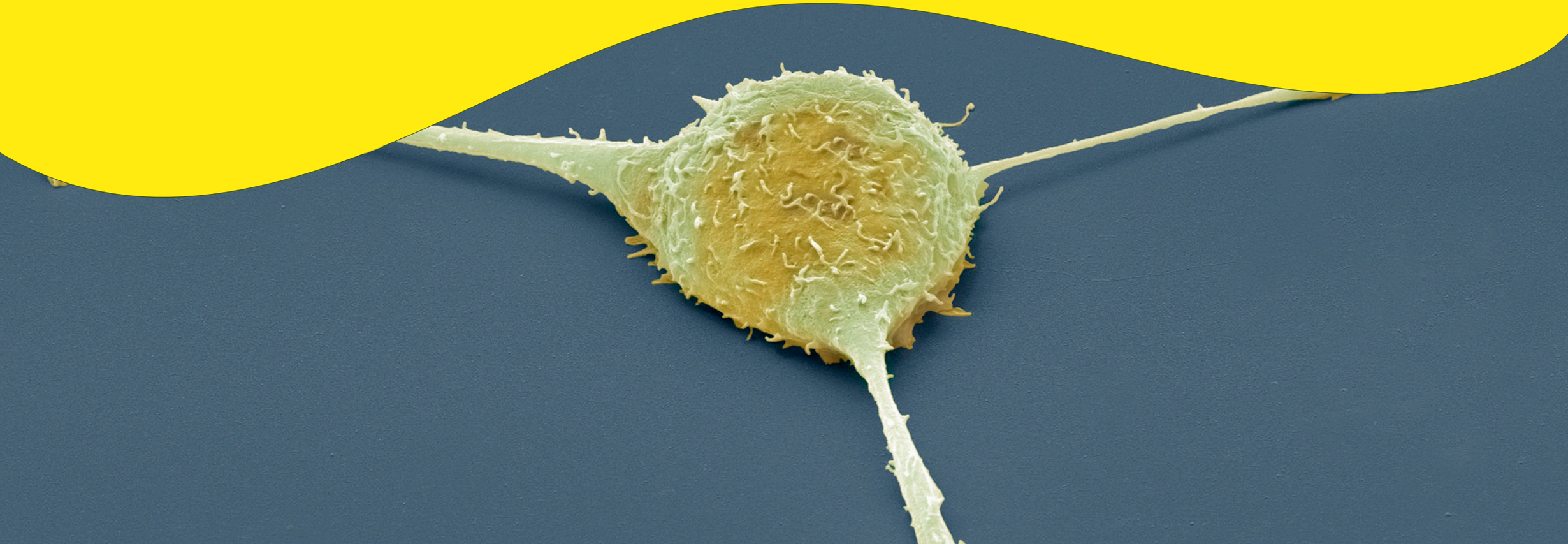
Tumor Stroma

Guide to how cellular actors of the tumor microenvironment challenge immunotherapies



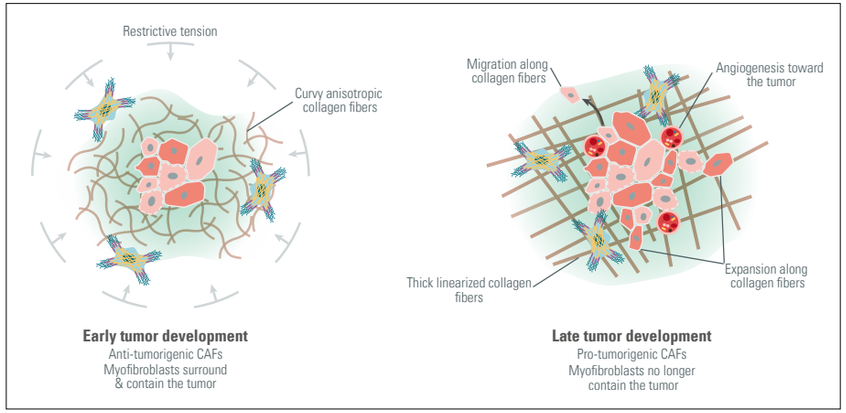
Introduction

Fibrogenesis in the immediate environment of tumors with signs of inflammation and tissue repair is closely associated with tumor development and growth. Interestingly, the link between chronic scarring and the onset of cancer was first observed as early as 1850[1]. In the 1980s, the notion that tumors are like wounds that do not heal and exhibit all the characteristics of tissue repair processes (leucocyte infiltration, neovascularization, fibroblast accumulation)[2, 3] highlighted extracellular matrix (ECM) remodeling as a companion process closely interacting with cancer and shone light on the role of tumor stroma or matrix environment in disease progression. Later evidence showed that both tumor progression and fibrogenesis are associated with myofibroblast proliferation and activity[4, 5], and that stiffer ECM remodeling is associated with tumors[6].

