulation is mediated by the secretion of prostaglandin E₂ (PGE₂), leading to an attenuated cytotoxic activity of NK cells [220, 221]. In addition, elevated secretion of matrix metalloproteinases (MMPs) by CAFs in melanoma reduces the expression of MHC class I chain-related protein A and B (MICA/B) at the surface of tumor cells and consequently decrease NKG2D-dependent cytotoxic activity of

NK cells [222]. CAFs also secrete adenosine that restricts NK cell function and immune protection against tumor development [223]. Finally, studies using endometrial cancer cells showed that CAF promoted NK dysfunction through contact dependent mechanisms involving downregulation, at the surface of CAFs, of poliovirus receptor (PVR/CD155), an important NK cell ligand [224]. Better understanding of the mechanisms through which CAFs manipulate NK cell activation will be needed as NK cells are attractive targets for novel adoptive cell immunotherapy [223, 225-227].

In conclusion, due to their ability to secrete immunomodulatory cytokines, chemokines or soluble factors, CAFs favor the recruitment of innate immune cells, such as monocytes or neutrophils and their acquisition of anti-inflammatory phenotypes like M2 macrophages or N2 neutrophils, but can also affect the cytotoxic activity of NK cells

3.3. Indirect effect of CAFs on anti-tumor immune response

One of the hallmarks of CAFs is the excessive production of extracellular matrix components, which results in a remodeling of the ECM, and affects tumor cell behavior [68, 228]. Increased ECM rigidity resulting from thickening of collagen fibers provides cues favoring migration, attachment, survival and proliferation of cancer cells [229]. It also restricts the access of immune cells to cancer cells, serving as a physical barrier [230]. Multiple findings have shown that CAF-mediated ECM remodeling and fibrosis contributed to the formation of an immunosuppressed microenvironment by multiple mechanisms [230].

In this regard, in pancreatic adenocarcinoma, dense collagen networks represented a physical barrier, inhibiting the migration of activated T cells, which can alter the development of T-cell-based immunotherapies [231-233]. Similar results were observed in human lung tumors, showing that the presence of dense matrix fibers surrounding the tumor islets prevents T cell infiltration [103].

In addition, ECM remodeling, mediated by CAFs acts directly on tumor-specific CD8⁺ T cells and F4/80 macrophages, either by reducing the number of CD8⁺ T cells within the tumor or by functionally reprogramming F4/80 macrophages differentiation toward M2 anti-inflammatory phenotype [234-238]. Thus, CAF-mediated ECM remodeling and fibrosis contribute to the formation of an immunosuppressed microenvironment that promotes tumor growth and metastasis formation.

4. CAFs: a promising target for cancer therapy

In situation in which the tumor stroma promotes cancer progression and induces resistance to anticancer therapies, the development of new therapies targeting the stroma could have curative outcomes. In addition, the recent characterization of CAFs, including identification of new CAFs subsets and their functional diversities, brings CAF-targeting therapies as promising tools for cancer treatment (Figure 3) [95, 106, 239].

4.1. CAF depletion via cell surface markers

As discussed above, FAP and SMA represent two cell surface markers for activated myofibroblasts. Based on this, targeting immunosuppressive CAFs using these markers was largely investigated in pre-clinical studies. Thus, targeting FAP by both genetic deletion or pharmacological inhibition inhibit tumor growth in two different mouse model of lung and colon cancer [240]. Similarly, conditional depletion of FAP⁺ stromal cells in a mouse model of pancreatic cancer re-sensitizes cancer cells to anti-PD-1 and anti-CTLA-4 immunotherapies [241]. Multiple other approaches were developed to target FAP⁺ CAFs, including an oral DNA vaccine targeting FAP [242], or an administration of a novel anti-FAP monoclonal antibody (FAP5-DM1) [243] that bound shared epitopes of mouse, human and monkey FAP and showed an excellent efficacy with a complete tumor regression without significant toxicity. In another metastatic breast cancer model, depletion of FAP⁺ stromal cells by FAP-targeting immunotoxin α FAP-PE38 alters the secretion of various growth factors, cytokines and matrix metalloproteinases by myofibroblasts and suppresses tumor growth [244]. Interestingly, genetic depletion of FAP⁺ CAFs is associated with increased CD8⁺ T cells infiltration in Lewis lung carcinoma and pancreatic cancer models [168, 173]. Similarly, administration of an oral mouse DNA vaccine targeting FAP increases CD8⁺ T cell infiltration and improves the intra-tumoral uptake of chemotherapeutic drugs in a model of colon cancer [242].

