Manuscript title: *Targeting cancer-associated fibroblasts: challenges, opportunities and future directions*

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Abstract

Cancer associated fibroblast (CAF) are a common cell in the tumour microenvironment with diverse tumour-promoting functions. Their presence in tumours is commonly associated with poor prognosis making them attractive therapeutic targets, particularly in the context of immunotherapy where CAF have been shown to promote resistance to checkpoint blockade. Previous attempts to inhibit CAF clinically have not been successful however, in part due to a lack of understanding of CAF heterogeneity and function, with some fibroblast populations potentially being tumour suppressive. Recent single cell transcriptomic studies have advanced our understanding of fibroblast phenotypes in normal tissues and cancers, allowing for a more precise characterisation of CAF subsets and providing opportunities to develop new therapies. Here we review recent advances in the field, focusing on the evolving area of therapeutic CAF targeting.

Keywords:

Tumour Microenvironment, Fibroblast, Myofibroblast, Cancer Associated Fibroblast, Stromal Targeting, Immunotherapy,

Abbreviations:

αSMA, alpha Smooth Muscle Actin; apCAF, Antigen Presenting CAF; ATRA, All-trans Retinoic Acid; CAF, Cancer Associated Fibroblast; CAR-T, Chimeric Antigen Receptor Expressing T cell; CCL, C-C Motif Chemokine Ligand; CTLA-4, Cytotoxic T-Lymphocyte Associated Protein 4; CXCL, C-X-C Motif Chemokine Ligand; CXCR, C-X-C Motif Chemokine Receptor; ECM, Extracellular Matrix; EMT, Epithelial-Mesenchymal Transition; FAP, Fibroblast Activation Protein; HA, Hyaluronic Acid; iCAF, Inflammatory CAF; IL, Interleukin; JAK, Janus Kinase; LIF, Leukaemia Inhibitory Factor; LOXL, Lysyl Oxidase-Like; LPA, Lysophosphatidic Acid; LPAR, Lysophosphatidic Acid Receptor; MDSC, Myeloid Derived Suppressor Cell; MHC, Major Histocompatibility Complex; myoCAF, Myofibroblastic CAF; NOX, NADPH Oxidase; PD-1, Programmed Cell Death Protein 1; PDAC, Pancreatic Ductal Adenocarcinoma; PDGF, Platelet Derived Growth Factor; PD-L1, Programmed Death Ligand 1; PSC, Pancreatic Stellate Cell; ROS, Reactive Oxygen Species; scRNA-Seq, Single-cell RNA Sequencing; Shh, Sonic Hedgehog; SMAD, Suppressor of Mothers against Decapentaplegic; STAT,

Signal Transducer and Activator of Transcription; TGF-β, Transforming Growth Factor beta; TME, Tumour Microenvironment; TNF-α, Tumour Necrosis Factor alpha; VDR, Vitamin D Receptor.

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1. INTRODUCTION

The importance of the interaction between cells of the tumour microenvironment (TME) and cancer was first identified in the early 20th century (Bashford, 1909) and since then, much effort has been put into deciphering the two-way communications between cancerous and surrounding cells during the process of tumour evolution (Calvo & Sahai, 2011; Chiodoni et al., 2019; Dominiak et al., 2020; Trosko, 1998). As cancer develops, the TME is constantly evolving, shaped through multiple mechanisms of two-way communication between cancer cells and the stromal compartment, to transition from a relatively tumour-suppressive environment towards a pro-tumourigenic network (Bremnes et al., 2011). One of the main driving forces behind this is the activation of cancer-associated fibroblasts (CAFs), which are one of the most abundant components of tumour stroma.

CAFs are now recognised as a heterogeneous and functionally diverse population of cells, and it is difficult to define a CAF succinctly. Previously, these cells have been characterised based on their elongated spindle shape, location within the tumour microenvironment (TME), and expression of α-smooth muscle actin (αSMA). Expression of other markers, including fibroblast activation protein (FAP), fibroblast-specific protein-1 (FSP1; also known as S100A4), platelet-derived growth factor receptor α or β (PDGFRα/β) and podoplanin (PDPN) have also been used as CAF markers yet, as with αSMA, none are CAF-specific and not all are regulated similarly or simultaneously by CAF. Even with recent advances in single-cell RNA sequencing (scRNA-Seq), no unique and conserved CAF marker has been identified. A recent consensus statement suggested the following criteria to define CAFs, whilst conceding this is not definitive: CAFs are cells located near a tumour, with spindle-like morphology, no cancer cell mutations, and are negative for epithelial, endothelial and leukocyte markers but positive for mesenchymal markers such as vimentin (Sahai et al., 2020). Recent studies have suggested several other markers which may classify CAFs and their subpopulations, but these require further validation in a wider range of human cancers. See reviews by (X. Chen & Song, 2019; Nurmik et al., 2020) for a more detailed description.

2. FIBROBLAST HETEROGENEITY

The study of normal fibroblast and CAF heterogeneity is a rapidly expanding area of research. In healthy tissue, resident fibroblasts are considered to exist in a quiescent, resting state showing minimal metabolic and transcriptomic activity

(Kalluri, 2016). While quiescent fibroblasts presumably function to maintain ECM homeostasis, there is no conclusive evidence of this activity (LeBleu and Neilson, 2020). Indeed, fibroblasts are considered to lay dormant within interstitial tissues, until their definitive function emerges as a consequence of activation by a plethora of stimuli. As postulated by Kalluri, it may be more appropriate to consider non-activated fibroblasts as tissue-resident mesenchymal stem cell precursors due to the shared activation responses to various ligands such as transforming growth factor-β (TGF-β), interleukin-6 (IL-6), platelet-derived growth factor (PDGF) (Öhlund, Elyada and Tuveson, 2014). Thus, our understanding of 'normal' fibroblasts is incomplete and much of the fibroblast literature likely reflects the study of activated counterparts.

Fibroblasts that fulfil specialised functions have been described in a number of tissues, including lung (Ramos et al., 2001), heart (Camelliti et al., 2005), and muscle (Kuhl et al., 1984). While normal fibroblasts are considered cells of mesenchymal origin (Kalluri et al., 2006), evidence from fate mapping studies in murine models indicates that they can arise from mesenchymal and non-mesenchymal sources (*e.g.* neural crest; Roulis et al., 2016; Driskell et al., 2015), and relative contribution of each source is likely to be dependent on anatomical site. For example, fibroblasts in ventral body skin have been shown to arise from mesoderm, whereas those of facial skin originate from the neural crest (Driskell et al., 2011). Lineage tracing experiments have shown that the fibroblasts of murine skin arise from two different lineages; one forms the upper dermis, including the dermal papilla that regulates hair growth; the other forms the lower dermis, including the reticular fibroblasts that synthesise the bulk of the fibrillar ECM (Driskell et al., 2013).

An increasing wealth of single-cell transcriptomic data has highlighted fibroblast heterogeneity in a variety of disease states and organs. However, the bias toward the analysis of fibroblasts in pathological conditions rather than healthy tissues has meant our understanding of normal fibroblasts (pre-activation) remains comparatively unclear. Recently, Buechler *et al* constructed an integrated fibroblast single-cell transcriptomic atlas, examining fibroblast subsets in healthy and perturbed states across multiple tissues and diseases. In murine tissues, the authors identified two universal fibroblast subsets found in nearly all tissues (identified by dominant cluster-specific genes Pi16+ and Col15a1+ respectively). Differentially expressed genes in the Pi16+ cluster (Pi16, Dpp4 and Ly6c1) suggested a similarity

to adventitial stromal cells found in vascular niches, whereas the Col15a1+ cluster exhibited an association with basement membrane (expression of Col4a1, Hspg2 and Col15a1). Both subgroups expressed dermatopontin (Dpt) which was highest in Pi16+ fibroblasts, and inversely correlated with fibroblast specialisation. Elevated levels of stemness-associated genes (Cd34 and Ly6a) and lineage inference analysis suggested that the Pi16+ subtype potentially serves as a resource cell that can differentiate into specialised fibroblasts, and function as a reservoir population giving rise to 'activated' fibroblast subsets in disease. However, anatomical site-specific fibroblasts were also identified in a number of sites, including bone, lung, liver, lymph nodes, spleen and intestine, and it appears that different tissues contain common fibroblast phenotypes but, in certain instances, functionally-specialised populations (Buechler et al., 2021). Analysis of human tissues showed concordance with murine fibroblast phenotypes, with conserved fibroblast phenotypes across cancers, fibrotic and inflammatory diseases.

3. CANCER ASSOCIATED FIBROBLASTS

The CAF literature has also been confounded by a lack of universal nomenclature. CAFs have been variably called peritumour fibroblasts, tumour-associated fibroblasts, myofibroblasts and tumour/cancer-associated stromal cells, with these terms used interchangeably (Bussard et al., 2016). In recent years the term CAF has become more widely accepted, the challenge now is to define CAF subgroups more accurately and characterise commonalities and differences between tumour types. The accurate characterisation of CAF phenotypes and functions will be critical to developing therapeutic strategies to precisely target these accurately.