While cancer originates from mutant cancer cells, its development is greatly dependent on non-mutant actors in the immediate environment, with tumor survival being typically related to how accessible they are to cytotoxic killer cells (NK and CD8+ T-cells) and how effective these cells are at identifying and removing tumor cells. Counter intuitively, the predictive power of stromal (matrix) gene expression on cancer outcomes is greater than that of tumor genes, which speaks volumes about the critical nature of the ECM and matrix-related cells to tumor development[7].

The definitive pro- or anti-tumorigenic nature of fibrosis is controversial, with evidence in favor of both. It is likely not the same at all stages of tumor development. In the early stages of tumor growth, fibrotic behaviors appear to contribute to the containment of cancer cells and the reduction of tumor expansion. This is achieved in similar ways to wound closing, with myofibroblasts surrounding and crossing the inflamed area with anisotropic collagens, then contracting around it. The application of mechanical force around the tumor results in a tighter stroma that collapses blood vessels and limits tumor growth. However, later events of ECM reorganization seem to shift these anti-tumorigenic effects to pro-tumorigenic ones[8].

On top of that reorganization, the regulation of the ECM is altered in the tumor environment, with collagen and matrix metalloproteinase (MMP) turnover increasing greatly, which both facilitates the reorganization into a linear architecture and creates space for cancer cells to invade[9, 10]. The increased turnover is also a source of growth factors which are released from the ECM and promote invading tumor cell growth in this already cell-free and nutrient rich medium[11].



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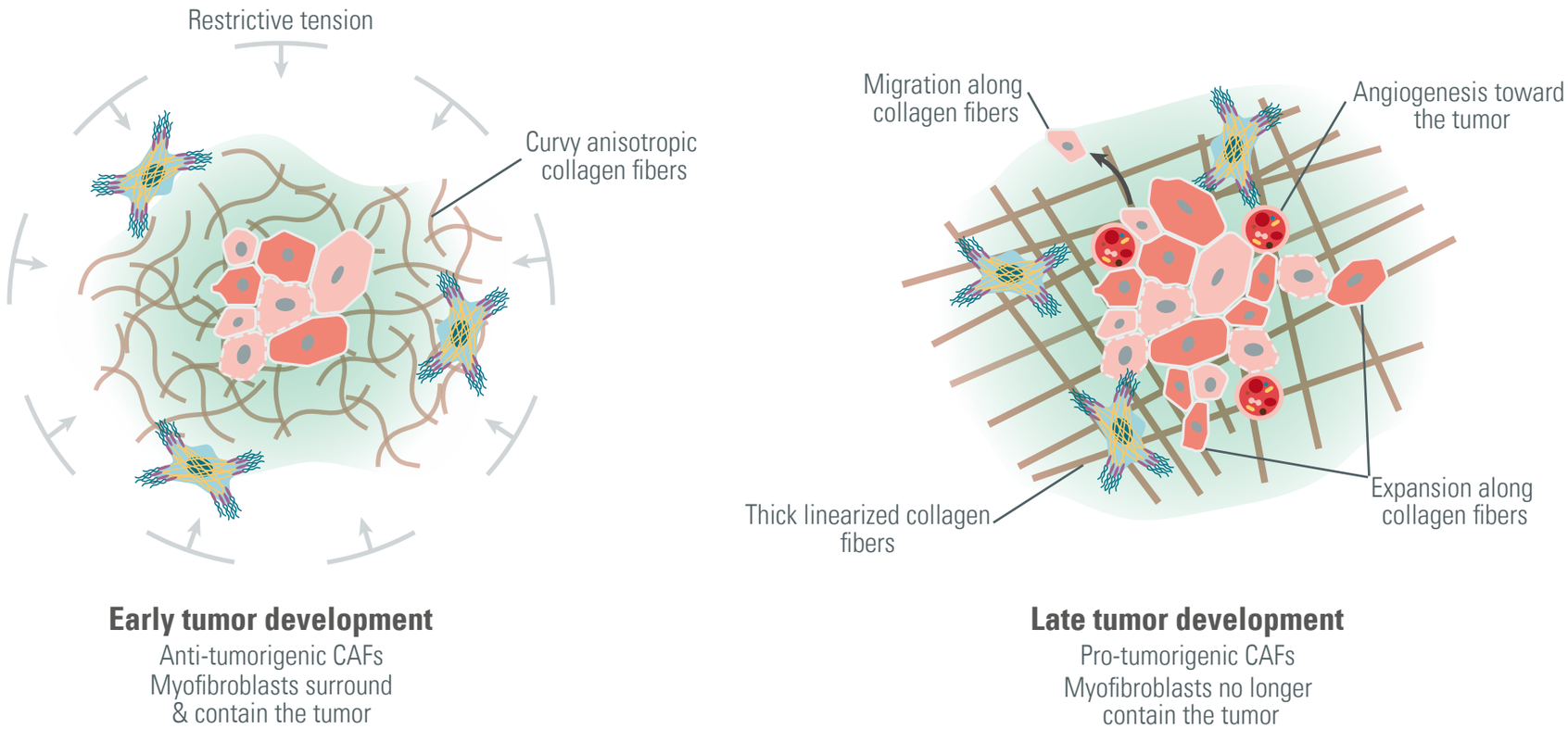