Based on these promising preclinical data, a phase I trial was started with a monoclonal antibody recognizing FAP (F19) in patients with colorectal cancer and small cell lung cancer [245, 246]. The results showed that this antibody was administrated safety to patients even after repeated infusions and was associated with tumor regression. However, it failed in patients with metastatic colorectal cancer [247]. Interestingly, CAR therapy was also used to target FAP⁺ CAFs in the tumor stroma. For this purpose, T cells are modified to express a FAP-specific chimeric antigen receptor and transferred to an established A549 lung cancer model. These CAR T cells promote a specific immune attack against FAP⁺ stromal cells inducing a significant decrease in tumor growth [169]. Similarly, adoptive transfer of

FAP-CAR T cells also decreases tumor vascular density and restrains growth of desmoplastic human lung cancer xenografts and syngeneic murine pancreatic cancers [248]. However, it is important to note that an opposite result came from another study and showed that depletion of FAP⁺ stromal cells led to loss of muscle mass and anemia confirming that using FAP as a universal target antigen to deplete stromal cells should still be investigated [249]. A recent study investigated the use of a bispecific antibody (RO6874281) consisting of an interleukin 2 variant domain that binds to IL-2 receptor and stimulates a local immune response by activating cytotoxic NK cells and lymphocytes T, associated to a FAP-specific domain targeting CAFs. This component showed an acceptable safety profile and displayed monotherapy activity in different tumors [250]. These promising results drive the development of multiple clinical trials consisting to combine RO6874281 together with immunotherapies [251-254]. Moreover, a second bispecific protein was developed in order to target simultaneously the T cell costimulatory receptor 4-1BB and FAP⁺ stromal cells (FAP-4-1BB). Interestingly, treatment with this compound in a colorectal cancer-bearing rhesus monkey, decreased tumor growth and enhanced accumulation and activation of intratumoral CD8⁺ T cells [255, 256]

SMA was also used to target myfibroblastic CAFs. In a mouse model of breast cancer, docetaxel conjugate nanoparticles that target SMA⁺ stromal cells suppress metastasis formation [257]. In parallel, in a mouse model of pancreatic cancer crossed with SMA thymidine kinase transgenic mice, depletion of SMA⁺ myfibroblasts results unexpectedly in more invasive tumors with enhanced hypoxia, EMT induction and diminished animal survival [37]. Moreover, suppressed immune surveillance with increased CD4⁺ Foxp3⁺ Tregs was observed in myfibroblast-depleted mice accompanied with a resistance to anti-CTLA-4 immunotherapy [38]. These results suggested that targeting CAFs using surface markers requires careful consideration and might be more challenging than initially thought.

More recently, two cell-surface molecules, CD10 and GPR77, were defined as new markers for a CAFs subset correlated with chemoresistance and poor survival in multiple cohorts of breast and lung cancer patients. Mechanistically, CD10⁺GPR77⁺ CAFs promote tumor formation and chemoresistance by providing a survival niche for cancer stem cells. Moreover, targeting these CAFs with a neutralizing anti-GPR77 antibody abolishes tumor formation and restored tumor chemosensitivity [35].