MYOFIBROBLASTS

Historically, the term CAF has mostly been used to describe cells that resemble wound healing myofibroblasts; contractile cells that express αSMA and secrete collagenous extracellular matrix (ECM). The origins of myofibroblasts/myofibroblastic CAF (myoCAFs) has been contested; resident quiescent fibroblasts, pericytes, mesenchymal stem cells, endothelial cells (endothelial-to-mesenchymal transition) and epithelial cells (epithelial-to-mesenchymal transition) can all undergo myofibroblastic differentiation and provide potential origins of myofibroblasts/myoCAFs (Potenta, Zeisberg and Kalluri, 2008; Kalluri and Weinberg, 2009; LeBleu and Kalluri, 2018; Figure 1). However, despite advances in cell lineage tracing and fluorescent tagging techniques, the lack of fibroblast-

specific markers means determining CAF origin remains problematic (Sahai et al., 2020). Currently, the majority of myoCAF are believed to originate from local fibroblast populations (Sahai et al., 2020), yet, it is clear that myoCAF can differentiate from other cell types, and the relative contribution of these other cells to myoCAF in tumours may be organ dependent (Humphreys et al., 2010; Kisseleva, 2017; Yavuz et al., 2020). It will be intriguing to determine if different progenitors develop the same myoCAF phenotype.

In healing wounds myofibroblasts facilitate wound contracture and collagen deposition (Darby et al., 2014). myoCAF share these features, as well as common activation mechanisms through TGF-β signalling, oxidative stress and mechanotransduction (Zent and Guo, 2018). Expression of collagen-1, fibronectin EDA (FN-EDA) and periostin reflect the ECM-depositing role of these cells; this, along with cell contractility, positively reinforces myofibroblast differentiation through increased tissue tension and integrin-mediated TGF-β activation (Hinz, 2016; Zent and Guo, 2018). Myofibroblasts ultimately disappear from a resolved wound, either through apoptosis, senescence or reversion to a quiescent fibroblast phenotype (Desmoulière et al., 1995; Kalluri, 2016; Hinz and Lagares, 2020); however, this reversibility is poorly understood. Nonetheless, in cancers myoCAFs persist, facilitated in part by epigenetic reprogramming and self-perpetuating activation, and possess many tumour-promoting functions (Hu et al., 2005; Franco et al., 2010; Albrengues et al., 2016).

CAF SUBTYPES AND PLASTICITY – MYOCAF, ICAF AND APCAF

Fibroblasts in wound healing, fibrotic diseases, and inflammatory conditions share overlapping features of distinct phenotypes that mediate these processes, and these can also be found in tumours (reviewed by Biffi and Tuveson, 2021). Whilst aberrant myofibroblast activation and persistence underlies fibrotic pathologies, including pulmonary, heart and kidney fibrosis (Phan, 2002; Desmoulière, Darby and Gabbiani, 2003; Hinz, 2007), other fibroblast states are found in inflammatory disorders. For example, rheumatoid arthritis and osteoarthritis contain inflammatory, immune cell-recruiting fibroblasts that express IL-6 and CXCL12 (Mizoguchi et al., 2018; Croft et al., 2019), and deletion of FAP+ fibroblasts has been shown to attenuate joint damage and inflammatory bone remodelling in mouse models of arthritis, highlighting the significant role of fibroblast subsets in mediating the inflammatory disease (Croft et al., 2019). Recent studies have also highlighted the presence of inflammatory CAF populations (iCAF) and antigen-presenting CAF

(apCAF) in several tumour types. This supports the concept that through the CAF spectrum, fibroblasts function to initiate, support and ultimately suppress inflammation, and have many immunomodulatory functions (Figure 2).

Öhlund and colleagues first described the existence of two functionally distinct CAF populations in pancreatic cancer (PDAC) using *in vitro* coculture models (Öhlund et al. 2017). They also found that the two CAF populations were spatially distinct; αSMA-high myoCAFs were found directly adjacent to tumour cells, whilst αSMA-low CAF were situated more distant to the neoplastic cells, and expressed inflammatory markers such as IL-6, LIF and CXCL12; these were consequently termed iCAFs. Subsequent scRNA-Seq studies have confirmed the presence of iCAFs in PDAC *in vivo*, as well as other tumour types, including breast (Kieffer et al., 2020) and lung cancer (Lambrechts et al., 2018). Notably, these iCAF populations share multiple markers with the PI16+ universal (adventitial) fibroblast subpopulation identified by Buechler *et al* under steady-state conditions (*e.g. C3*, *EFEMP1*, *HAS1*, *TNXB*, *CXCL12*, *OGN*, *PTX3*, *GSN*, *TNFAIP6*). To characterise the role of iCAF in the tumour microenvironment further it will be important to understand what features differentiate these cells from their steady-state counterparts (PI16+ adventitial fibroblasts). Importantly, myoCAF and iCAF are now recognised to be plastic cell populations rather than fixed states of terminal differentiation; these phenotypes are regulated by TGF-β and IL-1/JAK/STAT signalling respectively and are interconvertible depending on the biochemical and mechanical features of the *in vitro* culture environment, demonstrating the potential to manipulate these phenotypes therapeutically (Biffi et al., 2019; Figure 3).

CAFs have also been shown to express antigen presentation machinery (*e.g. CD74* and HLAs corresponding to MHC class II). Elyada *et al* first identified apCAFs as a distinct cluster in CAFs isolated from the KPC murine model of PDAC, and then found human CAFs with similar gene expression (Elyada et al., 2019). These cells were characterised by upregulation of MHC-II and CD74 but lacked the classical costimulatory molecules found on professional antigen-presenting cells. A subsequent study identified a discrete cluster expressing these apCAF markers in breast cancer (Kieffer et al., 2020) with *CCL19* also identified as the most significant marker of these cells. This gene expression profile is also consistent with fibroblast reticular cells (FRCs) associated with lymph nodes from steady state conditions in murine tissues (Buechler et al.) and head and neck cancer metastases (Puram et al.2018). The signalling pathways

regulating apCAF have yet to be identified, and it is not clear whether this phenotype is part of the plasticity spectrum that includes myCAF and iCAF.

Given that fibroblast plasticity is essential for our bodies to react to variable injury scenarios, classifying cells by their function may ultimately be preferable to using molecular markers, similar to the conclusion reached by researchers in the macrophage field (Murray et al., 2014). The recognition that CAF phenotypes are not fixed states of terminal differentiation opens up therapeutic opportunities to reprogram CAF from a tumour-promoting subtype towards a phenotype that could possibly function to suppress tumour progression or facilitate treatment efficacy (Öhlund et al., 2017; Biffi et al., 2019; Biffi and Tuveson, 2021), but currently more research is required to determine the functional roles of the newer CAF population identified in recent years.

MYOCAF – PROMOTING TUMOUR PROGRESSION AND THERAPY RESISTANCE

Pan-cancer analyses of bulk transcriptomic and single-cell data have shown that myoCAF are commonly found in most types of solid cancer (Qian et al., 2020; Dominguez et al., 2020; Beuchlar et al., 2021). MyoCAF accumulation in the tumour stroma is associated with poor prognosis in many cancer types (Hanley et al., 2018; Underwood et al., 2015), and consistent with this, myoCAF support many of the hallmarks of malignancy; including promoting tumour proliferation, invasion and metastasis; suppressing apoptosis and anti-tumour immunity (Sahai et al., 2020). Notably, and in contrast to most cancer types, studies in pancreatic cancer have shown that αSMA-positive CAF may have important tumour-restraining roles (Lee et al., 2014; Rhim et al., 2014; Özdemir et al., 2014). It is possible that αSMA-positive myoCAF derived from pancreatic stellate cells have suppressive, perhaps organ-specific functions that fibroblast-derived myoCAF lack but is also worth noting that αSMA is expressed in other cell types as well as other CAF subtypes i.e., it is not myoCAF specific.

Most CAF studies historically have focused on myoCAF. In part, this is because culturing fibroblasts on ‘stiff’ tissue culture plastic tends inadvertently to promote myofibroblast differentiation (Wang et al., 2012). Stellate cells for example remain quiescent when cultured on physiologically relevant soft matrices, yet are activated when cultured on stiff matrices, undergoing myofibroblast differentiation (Wells, 2008). Öhlund *et al* demonstrated that iCAF

phenotypes were lost (downregulation of IL-6. IL-11, LIF) and myoCAF markers upregulated when plating cells in monolayers rather than in soft Matrigel, further highlighting the importance of culture substrate for maintaining CAF phenotypes. Similarly, 2D culture of lung fibroblasts on tissue culture plastic has been found to upregulate genes associated with myofibroblast differentiation (ACTA2, COL1A1, COL3A1), whereas culture in 3D collagen-Matrigel® gels resulted in upregulation of IL6, a marker of inflammatory fibroblasts. Notably, 3D culture was not sufficient to recover *ex vivo* gene expression, emphasising the need to optimise conditions to accurately study fibroblast phenotypes *in vitro* (Waise et al., 2018).

Generally, it appears that many myoCAF-rich tumours are 'immune cold' and contain low levels of T-cells, and particularly are associated with T-cell exclusion. Chakravarthy and colleagues developed MethylCIBERSORT – a cell deconvolution tool based on methylation profiles – identifying a significant inverse correlation between myoCAF-and cytotoxic T-cells in head and neck cancer (Chakravarthy et al., 2018b). Pan-cancer analyses have also linked TGF-β1-associated ECM myoCAF genes with immunosuppression (Chakravarthy et al., 2018a). Studies have shown that myoCAF modulate immune evasion through numerous mechanisms, including secretion/activation of TGF-β1, which has suppressive effects on multiple immune cell subsets, including inhibiting CD8+ T-cell proliferation and cytotoxicity (reviewed by Li and Flavell, 2008). Perhaps this general dampening of the immune response reflects the role of myofibroblasts in the later stage of wound healing when the inflammatory phase is switched off. It is worth noting, however, that many myoCAF rich tumours show T-cell exclusion rather than a complete absence of T-cells. This suggests an active anti-tumour immune response, albeit with T-cells unable to access the tumour compartment effectively, raising the possibility that overcoming myoCAF-mediated T-cell exclusion could effectively potentiate immunotherapy response.