Figure 1: Cancer-Associated Fibroblasts (CAFs) exert nuanced anti- and pro-tumorigenic effects at different stages of tumor development. Initially, fibroblasts in the tumor microenvironment surround and cross tumor cells with anisotropic collagen fibers that densify and restrict tumor cells, providing little opportunity for migration and expansion. In later stages, differentiated CAFs switch to thick linearized collagen fibers that limit immune cell infiltration into the tumor and provide migration and expansion avenues for tumor cells.

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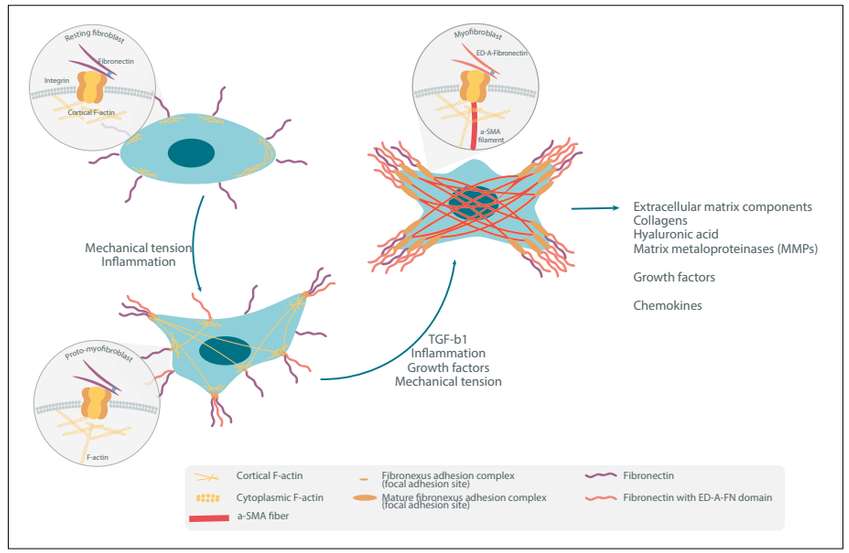
Cancer-associated fibroblasts