4.2. Restoration of quiescent fibroblasts phenotype

As mentioned above, CAFs can result from activation of resident fibroblasts. Thus, it was considered to revert CAF activated state to a quiescent state in order to reduce tumor growth. In pancreatic tissue, resident fibroblasts store retinol that is lost upon activation. Restoring retinol level in CAFs in a mouse model of pancreatic adenocarcinoma resets fibroblasts to an inactive state and results in enhanced tumor necrosis, increased vascularity and diminished hypoxia with a reduction in tumor size [258]. In a parallel study, treatment of pancreatic stromal cells with Vitamin D markedly reduces markers of inflammation and fibrosis [259]. Interestingly, Vitamin D-treated fibroblasts get back to quiescent cell features, resulting in stromal remodeling that facilitates the delivery of chemotherapeutic agents within tumors [260]. Interestingly, a phase II clinical trial of concomitant treatment with PD-1 inhibitor and Vitamin D analog is now underway [261]

4.3. Targeting downstream effectors and activation signaling of CAFs

Because depletion of CAFs or reversing their activated phenotype remains challenging, other therapeutic options have been proposed, such as targeting CAF downstream effectors. As described above, the IL6/JAK/STAT3 signaling pathway increases proliferation, survival and metastasis of tumor cells, accompanied with a strong suppression of anti-tumor immune response [262]. IL6 also induces production of pro-inflammatory and pro-angiogenic factors, including IL-1 β , IL8 and multiple chemokines that act on both immune and non-immune cells. Moreover, IL6 activates the JAK/STAT3 pathway, which negatively regulates the cytotoxic activity of NK and T cells. Agents targeting this pathway already received FDA approval for the treatment of inflammatory pathologies and myeloproliferative neoplasms, and their efficacy was also approved in patients with hematopoietic malignancies and solid tumors, as they reduce tumor growth and increase sensitivity to chemotherapies [263, 264]. The mTOR/4E-BP1 regulatory pathway is up-regulated in primary cultures of SMA⁺ CAFs isolated from human PDAC [265]. These cells abundantly expressed the somatostatin receptor 1, which is known to mediate inactivation of mTOR/4E-BP1 pathway. Thus, treatment with the somatostatin analog SOM230 in a murine xenograft model of pancreatic cancer down-regulates CAF-secreted molecules, including IL6, and abrogates CAF-mediated tumor growth [265, 266]. Importantly, combination of SOM230 with gemcitabine, the standard chemotherapy for pancreatic cancer, reduces tumor growth and facilitates the delivery of chemotherapeutic agents within the tumor. These results highlight a novel promising anti-tumor therapy indirectly targeting pancreatic cancer cell invasion through pharmacological inhibition of stromal cells [265, 266]. Additionally, PDGF signaling has a

key role in the recruitment and phenotypic changes of fibroblasts upon activation [25, 27, 52, 87, 267]. Interestingly, inhibition of stromal PDGF receptors in a mouse model of human cervical cancer reduces proliferation and angiogenesis, through a mechanism involving the suppression of expression of the angiogenic factor fibroblast growth factor FGF-2 and epithelial cell growth factor FGF-7 [267, 268].