The use of transcriptomic tissue analyses in clinical trials has highlighted the role of myoCAF in suppressing treatment response, particularly resistance to checkpoint immunotherapy (Mariathasan et al., 2018; Kieffer et al., 2020; Dominguez et al., 2020; Chakravarthy et al., 2018a). Mariathasan and colleagues analysed a cohort of anti-PDL1-treated patients with metastatic bladder cancer and found that lack of response was associated with a signature of TGF-β signalling in fibroblasts, which occurred particularly in patients with tumours that showed exclusion of CD8+ T-

cells from the tumour parenchyma (Mariathasan et al., 2018). Preclinical models that recapitulate the αSMA-positive, myoCAF-rich stroma found in human tumours have also re-produced this T-cell exclusion effect. Ford *et al* generated myoCAF-rich tumour models by co-injecting tumour cells with either TGF-β-treated (myo)fibroblasts or CAF expanded *ex vivo* and found that myoCAF specifically excluded CD8 T-cells from tumours; in turn this promoted resistance to anti-tumour vaccination and anti-PD1 therapy (Ford et al., 2020). MyoCAF were also shown to increase macrophage infiltration into tumours but intriguingly did not affect intratumoural CD4 T-cell numbers (Ford et al., 2020). Subsequent studies have drawn similar conclusions; Pan-cancer gene analysis has shown that TGF-β1-associated ECM myoCAF genes predict non-response to anti-PD1/PDL1 treatment, (Chakravarthy et al., 2018a). More recent single-cell transcriptomic studies have also described the relationship between myoCAF and immunotherapy resistance; Dominguez and colleagues analysed murine and human tumours using single-cell transcriptomics and identified a population of TGF-β-dependent CAF that surrounded tumour islands and expressed LRRC15 (Dominguez et al., 2020). Further analysis of immunotherapy trials across 6 cancer types showed that elevated levels of LRRC15 fibroblasts were associated with poor response to anti-PDL1 therapy (Dominguez et al., 2020).

In a recent study, Bagaev *et al* performed a detailed transcriptomic analysis of >10,000 cancer patients and identified four distinct tumour microenvironment subgroups that were conserved across 20 different types of cancer. These were termed fibrotic, immune-depleted, immune-enriched non-fibrotic and immune-enriched fibrotic. Fibrotic and immune-depleted tumours lacked lymphocyte infiltration, whereas immune enriched non-fibrotic and immune enriched fibrotic contained abundant lymphocytes (Bagaev et al., 2021). Fibrotic tumours were characterised by upregulation of typical myoCAF genes and prominent collagen deposition and were negatively correlated with survival across cancer types. These TME subgroups also predicted response to immunotherapy; analysis of two cohorts of melanoma patients treated with anti-CTLA4 therapy, revealed a response rate of 86% in the immune-enriched subgroup compared with only 10% in the fibrotic group. Similar findings were observed in three melanoma cohorts treated with anti-PD1 (response rates of 75% and 10% respectively). In PDL1-treated patients with bladder cancer, over 38% of bladder cancer patients with immune-enriched TME subtype were responders (with PFS >6 months). In contrast, the immunosuppressive fibrotic subtype bladder cancer patients had significantly lower response rates (less than 10%, with PFS less than 6 months; Bagaev et al., 2021).

It is intriguing that the study by Bagaev *et al* identified an immune-enriched fibrotic subgroup, suggesting that some CAF phenotypes may promote an anti-tumour immune response (or at least not be actively suppressive; Bagaev et al., 2021). Kieffer et al. (2020) performed scRNA-Seq on flow-sorted FAPhi/CD29med-hi (CAF-S1) fibroblasts from breast cancers that had previously been shown to be associated with immunosuppression (Costa et al., 2018). They identified eight CAF-S1 subclusters; of these subclusters, myoCAF populations (ecm-myoCAF and TGF-β-myoCAF) were associated with an immunosuppressive environment, low CD8+ T-cells and poor response to immunotherapy. Conversely, two iCAF populations (detox-iCAF and IL-iCAF) were found to correlate positively with CD8+ T-cell infiltration and cytotoxicity. Given the plasticity of the CAF population and the spectrum of fibroblast function through inflammation to fibrosis, it is possible that immune-promoting and immune-suppressing iCAF subgroups exist; certainly, iCAF can express chemokines associated with immune suppression, including CXCL12 and IL-6 (Öhlund et al., 2017; Bernard et al., 2019), and further work is required to define these roles precisely. For example, a recent study by Nicolas and colleagues showed that IL-1-dependent signalling in iCAF elevates oxidative DNA damage, resulting in cell senescence and therapy resistance following irradiation (Nicolson et al., 2022). Therefore, simply skewing a CAF from a myoCAF to an iCAF phenotype may inadvertently promote tumour progression through different mechanisms in certain circumstances. The literature is confounded by the fact that many previous studies examining fibroblast immune function have not characterised the CAF subtype in any detail, and future research will need to incorporate detailed fibroblast phenotyping.

RECAPITULATING A CAF-RICH TUMOUR MICROENVIRONMENT IN MURINE TUMOUR MODELS

The recent explosion in interest for CAF targeting has resulted primarily from the findings that myoCAF -rich tumours are commonly resistant to anti-PD1/PDL1 checkpoint blockade (Ford et al., 2020; Mariathasan et al., 2018), suggesting that targeting myoCAF could potentiate immunotherapy. It is perhaps unsurprising that the role of CAF in promoting immunotherapy resistance was not identified sooner, given that subcutaneous syngeneic murine tumour models typically used for preclinical immunotherapy testing (e.g., TC1-lung; MC38, CT26 – colorectal; 4T1 – breast; B16F10 – melanoma; RENCA – renal) contain low levels of myoCAF, unlike their human equivalents. Ford *et al* analysed TC1, MC38 and 4T1 tumours, and found a typical myoCAF content of < 5% (tumour area), compared with 20-30% in human

myoCAF rich tumours (Ford et al., 2020). Co-injecting tumour cells with murine fibroblasts (either TGF-β-treated primary fibroblasts or myoCAF cultured *ex vivo)* produce a similar stromal appearance to that of human tumours and produced similar changes in the tumour immune microenvironment to those observed in human tumours; excluded CD8 T-cells and increased macrophage numbers. Similar approaches could be used for iCAF and apCAF once understanding of how to maintain these phenotypes in culture improves. Several syngeneic models develop an endogenous myoCAF stroma; the MCa-M3C murine breast cancer cell line derived from an MMTV-PyVT spontaneous mouse model develops an endogenous stroma that recapitulates the fibrosis seen in human tumours when implanted orthotopically (I. X. Chen et al., 2019). Pancreatic cancer cell lines derived from the KPC mouse also retain a variable degree of desmoplasia when injected either subcutaneously or orthotopically and contain a high abundance of immunosuppressive macrophages and a relative lack of T-cell infiltration (Pham et al., 2021).

4. CAF TARGETING STRATEGIES

In recent years there has been a resurgence in interest in targeting CAF therapeutically, driven chiefly by the association of myoCAF with checkpoint immunotherapy resistance. Different strategies are being investigated including: i) suppressing CAF formation; ii) Reprogramming CAF; iii) depleting CAF; iv) targeting CAF functions (Figure 4).

REPROGRAMMING CAF

With recent studies indicating that CAFs show phenotypic plasticity and that some CAF phenotypes may be tumour suppressive, there is excitement in the idea of CAF reprogramming –reverting myoCAF to a quiescent or even immune-permissive phenotype. Clearly a greater understanding of how different CAF subgroups function is required for this approach to be effective.

TGF-β inhibition

TGF-β signalling is the main pathway regulating myoCAF activation (Pohlers et al., 2009); myoCAFs also promote secretion and activation of TGF-β, which has numerous immunosuppressive effects, including inhibiting CD8+ T-cell proliferation and cytotoxicity (Li and Flavell, Cell 2008). Targeting myoCAF through inhibiting TGF-β signalling would

therefore seem a logical approach, and a number of different strategies have been developed to target the pathway, including monoclonal neutralizing antibodies directed against the TGF-β ligand and its receptor; bifunctional antibodies, such as dual-targeting anti-TGF-β/programmed death-ligand 1 (PD-L1) antibodies; antisense oligonucleotides; TGF-β-related vaccines; and receptor kinase inhibitors (reviewed by Ciardiello et al., 2020), and there are numerous ongoing clinical studies testing TGF-β targeting agents in combination with anti-PD1/PDL1 checkpoint blockade (reviewed by Kim et al., 2021). Mariathasan and colleagues found that lack of response to anti-PDL1 in patients with metastatic urothelial cancer was associated with a CAF TGF-β signalling gene signature and with CD8+ T-cell tumour exclusion. They further showed that in preclinical murine tumour models (MC36, EMT6) that co-administration of TGF-β-blocking antibodies with anti-PDL1 antibodies improved treatment response and improved intratumoural T-cell infiltration (Mariathasan et al., 2018). Tauriello *et al* generated mice bearing four of the key mutations in human colorectal cancer (Apc, Kras, Trp53, Tgfbr2) and found that these developed CAF-rich, T-cell-excluded tumours that were metastatic and resistant to anti-PD-L1 therapy (Tauriello et al., 2018). Combining anti-PD-L1 therapy with a TGF-β receptor inhibitor (Galunisertib) eradicated most metastases and prolonged recurrence-free survival for over a year following cessation of treatment (Tuariello et al., 2018).