Fibroblasts are one of the most abundant cell types in connective tissues, where they reside in a widely spaced manner and anchor themselves via focal adhesion points involving integrin and fibronectin complexes connected to their actin cytoskeleton. They exhibit a typical elongated spindle shape or a stellate shape with indented nucleus morphology that is conserved throughout the whole body[12, 13]. Despite their conserved morphology, the gene expression profile of fibroblasts is dependent on their location and immediate environment, which results in a collection of phenotypically different subpopulations[14, 15]. The main variables between these subpopulations include surface markers, cytoskeletal characteristics, and cytokine profiles[16]. It is worth noting that fibroblasts are not a terminally differentiated cell type, but rather quiescent precursors to active specialized cells like myofibroblasts. The latter are promoted by TGF-β1 signaling and express significant amounts of cytokines, ECM proteins (extracellular matrix), and α-SMA (α-smooth actin). They have roles related to inflammation, connective tissue elaboration (ECM), and tissue mechanics[17, 19]. This plasticity and their multiple functions in tissue remodeling and involvement at inflammation sites result in a rise in a tumor-specific cell type called cancer-associated fibroblasts (CAFs). Beyond their shape and functions, defining fibroblasts is not as straightforward as most specialized cells because they share a mesenchymal lineage with other cell types and generally lack exclusive identifying markers. However, fibroblast function and activation can be specifically identified thanks to some of their biomarkers. In particular, α-SMA and the alternatively spliced extra domain A (EDA) form of fibronectin are almost exclusively found in active fibroblasts and the fully differentiated myofibroblast subtype. Fibroblasts' matrix products such as collagens are also used to identify and monitor fibroblasts. CAFs are equally arduous to define and they are usually understood as being the fibroblast-like cells in the vicinity of tumor cells, while not matching epithelial, endothelial, and leukocyte markers[20].



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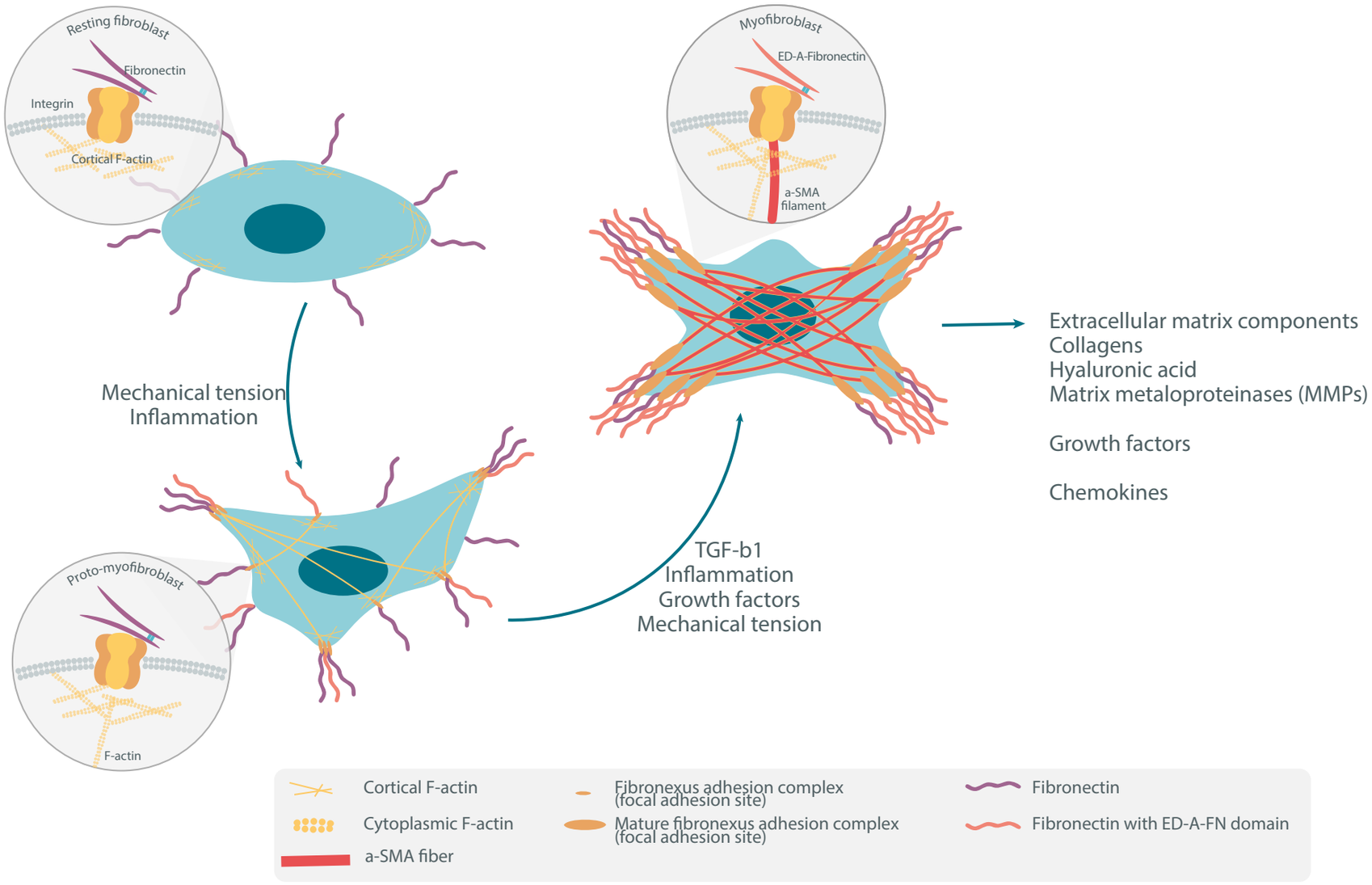