FAP⁺ CAF are a major source of CXCL12 secretion, which has a crucial role in immunosuppression and in resistance to anti-PD-1 and anti-CTLA-4 immunotherapies in pancreas, breast and ovarian cancer [20, 25, 27]. Moreover, administration of AMD3100, an inhibitor of CXCR4, the receptor of CXCL12, provides therapeutic interest in HER2 breast cancer patients [269]. Moreover, AMD3100 enhances T cell accumulation and acts synergistically with anti-PD-L1 antibody to diminish cancer progression in a pancreatic cancer mouse model [20]. Similarly, other proteins secreted by CAFs were also targeted in order to restrain their immunosuppressive capacities, such as TGF β . In many preclinical studies, TGF β -targeting agents have shown potent anti-tumor effects. For example, disruption of TGF β signaling using the TGF β receptor kinase inhibitor (LY2157299) or a TGF β neutralizing antibody (1D11) reduces the formation of bone metastasis from breast tumors [270, 271]. In addition, trihydroxyphenolic compounds were identified as potent blockers of TGF- β 1 in *in vivo* models of pulmonary fibrosis and lung cancer metastasis. Remarkably, trihydroxyphenolics functional effects require the presence of active lysyl oxidase-like 2 (LOXL2), only produced by fibroblasts and cancer cells. Administration of these compounds resulted in potent blockade of pathological collagen accumulation *in vivo* without toxicity [272]. In addition, the use of Tranilast (a known suppressor of fibroblast proliferation and TGF β secretion) in mice bearing E.G7 lymphoma, LLC1 Lewis lung cancer, or B16F1 melanoma decreases infiltration of immune suppressor cells, such as Tregs and MDSC, and activates CD8⁺ T lymphocytes [170]. Similarly, Artemisinin, identified as an inhibitor of TGF β signaling that reverts CAFs from an activated to an inactivated state, suppresses CAFs-induced breast cancer growth and metastasis by blocking the interaction between tumor cells and their microenvironment [273]. In addition, TGF β inhibitor is able to synergize with the anti-OX40 antibody to elicit a potent anti-tumor effect, associated with an overall accumulation of CD4⁺ and CD8⁺ effector T lymphocytes [274]. Beside the highly positive data observed in animal models, clinical trials with some agents targeting TGF β have been recently initiated [275-277], although TGF β can act as a tumor suppressor at early tumor stages and TGF β inhibitors are not cytotoxic [274].

4.4. Targeting CAF-derived ECM proteins

As mentioned earlier, fibroblasts activation results in ECM remodeling through production of several components, including collagen fibers and ECM-degrading proteases, such as MMPs. This remodeling increases tissue stiffness and matrix rigidity and serves as a physical barrier that inhibits the access of anti-tumor immune cells and impedes the delivery of therapeutic drugs [29, 30, 65, 70, 103, 104] . Thus, targeting the ECM proteins or degrading ECM could be a new therapeutic option. Hyaluronan, produced by CAFs, is considered as the primary matrix molecule responsible for vessel compression, in a collagen-dependent manner [278, 279] . In this sense, the angiotensin inhibitor losartan, reduces stromal collagen and hyaluronan production and decreases the expression of profibrotic signals (TGF β 1, CCN2) [280]. Consequently, losartan increases vascular perfusion and improves drug and oxygen delivery to tumors, thereby potentiating chemotherapy in breast and pancreatic cancer models. Similarly, an enzymatic depletion of hyaluronic acid using a clinically recombinant PH20 hyaluronidase (PEGPH20) facilitates the intra-tumoral penetration of standard chemotherapeutic agents [281, 282]. This component, in combination with gemcitabine shows therapeutic benefit in patients with advanced pancreatic cancer [283]. In addition, as pancreatic adenocarcinoma is poorly vascularized, the administration of IPI-926, a specific inhibitor of Hedgehog signaling pathway, reduces tumor-associated stromal tissue, enhances intra-tumor vascular density and increases concentration of gemcitabine [284-286]. Furthermore, CAF-mediated ECM remodeling cannot be achieved without the production of MMPs that influence ECM degradation and facilitate cell migration. Despite promising preclinical data supporting the blockage of MMPs as a treatment for cancer, phase III clinical trials failed [287-290]. This was due principally to inadequate clinical trial design and poor knowledge of the complexity of the MMPs. Still, better and more specific inhibitors have been developed but are not yet ready for clinical use [291, 292].

Collectively, drugs that target stromal CAFs have emerged as an important option to improve anti-cancer therapies and drug resistance. However, the functional complexity and heterogeneity of CAF sub-populations within the same tumor might be taken into consideration to avoid off-target side effects.