While inhibiting myoCAF differentiation is potentially useful, in an established tumour, myoCAF differentiation has already taken place, and it is not clear how targeting TGF-β affects the established phenotype. Ford *et al* found that inhibiting TGF-β signalling in myoCAF *in vitro* does not reverse myoCAF differentiation. Although TGF-β inhibition was found to improve the efficacy of anti-PD1 immunotherapy *in vivo*, this occurred in both CAF-low and CAF-high tumours (TC1, MC38), suggesting that the potentiating effect was largely independent of myoCAF (Ford et al., 2020). Grauel and colleagues showed that treatment of mice bearing orthotopic 4T1 breast tumours with TGF-β1-neutralising antibodies generated a novel population of Nt5e+ 'interferon-licensed' CAF (ilCAF); these were not present in control tumours, distinct from iCAF and with superior immunomodulatory properties, resulting in tumours with greater infiltration and activation of CD8+ T-cells (Grauel et al., 2020). Whether ilCAFs arise from pre-existing myoCAF or emerge from an alternative trajectory of progenitor cells in the absence of TGF-β signalling is unclear, but given that mice were treated with anti-TGF-β antibodies at an early stage of tumour development (2 days post-injection) and throughout tumour growth (treatment days 2-12; endpoint analysis day 14), the latter seems more likely. Intriguingly,

iCAF populations remained relatively unchanged following TGF-β-blockade (Grauel et al., 2020). A clearer understanding of the dynamics of TGF-β inhibition on CAF phenotypes is needed.

NOX4 inhibition

Systemic targeting of TGF-β signalling also carries significant risk; TGF-β has both tumour-promoting and tumour-suppressive functions and also plays an important role in tissue homeostasis. Broad targeting of the pathway has resulted in cardiac toxicity and the development of cutaneous tumours (de Gramont et al., 2016; Lacouture et al., 2015). Intracellular reactive oxygen species (ROS) play an important role in myofibroblast differentiation, and the ROS-producing enzyme, NADPH oxidase-4 (NOX4), has been shown to be most accountable for ROS-induced fibroblast activation in fibrosis and cancer (Barnes and Gorin 2011; Sampson et al., 2011; Hanley et al., 2018). As a downstream target of TGF-β, NOX4 mediates myofibroblast differentiation through a delayed phase of intracellular reactive oxygen species generation. Inhibition of NOX4 abrogates TGF-β-dependent ROS production and myoCAF activation (Hanley et al., 2018). Notably, myoCAF cultured *ex vivo* continue to express high levels of NOX4 and ROS, and inhibition of the enzyme significantly reduces the expression of myoCAF genes, suggesting a more 'normal-like' fibroblast phenotype (Hanley et al., 2018; Ford et al., 2020), further evidence of CAF plasticity. Ford *et al* used myoCAF-rich murine tumour models (TC1, MC38, 4T1) to evaluate the potential of targeting NOX4 to overcome immunotherapy resistance. The study showed that the NOX1/4 inhibitor, GKT137831 (setanaxib), suppresses/reverses myoCAF differentiation in tumours, promotes intratumoural infiltration of CD8+ T-cells and overcomes myoCAF-mediated resistance to anti-tumour vaccination and anti-PD1 therapies (Ford et al., 2020). The first cancer clinical trial testing setanaxib in combination with anti-PD1 in relapsed/metastatic head and neck cancer opens in 2022 (EudraCT number: 2021-004627-3).

Vitamins A and D

One of the first reports of tumour stromal reprogramming came from the use of all-trans retinoic acid (ATRA), a vitamin A metabolite, in pancreatic cancer *in vivo/in vitro* models (Froeling et al., 2011). Observations that vitamin A deficiency in PDAC patients leads to PSC activation led to the investigation of ATRA as an agent to replenish physiological retinol and potentially inactivate pancreatic stellate cells (PSCs; Froeling et al., 2011), which are the major cell type

differentiating into myoCAF and initiating desmoplasia in PDAC (Vonlaufen et al., 2008; Tian et al., 2019). Froeling and colleagues showed that ATRA induced quiescence in PSCs along with broad transcriptional alterations and reduced motility. Strikingly, quiescent PSCs reduced Wnt-β-catenin signalling in cancer cells through the expression of secreted frizzled-related protein 4 (SFRP4), suppressing proliferation and invasion, and increasing apoptosis of PDAC cells, demonstrating the potential for indirectly targeting malignant cells through modulating tumour-stroma crosstalk. Notably, ATRA administration and consequent PSC quiescence have been shown to result in increased intratumoural CD8+ T-cell and improved survival (Ene-Obong et al., 2013). ATRA has also been tested in combination with gemcitabine in 3D organotypic culture and mouse models of PDAC (Carapuça et al., 2016). ATRA treated PSCs had significantly lower fibronectin and collagen I deposition indicative of stromal remodelling while increasing vascularity – an exciting finding in the context of stromal normalisation. The combination of ATRA and gemcitabine-nab-paclitaxel in pancreatic cancer has undergone successful phase 1 clinical testing, and a phase 2b trial is underway (Kocher et al., 2020; NCT04241276). Whether this approach could work in other cancers where the source of myoCAF are not PSCs remains to be seen.

Similar to vitamin A, vitamin D receptor (VDR) ligands have also been shown to play a role in regulating the balance between quiescence and activation of stellate cells (Ding et al., 2013). This work uncovered the interplay between VDR and TGF-β/SMAD signalling mechanisms regulating fibrogenic gene expression and demonstrated the therapeutic potential of synthetic superagonist derivatives of calcitriol such as calcipotriol and inecalcitol (which display up to 100-200-fold less hypercalcemic activity). In the context of the effect on CAF phenotype, VDR is thought to act as a negative regulator of TGF-β signalling (Ding et al., 2013; Sherman et al., 2014). Studies have also shown that vitamin D plays a key role in suppressing intracellular oxidative stress by modulating Nrf2 and maintaining normal mitochondrial function (reviewed by Wimalawansa, 2019); given the major role of intracellular ROS in promoting myofibroblast/myoCAF differentiation, this may also play a role in modulating the CAF phenotype (Hanley et al., 2018). In pancreatic cancer, targeting VDR with calcipotriol has been found to suppress PSC activation and revert cells to a quiescent phenotype, decreasing expression of typical myoCAF genes (e.g., αSMA, ECM genes), also suppressing inflammatory genes and enhancing angiogenesis (Sherman et al., 2014). VDR-induced transcriptional reprogramming of PDAC stroma was sufficient to increase levels of intratumoural gemcitabine and improve survival compared to

chemotherapy alone (Sherman et al., 2014). This murine study became the basis for several clinical trials testing vitamin D agonists as stroma-targeting agents in patients with pancreatic cancer (e.g., NCT03520790). Recent reports have also provided positive evidence for the use of VDR targeting in colorectal cancer despite variation in stromal VDR expression (Ferrer-Mayorga et al., 2017). However, questions remain regarding the use of this strategy to improve immunotherapy response due to potential VDR-mediated dampening of effector T-cell function (Gorchs et al., 2020).

Hedgehog inhibition

Aberrant activation of the Hedgehog (Hh) signalling pathway has been shown to promote the development of stromal desmoplasia in pancreatic cancer (Bailey et al., 2008; Tian et al., 2009). Olive *et al* treated KPC mice with a Smoothened (Smo) inhibitor (IPI-926) and found that the drug significantly reduced desmoplasia and increased vessel density; in turn this improved gemcitabine delivery to tumours and increased survival (Olive et al., 2009). However, deletion of sonic hedgehog (Shh) from pancreatic epithelial cells in PKCY mice promoted earlier tumour development and resulted in more aggressive, poorly differentiated tumours with significantly increased EMT (Rhim et al., 2014). These tumours also had significantly reduced stroma. Notably, chronic treatment of KPC mice with IPI-926 at the premalignant (panin) stage of tumour development produced a similar effect. Activating Hh signalling in fibroblasts also has been shown to markedly reduce tumour load and disease progression in a colon cancer model (Gerling et al., 2016). Clinical trials testing hedgehog inhibitors for stromal targeting have produced disappointing results (Catenacci et al., 2015; de Jesus-Acosta et al., 2020), and the factors regulating the pro- and anti-tumour effects regulated by Shh stromal signalling have yet to be identified.

Viral vaccines

A recent study by Ring *et al* demonstrated that CAF can be reprogrammed to an immune-permissive phenotype using a viral vector (Ring et al., 2021). An intratumoural LCMC-based vaccine vector incorporating the melanoma antigen TRP2 was used to treat mice bearing TRP2-expressing B16F10 melanomas. Notably, although the vaccine was not designed to target fibroblasts specifically, the analysis showed that the vector was predominantly found in tumour cells and CAF. This had the effect of reprogramming Pdpn-positive fibroblasts into an immunostimulatory iCAF subset characterised by expression of Ly6c, Cd34, Cxcl13 and Ccl19 which sustained T-cell activity through Il33 signalling.

Currently, an LCMV vector targeting human papillomavirus (HPV) E6/E7 antigens is in clinical testing for the treatment of patients with HPV-induced head and neck cancer (NCT04180215).

CAF DEPLETION

A different approach for CAF-directed therapy is to deplete the CAF population within a tumour. This approach has been hampered by the lack of specific targets that can be used to selectively kill CAF. αSMA is the classical activated CAF (myoCAF) marker and seems the most obvious target for ablating CAFs. As with other markers, αSMA expression is not CAF-specific; it is also highly expressed by smooth muscle cells, pericytes and across different CAF subsets. Whilst αSMA cannot be regarded as a potential therapeutic target, preclinical models using various methods of ablating αSMA-expressing cells have produced intriguing insights into CAF biology. Murakami *et al* found that docetaxel-conjugate nanoparticles (synthesized by covalently conjugating docetaxel and polyethylene glycol to acetylated carboxymethylcellulose; Cellax), although not designed to specifically target CAF, preferentially accumulated in αSMA-positive stromal cells (Murakami et al., 2013). In breast tumour models (4T1, MDA-MB-231), this led to a significant reduction in αSMA-expressing cells (>70%), increased vascular permeability and decreased lung metastasis (7-24%). Notably, stromal depletion occurred rapidly post-injection (50% depletion within 16 hours), and the αSMA stromal population was almost undetectable after 1 week. nab-Paclitaxel, a nanoparticle formulation of paclitaxel bound to albumin, has also been shown to exert anti-tumour effects through several mechanisms, including CAF depletion, and is commonly used as a first-line treatment for pancreatic, breast and other solid cancers (Choi et al., 2014; Montero et al., 2011; Yardley, 2013). Studies have reported a reduction in CAF numbers in human pancreatic cancer using nab-paclitaxel/gemcitabine combination although the effect on αSMA CAF population specifically is less clear (Alvarez et al., 2013; Miyashita et al., 2018).