Figure 2: Fibroblast activation and differentiation process. Resting fibroblasts are activated by inflammatory or mechanical stress. They differentiate with growth factors like TGF-β1 and become proto-myofibroblasts that organize their cytoskeleton differently and incorporate more cytoplasmic actin filaments. Proto-myofibroblasts organize extracellular fibronectin into fibronexus-adhesion complexes (extracellular fibronectin associated to cytoplasmic actin filaments) and begin to include focal adhesion sites tying the new actin filaments (intracellular) and fibronectins (extracellular) together. Three elements have been identified as critical to the differentiation third stage. First, TGF-β1 availability remains necessary. Second, the mechanical tension proto-myofibroblasts receive is significantly increased by the more rigid actin cytoskeleton and anchorage points in the form of fibronexus-adhesion complexes as the tension triggers the increased polymerization of actin filaments which modulate more cytoskeletal modifications. Third, in the resting fibroblast state, fibronectin is usually expressed without its EDA domain. When in proto-myofibroblast form, fibronectin is spliced into EDA fibronectin that increases TGF- β 1 uptake. Fully differentiated myofibroblasts exhibit an α-SMA-rich cytoskeleton with multiple fibronexus-adhesion complexes as anchorage points, strongly expressed EDA-FN, and enhanced ECM secretion abilities.

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Cancer-associated fibroblasts

CAFs are key components of tumors at the microenvironment level, where they fulfill the expected roles of fibroblasts (matrix assembly, remodeling) but also exhibit a particular extensive and intensive crosstalk with cancer cells and infiltrating immune cells. The critical role of tumor stroma in cancer development and the direct relation between stroma elaboration and CAFs make these cells and the crosstalk they partake in potential therapeutic targets. CAFs are major regulators of tumors, regulating parameters such as growth factor availability, circulation and infiltration of immune cells, growth and state of blood vessels, tumor exclusion, cancer migration, etc. This array of functions makes for a somewhat confusing understanding of how CAFs affect tumors, and there currently is an uncertainty on their pro- and/or anti-tumorigenicity. The current consensus is that CAFs appear to start as anti-tumor actors and evolve over time into pro-tumor cells. Unraveling the different CAF phenotypes and function currently stands as the main challenge to make them a plausible therapeutic avenue for cancer[21].

Among the most noticeable pro-tumorigenic functions of CAFs, they appear to be responsible for the epithelial-mesenchymal transition (EMT) by which cancer cells acquire migratory and invasive properties. This results from EMT being a key process of tissue remodeling (especially in wound healing) which fibroblasts specialize in and promote as part of their scarring and healing roles[22]. The other key function of CAFs that is critical to tumor development is the mis-regulated chronic ECM deposition, which is mostly performed by the myCAF (myofibroblast CAF) subtype of CAFs. Like regular myofibroblasts, they have a connected cytoskeleton of actin and contractile α-SMA fibers that reach out to the outer layer of the membranes via transmembrane integrins and exert their contractility upon the microenvironment. Overall, their matrix-deposition role stiffens the local matrix with collagen[23]. The consequence of matrix deposition in tumor stroma could be anti-tumorigenic at first as the tumor cells are effectively engulfed, contained, and choked in collagen, but seem to eventually turn pro-tumorigenic. Stromal density increases, polarized fibers act as migration roads for cancer cell invasion, local pressure increases with ramifications into the local vascular system state and collapsing vessels (locally altered blood composition, hypoxia, pH regulation, impaired removal of metabolic wastes, and infiltration of monocytes and other immune cells from the blood). On top of the local dysregulation, such abnormal ECM around tumors acts as a physical and chemical barrier for killer cells (NK and CD8+ T-cells) which allows tumor cells to thrive[24].