5- Conclusion

It is nowadays commonly accepted that CAF are one of the major components of tumors that mainly support different steps of cancer progression. However, the precise definition of CAF

heterogeneity and CAF subpopulations, with the complex identification of specific markers and specific CAF subpopulation functions, remain the most challenging aspects in the study of CAFs. Recently, rapid advances have been made in the molecular characterization of CAFs. This provides a better understanding on the mechanisms underlying CAF subsets-mediated tumor promotion and immunosuppression. Subsequently, these recent knowledges have facilitated the development of novel therapeutic strategies for cancer treatment. On the whole, the aforementioned anti-CAF therapies are designed to target immunosuppressive CAFs functions, which promote cancer development. Nevertheless, other CAFs subtypes have also been recently identified, which display tumor-suppressive capacities and can hold therapeutic potential.

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Declaration of interest

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Legends of figures

Figure 1: Schematic representation of CAF heterogeneity

CAFs represent a heterogeneous cellular population within the tumor microenvironment. By combining the analysis of six CAF markers, four different CAFs populations (referred as CAF-S1 to CAF-S4) were discovered recently in breast and ovarian cancers. While CAF-S1 present an immunosuppressive function, CAF-S4 promote metastasis formation. Later on, single cell analysis allows the identification of 8 different clusters within the CAF-S1 subset with 3 clusters belonging to the iCAF subgroup and 5 clusters to the myCAF subgroup. Both ecm-myCAF and TGF β -myCAF exhibit an immunosuppressive function and are associated with resistance to immunotherapy.

Figure 2: CAFs-mediated immunosuppression

Due to their secretion of cytokines, chemokines, or other soluble factors, CAFs shape the immune response within the tumors toward a pro-tumorigenic microenvironment, by affecting both innate and adaptive immune cells

Figure 3: Principal strategies for CAF-directed anticancer therapy:

Multiple approaches have been developed to target CAFs for cancer treatment. CAFs can be directly depleted by targeting CAFs surface markers (FAP, GPR77). This depletion is mediated by either transgenic technologies or CAR-T cell therapies. Moreover, blocking secreted cytokines and specific effectors of CAFs (IL-6, TGF-b, CXCL12) or other growth factor pathways (PDGFR, mTOR) can be also used to inhibit the activation and function of CAFs.

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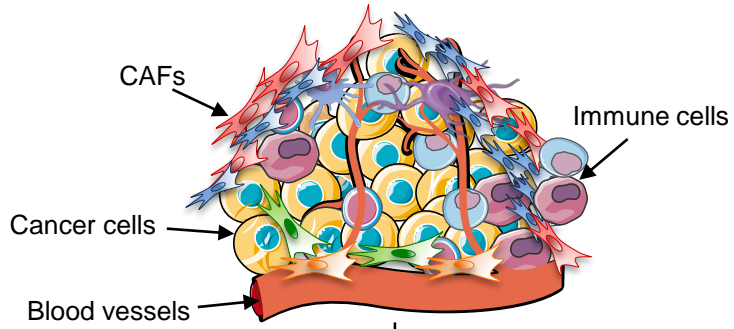
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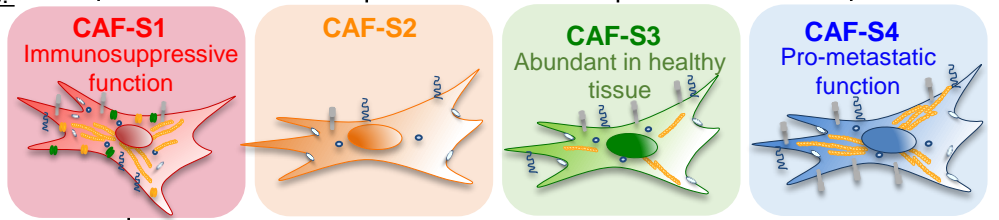
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Tumor microenvironment



CAF markers:

- CD29
- FAP
- FSP1
- α SMA
- PDGFR β
- CAV1



Myofibroblastic "myCAF"



Inflammatory "iCAF"



Immunosuppressive function and resistance to immunotherapy