Although in most tumour types, αSMA-positive myoCAF are regarded as tumour-promoting, there have been several studies in pancreatic cancer that have identified potential tumour suppressive functions for these cells (Y. Chen et al., 2021; Özdemir et al., 2014). Özdemir *et al* generated αSMA-tk PKT transgenic mice and depleted αSMA-positive cells at different stages of tumour development. Unexpectedly, they found that this accelerated disease progression, and

led to more aggressive, invasive tumours and reduced survival. These tumours were characterised by increased EMT and regulatory T-cells associated with a decline in immune surveillance. A subsequent study by the same group showed that depleting type 1 collagen in αSMA positive cells also resulted in accelerated tumour progression and decreased survival, further suggesting that fibrotic response associated with PDAC is a host defence mechanism against tumour progression. It is possible that these results may reflect opposing roles of αSMA+ CAF in different cancer types or at different stages of tumour development. αSMA-positive pancreatic CAF are derived mostly from stellate cells and the question as to whether myoCAF derived from different progenitor populations function differently remains unanswered.

FAP has been a primary focus of CAF-directed therapeutic studies, both preclinical and clinical. This type II transmembrane serine protease is induced in activated fibroblasts at sites of tissue remodelling and is highly expressed by fibroblasts in the TME (Hamson et al., 2014). Studies have shown that FAP+ CAFs promote an immunosuppressive tumour microenvironment through several mechanisms; interfering with differentiation and maturation of dendritic cells, recruiting MDSCs, and inhibiting intratumoural T-cell infiltration and activation (L. Chen et al., 2017; Feig et al., 2013; Kumar et al., 2017). Kraman *et al* investigated the effect of systemic depletion of FAP-expressing stromal cells; this resulted in hypoxic necrosis of tumours with increased infiltration and activation of T-cells (Lewis lung carcinoma and PDAC murine tumour models; Kraman et al., 2010). However, FAP expression is not CAF specific; there is widespread FAP expression in most murine tissues, including skeletal muscle, and systemic depletion of FAP-expressing cells results in cachexia and anaemia (Roberts et al., 2013). Tran *et al* found FAP expression in pluripotent stem cells in the bone marrow stroma of mice and humans (Tran et al., 2013). FAP is also expressed on different CAF subsets, which may not all be pro-tumorigenic. Nevertheless, interest in FAP as a CAF target remains, particularly since it is also commonly expressed by tumour cells, and different approaches have been adopted including vaccines (vector-based, cell-based and DNA vaccines), chimeric antigen receptor (CAR) therapy and immunotoxins (G.-M. Jiang et al., 2016). These either aim to deplete CAF or utilise FAP expression on CAF/tumour cells to improve drug delivery or localise drug activation in tumours.

Loeffler *et al* tested a DNA vaccine directed against FAP in murine models of breast and colon cancer. The vaccine triggered CD8+ T-cell killing of the FAP+ CAFs, resulting in reduced tumour growth and metastasis, and increased survival (Loeffler, 2006). Additionally, FAP-vaccinated mice showed up to 70% higher intratumoural uptake of chemotherapeutic drugs, thought to be due to a decrease in type I collagen resulting from CAF depletion. Coupling anti-FAP vaccination with chemotherapy further promoted the anti-tumour effect and increased the lifespan of tumour-bearing mice 3-fold. Several other anti-FAP DNA vaccines have been tested in pre-clinical models of breast, pancreatic and colon cancers, all showing CAF depletion, inhibition of tumour growth, and associated improved survival (Liao et al., 2009; Wen et al., 2010). Duperret *et al* treated murine tumours (TC-1, TRAMP-C2) with a combination of DNA vaccines targeting FAP and tumour antigens (TC-1 – TERT; TRAMP-C2 - PSMA) and found a synergistic effect, generating a more robust immune response than either anti-CAF or anti-tumour vaccine used independently (Duperret et al., 2018). FAP DNA vaccines have also been combined with other anti-cancer agents to successfully enhance anti-tumour immunity, including cyclophosphamide and curcumin (G.-M. Jiang et al., 2015; Xia et al., 2017). Other vaccine platforms have proved similarly effective. Zhang and Ertl used an AdC68-mFAP adenovirus vector-based vaccine to target FAP+ stromal cells in murine melanomas and showed that this improved CD8+ T-cell recruitment and function (Zhang & Ertl, 2016). Chen *et al* designed a whole-cell tumour vaccine (WCTV) expressing FAPα, which inhibited tumour growth by simultaneously targeting cancer cells and CAFs, and subsequently improved the vaccine using xeno-antigens to overcome immune tolerance. This reduced CAF numbers in the TME and effectively delayed tumour growth and prevented recurrence (M. Chen et al., 2019).

Depletion of FAP+ CAF has also been achieved using FAP-directed CAR-T-cells. Lo *et al* used the adoptive transfer of FAP-targeted CAR T-cells in stromal-rich, weakly immunogenic ('immune desert') lung and pancreatic murine tumour models and showed that this decreased vascular density and limited tumour growth. These effects were not modulated by enhanced anti-tumour immunity, consistent with CAF tumour-promoting functions beyond immune suppression (Lo et al., 2015). Kakarla *et al* transduced T-cells with a retroviral vector encoding mhFAP-CAR to generate FAP-specific T-cells, combined these with T-cells targeting tumour cell antigen EphA2 and found that this approach significantly decreased A549 tumour growth, reduced FAP-positive stromal cells and increased overall survival (Kakarla et al., 2013). However, the risk of on-target/off tumour effects using this approach was highlighted by Tran and

colleagues, who found that adoptive transfer of FAP-reactive CAR T-cells into mice bearing a variety of subcutaneous tumours (B16, MC38, MC17-51, 4T1, CT26, Renca) had limited anti-tumour effect but induced significant cachexia and lethal bone marrow toxicity (Tran et al., 2013). The evolution of CAR T-cell design has resulted in modifications that increase tumour penetration, killing efficiency and persistence, as well as incorporating approaches that enable more precise targeting of cell populations – a key requirement for CAF targeting (reviewed by Bughda et al., 2021). For example, synNotch-CAR-T-cells can target multiple antigens in the TME and utilise prime-and-kill circuits to achieve specific and controlled killing of target cells (Roybal et al., 2016).

CAF-DIRECTED DRUG CONJUGATES

Antibodies directed against FAP have also been used to target CAF and have been broadly successful in preclinical testing (Xin et al., 2021). Similar to CAR T-cells, the design of antibody bi-specifics, conjugates and delivery systems have also become more sophisticated to improve tumour delivery, uptake, and specificity. Ostermann *et al* developed a novel antibody-maytansinoid conjugate, FAP5-DM1, to target FAP, which induced tumour regression in xenograft models of lung, pancreas, and head and neck cancers (Ostermann et al., 2008). Tansi et al. conjugated a FAP single-chain antibody fragment with anti-HER2 Trastuzumab, delivered in a liposome package. This Bi-FAP/Tras-IL showed significantly improved delivery of the drug into both cancer cells and FAP+ CAFs, and the bispecific targeting improved tumour cell death compared to free Trastuzumab in human breast cancer xenograft models (Tansi et al., 2017). Fang *et al* used a FAP-targeting immunotoxin, αFAP-PE38, to deplete FAP+ stromal cells and in 4T1 breast tumours (Fang et al., 2016). This reduced tumour growth and altered levels of various growth factors, cytokines, and chemokines, increasing TNFα and decreasing the expression of TGF-β, CCL5, SDF-1 and MCP-1. Recruitment of tumour-infiltrating macrophages was also significantly reduced. Notably, combining αFAP-PE38 with paclitaxel potently inhibited tumour growth compared with either treatment alone. Phase I clinical testing of a humanised anti-FAP antibody (Sibrotuzumab) showed that it was well tolerated in humans and specifically concentrated in the tumour stroma. However, subsequent phase II testing in patients with advanced colorectal cancer produced no effect on disease progression (Hofheinz et al., 2003).

A different approach to FAP targeting is to use FAP protease activity to selectively activate pro-drugs at tumour sites in order to improve reduce toxicity and improve drug efficacy. Conjugation of arenobufagin with FAPα-specific tripeptides has been shown to have good anti-tumour activity in human breast cancer models, and notably with negligible cardiac toxicity, even at 3 times the dose of standard arenobufagin treatment (Deng et al., 2017). Brennen and colleagues generated ERGETGP-S12ADT, a FAP-activated analogue of the highly potent cytotoxic agent thapsigargin. The pro-drug showed a 100-fold greater therapeutic window over the parent thapsigargin analogue compound in human breast and prostate cancer xenograft models (Brennen et al., 2014). The FAP-activated pro-drug showed comparable anti-tumour efficacy to docetaxel using standard dosing regimens for both compounds, but notably, FAP prodrug treated mice had significantly fewer signs of toxicity. Wang *et al* produced similar results using epirubicin conjugated with a FAP-specific dipeptide (J. Wang et al., 2017). They observed a substantial anti-tumour effect in breast cancer 4T1 cells overexpressing FAP, and mice treated with the pro-drug showed fewer side effects compared to free epirubicin-treated mice.