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Tumor associated macrophages (TAM), partners in crime to CAFs?

Macrophages are phagocytic immune cells that infiltrate tissues where they become resident sentinels. They are plastic cells that respond to their microenvironment and fulfill homeostasis-maintenance missions that include tissue modeling, recycling, and clearance of dead cells and the removal of pathogens.

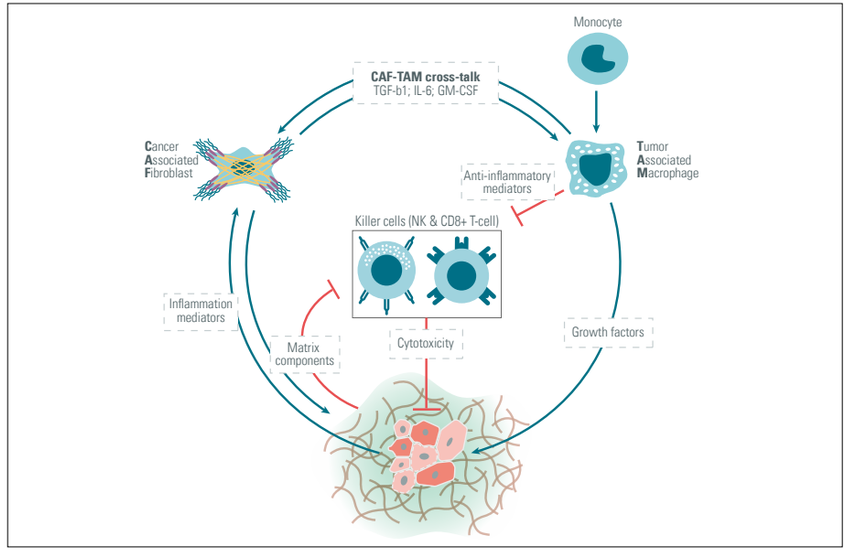
Macrophages are divided into two main categories which exhibit opposed and complementary phenotypes. Classically activated macrophages (CAM) or M1 are induced by a combination of TLR-signaling and IFN-γ. They are potent antigen-presenting cells with strong pro-inflammatory cytokine expression (IL-6 & TNF-α), and also exhibit high levels of lysis compounds and regulator nitric oxide that empower them against pathogens. CAMs are the pathogen-clearing pro-inflammatory population of macrophages.

Alternatively activated macrophages (AAM) or M2 are induced by IL-4 or IL-13. They express high levels of arginase (an enzyme required for DNA synthesis), ECM proteins, TGF- β1 and anti-inflammatory IL-10. They are the wound-healing pro-fibrogenesis population of macrophages[13,14,25,26].

It is important to note that due to their highly plastic nature, macrophage categories of phenotypes are not homogeneous and clearly defined, but rather exist in a spectrum of populations which are not restricted to the characteristics of their most prominent phenotype[26,27].

In tumor settings, these dual phenotypes seem to be an important factor as the M2 macrophages exhibit a close relationship with fibroblasts and enhance the pro-tumorigenic effects of the latter. These tumor-associated macrophages (TAM) have also been identified as contributors to tumor immune exclusion in their own right, with mechanisms not unlike that of CAFs or unrelated to them[28,29].

Research suggests there is a two-way self-amplifying crosstalk between fibroblasts and macrophages in the microenvironment of tumors, where local inflammation makes CAFs secrete messengers that draw monocytes (including pro-inflammatory lipid mediator prostaglandin E2; PGE2) from the circulation and orient their differentiation toward the M2 phenotype. M2 macrophages then stimulate CAFs with locally expressed TGF-β1 and impede immune cell activity on site with anti-inflammatory IL-10 secretion. M2-stimulated CAFs are also prone to expressing levels of growth factors that benefit macrophages but may also increase tumor aggressiveness[30]. Experiments in monocyte cocultures with cancer-stimulated CAFs suggest the key mediators in the aforementioned crosstalk are IL-6 and GM-CSF[31].



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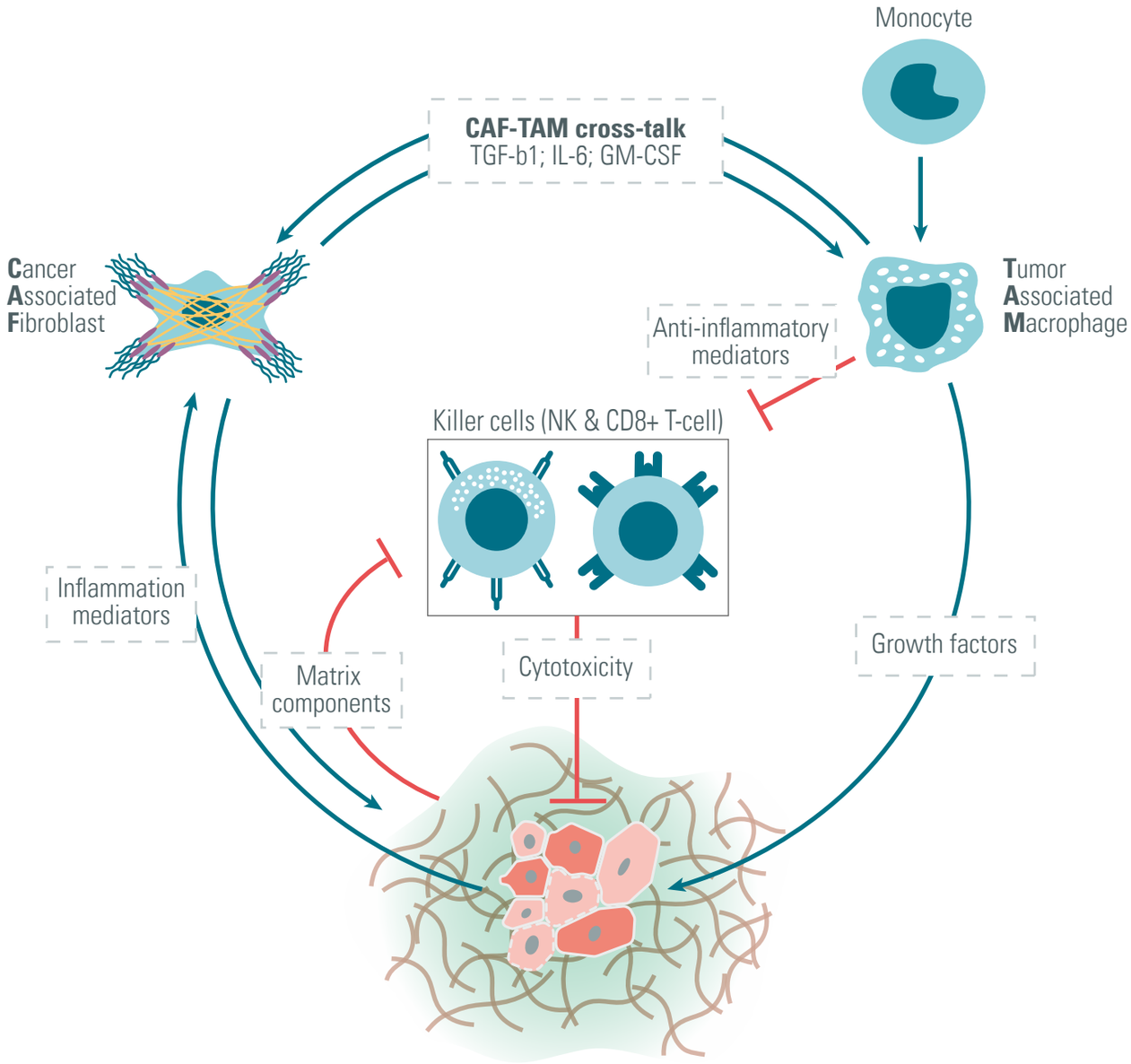

Figure 3: Pro-tumorigenic CAF-TAM crosstalk. Cancer-Associate Fibroblasts (CAFs) and Tumor Associated Macrophages (TAMs) promote each other with a crosstalk involving inflammatory mediators, pro-fibrotic signals, growth factors, and monocyte-attracting chemokines. Both cell types are sustained and benefits from an increase in number and/or activity over time. Tumor cell growth is promoted by TAM-secreted growth factors while killer cells are inhibited by macrophage-secreted anti-inflammatory mediators and prevented from infiltrating the tumor by CAF-deposited dense matrix.