Therapeutics have also been developed that inhibit FAP dipeptidyl peptidase activity (rather than harness its enzymatic function to activate a pro-drug). Small molecule inhibitors of FAP enzyme activity have been extensively researched and several have been clinically tested. Talabostat was one of the first small molecule inhibitors designed to inhibit the dipeptidyl peptidase activity shared by DPP4 and FAP. Oral administration was found to slow tumour growth in several syngeneic murine tumour models, including fibrosarcoma, melanoma, and bladder carcinoma, sometimes causing complete regression (Adams et al., 2004; Walsh et al., 2013). Talabostat also enhanced the effect of oxaliplatin in murine models of colorectal cancer (Li et al., 2016). However, despite these encouraging preclinical results, phase I/II clinical testing of Talabostat, either alone (relapsed/refractory paediatric tumours, metastatic colorectal cancer) or in combination with standard of care chemotherapies (stage IV melanoma [with cisplatin]; late-stage lung cancer [with docetaxel]) was largely unsuccessful, and its clinical development was discontinued (reviewed by Fitzgerald & Weiner, 2020).

TARGETING CAF IMMUNE EVASION MECHANISMS – CYTOKINES, CHEMOKINES, AND EXTRACELLULAR MATRIX

A potential problem with targeting specific immunomodulatory pathways is that CAFs contribute to immune escape through multiple mechanisms; upregulation of immunosuppressive cytokine production, immune checkpoint ligands and ECM proteins, suppressing infiltration and cytotoxic activity of CD8+ T-cells in tumours, and by affecting the functional differentiation of tumour infiltrating immune cells, including regulatory T-cells, macrophages and neutrophils (Reviewed by Monteran & Erez, 2019). Nevertheless, preclinical studies have shown that targeting specific CAF immunological functions can potentiate immunotherapy response.

Inhibiting CXCL12/CXCR4

iCAF secrete a number of immunosuppressive cytokines and chemokines, including IL-6, IL-8, IL-11, LIF, CXCL1, CXCL2, CCL2 and CXCL12 (Elyada et al., 2019; Öhlund et al., 2017). FAP+ CAFs are the main source of CXCL12 in tumours, which has been shown to mediate T-cell exclusion from tumours by coating the surface of cancer cells as a covalent heterodimer with keratin-19, formed by transglutaminase-2 (Biasci et al., 2020; Feig et al., 2013; Z. Wang et al., 2022). Blockade of the CXCL12-CXCR4 signalling pathway using AMD3100 (plerixafor), a CXCR4 inhibitor, has been shown to alleviate CAF-mediated immunosuppression and potentiate the response to anti-PD1 immunotherapy. Genetically deleting CXCR4 in αSMA+ stromal cells or using AMD3100 has also been shown to decrease fibrosis, increase T-cell infiltration and improve response to checkpoint inhibition in MCa-M3C and E0771 models of murine breast cancer (I. X. Chen et al., 2019). A positive effect on anti-PD1 response achieved through targeting this pathway has also been attributed to the inhibition of myeloid-derived suppressor cells (K. Jiang et al., 2019).

Inhibiting CTLA-4

To investigate potential mechanisms promoting myoCAF-mediated CD8+ T-cell exclusion, Ford *et al* performed RNA sequencing on flow cytometry-sorted CD8+ T-cells from myoCAF-rich and control TC1 tumours (Ford et al., 2020). They found one of the most highly upregulated genes was CTLA-4, a negative regulator of T-cell response and found a similar expression pattern in myoCAF-rich human tumours. Intriguingly, other CD8+ T-cell exhaustion/activation markers were not significantly changed (PD-1, granzyme B and Ki67) and the authors postulated that upregulation of CTLA-4 in the absence of additional exhaustion markers, raised the possibility that CD8+ T-cell exclusion could be mediated by CTLA-4 regulation of lymphocyte adhesion/migration (Schneider et al., 2005; Zell et al., 1998). Notably, inhibiting CTLA-

4 in CAF-rich TC1 tumours using blocking antibodies increased CD8+ T-cell infiltration and decreased tumour growth without affecting intratumoural T-reg levels or growth of CAF-low tumours (Ford et al., 2020).

Targeting the ECM

MyoCAF-rich tumours are often characterised by a desmoplastic stroma rich in collagens, fibronectin and various proteoglycans (hyaluronan, versican, decorin), which have been shown to 'trap' T-cells and inhibit T-cell motility (Evanko et al., 2012). A dense network of collagen fibres has been shown to limit T-cell access to tumours; in pancreatic and lung cancers, T-cells have been found to be heterogeneously distributed within the stroma, aggregating in areas of low collagen density (Salmon et al., 2012; Hartmann et al., 2014). Additionally, the protease-independent nature of T-cell amoeboid migration leads to contact guidance where T-cells follow a path-of-least-resistance along collagen fibres (Friedl and Weigelin, 2008). In fact, the alignment of fibrillar ECM components within tumours has been strongly associated with invasiveness and prognosis (Lee et al., 2011; Hanley et al., 2015; Erdogan et al., 2017; Bourgot et al., 2020). In preclinical studies, degradation of collagen using bacterial collagenase has been shown to improve drug delivery and efficacy (Goodman, Olive and Pun, 2007; Magzoub, Jin and Verkman, 2008; Dolor and Szoka, 2018), but it is not established whether this approach could be used clinically, particularly given effects on normal tissues and potential tumour dissemination (Dolor and Szoka, 2018; Paolillo and Schinelli, 2019).

Many of the pathogenic attributes of tumour desmoplasia result from alterations to the modification, orientation and biomechanical properties of 'core' ECM components. The lysyl oxidase-like (LOXL) enzymes catalyse elastin and collagen cross-linking, creating a 'stiffer' tissue and activating mechanotransduction signalling pathways (Chen, Li and Li, 2019; Levental et al., 2009; Chaudhuri et al., 2014). Additionally, LOXL2 has been implicated directly in myoCAF activation through activation of integrin-mediated focal adhesion kinase signalling (Barker et al., 2013); its overexpression is associated with poor survival in colorectal and gastric cancer (Torres et al., 2015 Kasashima et al., 2014). Chang *et al* used *in vivo* and *in vitro* models of human breast cancer to evaluate the efficacy of small molecule LOXL2 inhibitors. Inhibition of tumour growth and progression was observed with decreased angiogenesis and reduction in CAF activation indicative of mediating this. In mouse models of pancreatic cancer, LOX inhibition was found to increase sensitivity to gemcitabine and was associated with stromal alterations involving reduced fibrillar

collagen and increased vasculature and immune cell infiltration (Miller et al., 2015; Le Calvé et al., 2016). Simtuzumab, a humanised IgG4 mAb, allosterically inhibits LOXL2 however, in clinical trials there was no observed increase in progression-free survival in pancreatic and colorectal adenocarcinoma patients when administered alongside chemotherapy compared to chemotherapeutic drugs alone (Benson et al., 2017; Hecht et al., 2017).

Alongside collagens, hyaluronic acid (HA) is one of the most abundant components of the tumour ECM. This highly hydrophilic glycosaminoglycan forms viscous gels, increasing intratumoural interstitial pressure, causing vascular compression, and limiting penetration of drugs and immune cells into tumours (Jacobetz et al., 2013; Theocharis et al., 2000; Lokeshwar, Mirza and Jordan, 2014; Sato et al., 2016). Pegylated recombinant human hyaluronidase (PEGPH20) was found to increase infiltration of CD8+ T-cells and improve delivery and efficacy of anti-PD-L1 in murine EMT-6 and HAS3-transduced 4T1 breast cancers (Clift et al., 2019). Similarly, PEGPH20 increased the efficacy of anti-PD1, anti-PDL1 and anti-CTLA4 in murine models of colorectal cancer (CT26 cells) and pancreatic cancer (MH194 cells) that had been engineered to overexpress HAS3 (Rosengren et al., 2016). However, initial phase 1b/2 clinical testing of PEGPH20 combined with chemotherapy (modified fluorouracil, leucovorin, irinotecan, and oxaliplatin) in patients with metastatic pancreatic cancer revealed significantly increased toxicity and inferior outcomes in all efficacy measures (Ramanathan et al., 2019). A recent phase 3 study investigated the treatment of treatment-naïve HA-high metastatic pancreatic ductal adenocarcinoma with PEGPH20 in combination with gemcitabine and nab-paclitaxel (Tempero et al., 2020). PEGPH20 did not improve clinical outcomes versus chemotherapy alone.

Losartan, an FDA approved angiotensin II type 1 receptor (AGTR1) inhibitor antihypertensive can reduce/reverse fibrosis in a variety of animal models (Johnston, 1995; Lim et al., 2001; Yao et al., 2006). Treatment of murine E0771 and 4T1 breast tumours decreased stromal collagen and hyaluronidase, increased vascular perfusion and improved chemotherapy delivery and efficacy (Chauhan et al., 2013). The antifibrotic function of losartan stems from the inhibition of TGF-β/SMAD signalling via inhibition of thrombospondin-1 (Cohn et al., 2007; Diop-Frimpong et al., 2011). Losartan has additionally been suggested to destabilise the existing extracellular matrix through CCN2/CTGF inhibition (Chauhan et al., 2013). Zhao et al. (2019) used xenograft ovarian tumour models (SKOV3, Hey-8), and found that losartan enhanced the anti-tumour effects of paclitaxel, significantly decreasing numbers of αSMA+ stromal cells and

collagen I/III; this effect was mediated by antifibrotic microRNAs (upregulated expression of miR-1-3p, miR-133a-3p (miR-133), miR-29b, and miR-26b-5p). Recently, angiotensin receptor blockers (ARB)-nanoconjugates have been developed, activating ARB selectively in the TME, by the low tumoral pH (Chauhan et al., 2019). ARB-nanoconjugates downregulated TGF-β, hypoxia and inflammatory response pathways and improved the response to immune checkpoint blockers.

5. CLUES FROM THE FIBROSIS LITERATURE

Clearly, there is overlap between the pathological pathways underlying accumulation of myofibroblasts in fibrotic disease and myoCAF in stromal-rich cancers, both characterised by aberrant ECM deposition. The biological parallels suggest that important clues for the treatment of myoCAF-rich tumours can be found in the extensive fibrosis literature. Pirfenidone, an FDA-approved anti-fibrotic agent for the treatment of pulmonary fibrosis, demonstrates both anti-fibrotic and anti-inflammatory effects (Cottin and Maher, 2015), and has been shown to modulate TGF-β, PDGF, CXCL12 and TNF-α (Schaefer et al., 2011), all of which are important signalling pathways in the context of the TME and fibrosis. A proposed mechanism underlying the anti-fibrotic effect of pirfenidone is inhibition of TGF-β signalling by suppressing of SMAD2/3 nuclear accumulation, thereby preventing myofibroblast activation and ECM accumulation (Choi et al., 2012). In preclinical murine models of pancreatic and lung cancer, pirfenidone treatment reduced tumour desmoplasia and synergised with chemotherapy (Kozono et al., 2013; Mediavilla-Varela et al., 2016). The potential for repurposing pirfenidone as a cancer therapy would seemingly stem from its ability to target CAFs (Fujiwara et al., 2020; Aboulkheyr Es et al., 2020), and clinical trials are investigating the potential for pirfenidone to increase the therapeutic efficacy of chemotherapy and immunotherapy in advanced non-small cell lung cancer (NSCLC) (NCT04467723; NCT03177291).

Nintedanib, used for the treatment of idiopathic lung fibrosis (IPF), is a broad tyrosine kinase inhibitor (with activity against PDGFRs, FGFRs and VEGFRs), which has been shown to cause a reduction of pro-fibrotic gene expression and myofibroblast differentiation in lung fibrosis models (Myllärniemi and Kaarteenaho, 2015). Studies investigating combination of nintedanib with conventional chemotherapy have shown clinical benefit in NSCLC (Reck et al., 2014). Kato and colleagues evaluated nintedanib in murine melanoma models, and found that it reduced tumour growth and

improved survival, and was associated with TME remodelling and increased intratumoural infiltration of CD8+ T-cells (Kato et al., 2021). Furthermore, combining nintedanib with anti-PD-1 immune checkpoint blockade led to increased anti-tumour efficacy (Kato et al., 2021). Nintedanib may therefore have utility as a CAF-targeting drug (Yamanaka et al., 2020), and clinical trials (NCT03377023; NCT02856425) have reported encouraging increases in anti-tumour effect and response in anti-CTLA4/PD-1 combined with nintedanib administration (Varga et al., 2018; Puri et al., 2019).

Recently, the lysophosphatidic acid (LPA) pathway has been implicated in promoting both cancer and organ fibrosis. Autotaxin, a secreted lysophospholipase D-like enzyme, generates LPA from lysophosphatidylcholine (Yung, Stoddard and Chun, 2014). LPA signalling has been shown to modulate murine lung fibrosis; LPA1 (LPA receptor) deficient mice are protected from bleomycin-induced pulmonary fibrosis/mortality (Tager et al., 2008). Intriguingly, PA1-deficient fibroblasts were still responsive to TGF-β, with protective effects attributed to the reduced chemoattractant-mediated migratory response of LPA1-deficient fibroblasts to the site of injury, reducing fibroblast accumulation and subsequent myofibroblast-facilitated fibrosis (Tager et al., 2008). Inhibition of both autotaxin and autotaxin receptors (LPAR) has shown promising efficacy in phase 2a studies in patients with IPF (Maher et al., 2018; Kihara, Mizuno and Chun, 2015). Autotaxin/LPAR inhibitors have also been proposed to improve response to cancer immunotherapy due to the immunosuppressive response of LPA-signalling, dampening effectors of immunotherapy within the TME (Matthew et al., 2019; Matas-Rico et al., 2021; Tigyi et al., 2021). The autotaxin—LPA axis is associated with pro-tumourigenic functions in PDAC, with activated CAFs fuelling this axis – as co-opted by PDAC cells' exploitation of normal wound healing processes (Auciello et al., 2019). LPA signalling has been linked to carcinogenesis, tumour progression and therapy resistance in a range of malignancies (Geraldo et al., 2021). Targeting autotaxin/LPAR therefore has the dual benefit of inhibiting tumour cells and stromal cells (tumour-stroma crosstalk). While targeting this axis attenuates fibrosis progression and a known pro-cancer pathway, it remains to be seen whether targeting autotaxin/LPAR will be beneficial in tumours displaying pronounced fibrotic stroma and immune cold/excluded phenotypes. Perhaps the success of this approach will be conferred by adjuvants to chemo/radiotherapy – preventing treatment-associated side effects such as radiation-induced fibrosis.

6. CONCLUSION

Fibroblast heterogeneity reflects a highly responsive and plastic cell, and the discovery of common fibroblast phenotypes in normal tissues, inflammatory conditions and tumours, emphasises the likely functional similarities across disease states i.e CAF phenotypes and functions are not unique to cancer, and perhaps fibroblast nomenclature should reflect this. There is significant preclinical data to suggest that CAF targeting could increase immunotherapy efficacy. Although it remains unclear what aspects of the CAF phenotype are most important to their role in immune evasion in human tumours, strategies that reprogramme CAF to an immune permissive state are possibly the most attractive. For this to be effective, we require a clearer understanding of how distinct subtypes shape the tumour immune microenvironment. The clear overlap with fibroblast inflammation and fibrosis literature may provide clues for this. It is also important that we are able to learn from the many ongoing immunotherapy clinical trials by establishing reliable and consistently used biomarkers for important CAF phenotypes to establish a more complete perspective on the role of CAF in therapy resistance and identify areas where CAF targeting could be useful. Given that most solid tumours contain a CAF-rich subgroup, CAF-directed therapy has great potential.

7. CONFLICT OF INTEREST STATEMENT

C.J. Hanley and G.J. Thomas are co-inventors on patent WO2019086579 for the use of NOX inhibitors in cancer.

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10. FIGURES

Figure 1

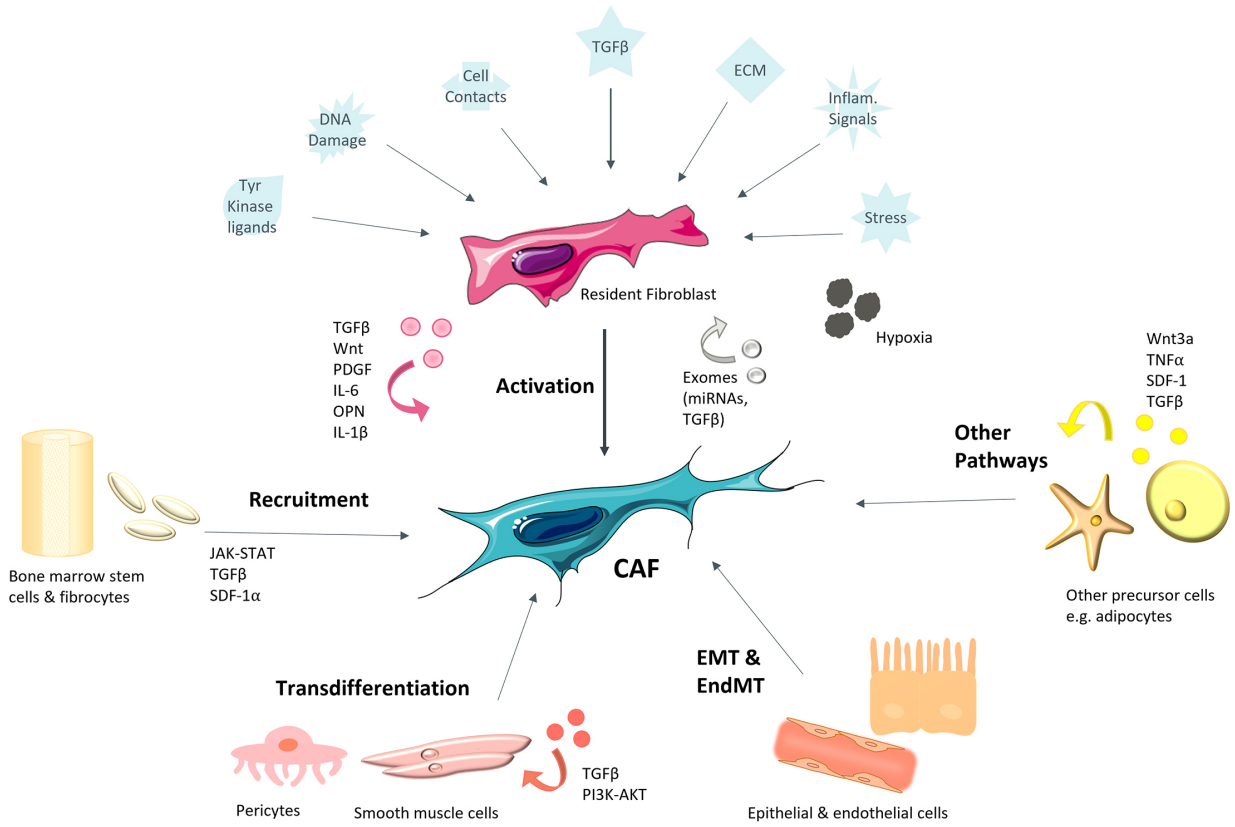

Figure 1 - CAF Progenitor Cells and their Activation. Cancer associated fibroblasts can be formed from a variety of precursor cells through multiple mechanisms. Activation of resident fibroblasts is the most well-studied mechanism, whereby a range of stimuli, from tumour-secreted factors to physical TME characteristics can trigger activation.

Figure 2

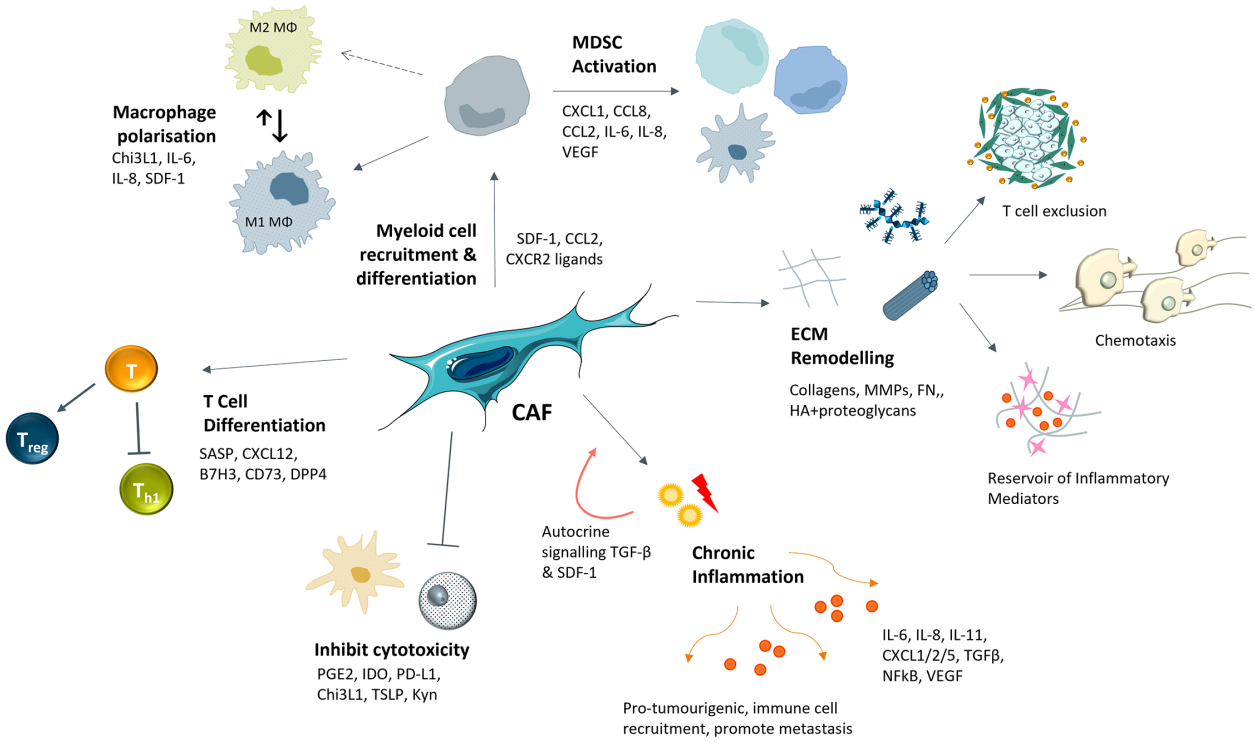

Figure 2 - CAF Immune Effects. Cancer associated fibroblasts mediate a complex variety of immunological effects that affects most immune cell populations, generally producing an immunosuppressive tumour microenvironment that contributes to immunotherapy resistance.

Figure 3

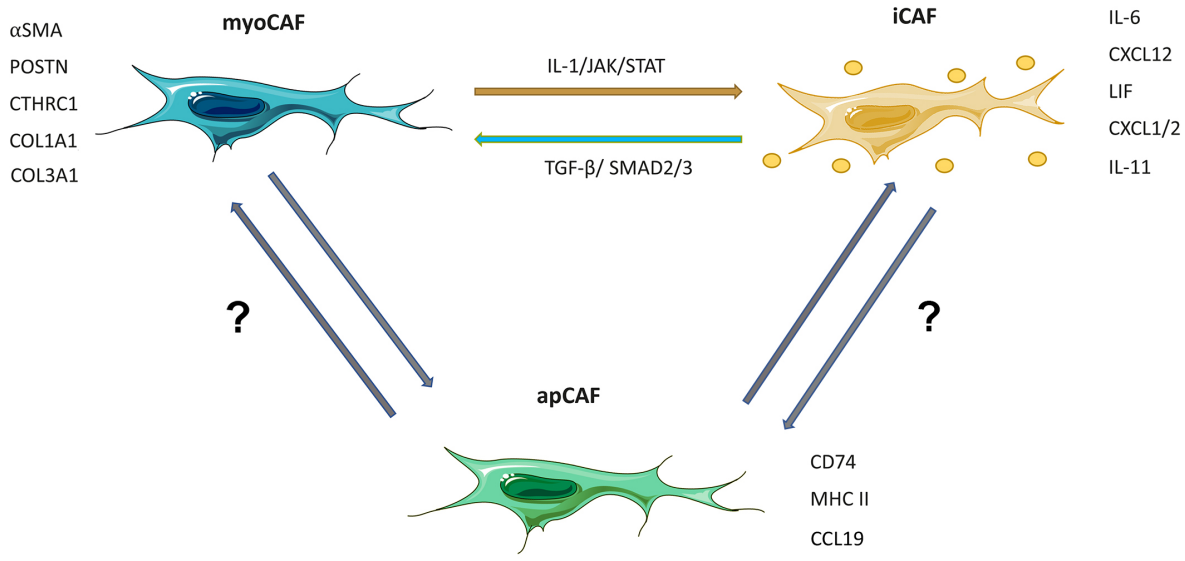

Figure 3 - CAF Subtypes. CAFs are heterogenous, with a variety of subtypes/phenotypes observed across different cancer types. Myofibroblastic CAF (myoCAF) have been extensively noted in various types of solid tumours for many years, other CAF subtypes have recently been described. Currently, three broad CAF subtypes have emerged from scRNASeq analyses; myoCAF, iCAF (inflammatory) and apCAF (antigen-presenting). myoCAF and iCAF have been found to be regulated by TGF-β/SMAD and IL-1/JAK/STAT signaling respectively, with interconversion demonstrated, underlining CAF plasticity. Whether apCAF form part of this plastic spectrum remains to be determined.

Figure 4

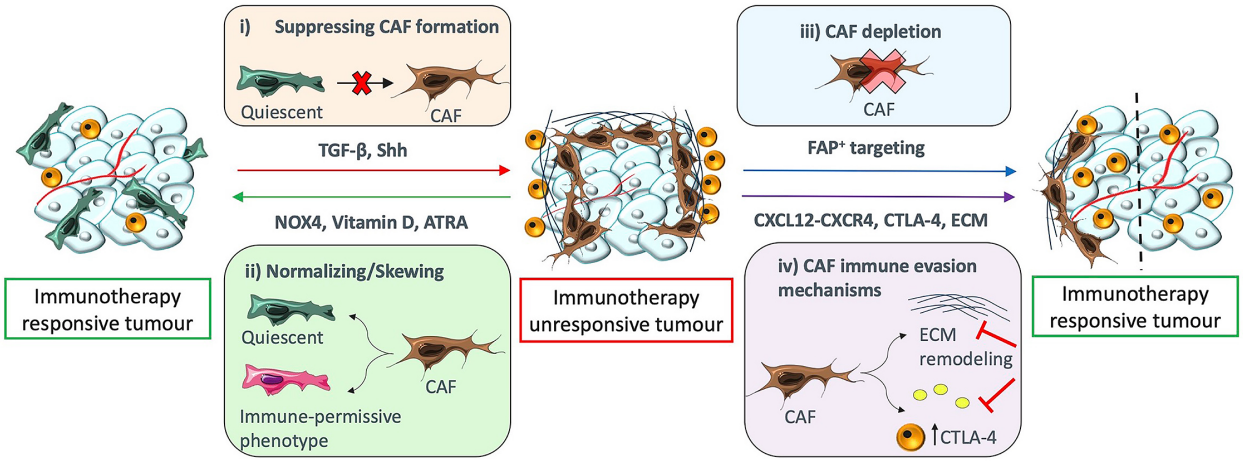

Figure 2 – Strategies for CAF Targeting. Here we summarize four strategies for CAF targeting to facilitate immunotherapy response. Suppressing CAF differentiation through inhibiting key activation pathways (TGF-β, Shh) driving CAF activation. Reprogramming CAF to a more 'normal' phenotype via NOX4 inhibition, Vitamin D agonists and All-trans Retinoic Acid (ATRA). Additionally, exploitation of CAF plasticity by reprogramming CAF to an immune-permissive phenotype (LCMC-based vaccine vector) may offer an alternative method to potentiate immunotherapy. Depletion of CAF expressing specific markers (FAP) using vaccination (vector-based, cell-based and DNA vaccines), chimeric antigen receptor (CAR) therapy and immunotoxins/mAb. Targeting immune evasion mechanisms granted by CAF including extracellular matrix (ECM; collagen, collagen-cross linking, hyaluronic acid), immunosuppressive cytokines/chemokines (CXCL12-CXCR4 axis) and CD8+ T-cell exclusion mechanisms (CTLA-4).