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Therapeutic research

The key interest that CAFs and TAMs present for oncology research is not only the pro-tumorigenic effect they may have on their own, but also the impact of their functions on the applicability of some of our most promising strategies to address cancer. As master regulators of the tumor stroma, CAFs and TAMs define the immediate environment of cancer cells, and that parameter is critical for immunotherapeutic strategies that rely on immune cells having physical and chemical access to tumors.

The case of immune checkpoint inhibitors is an interesting one. They are a successful immunotherapy strategy that strengthens the already existing anti-tumor immune strategies of killer cells (NK, CD8+ T-cells), with much improved outcomes in several cancers[32].

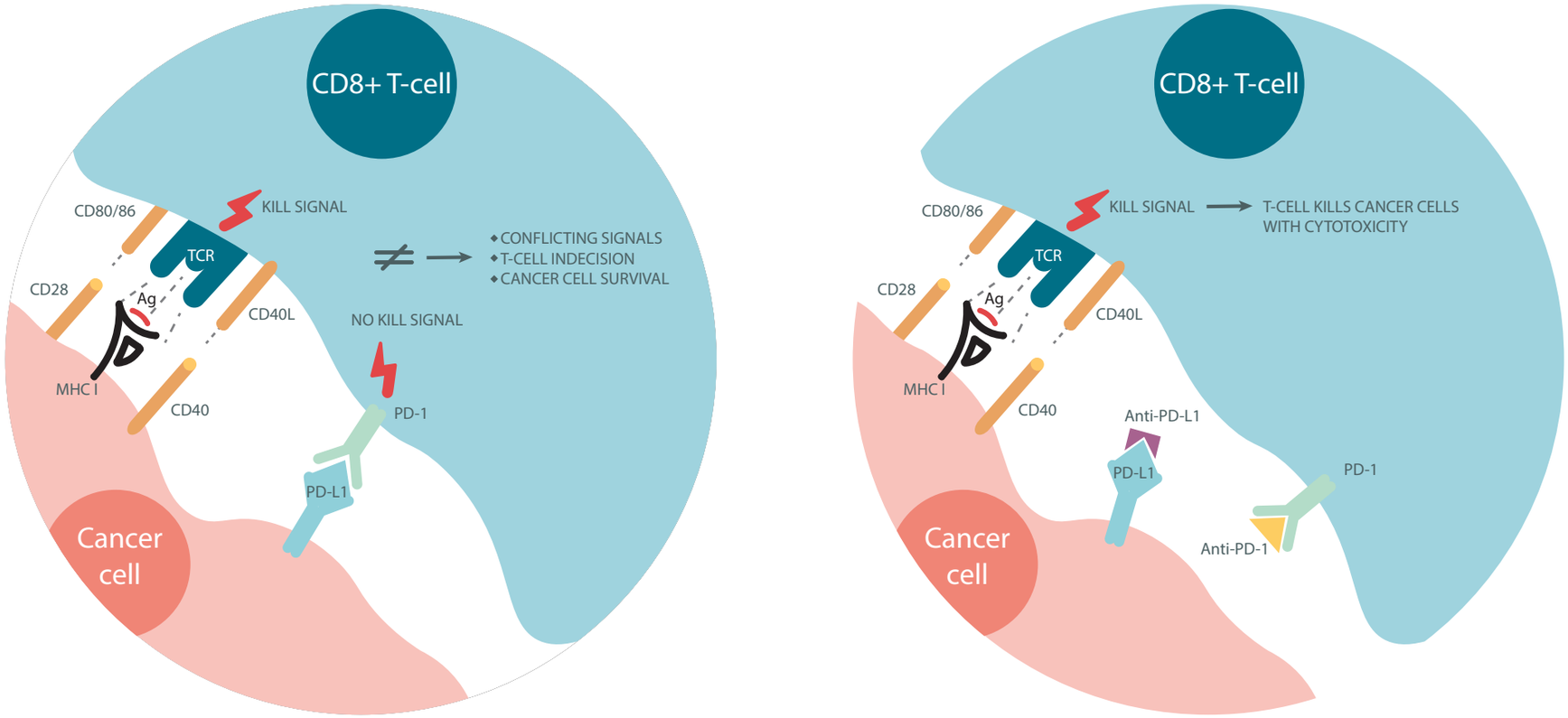

Figure 4: Principle of immune checkpoint inhibitor therapy. Case of PD-1/PD-L1.

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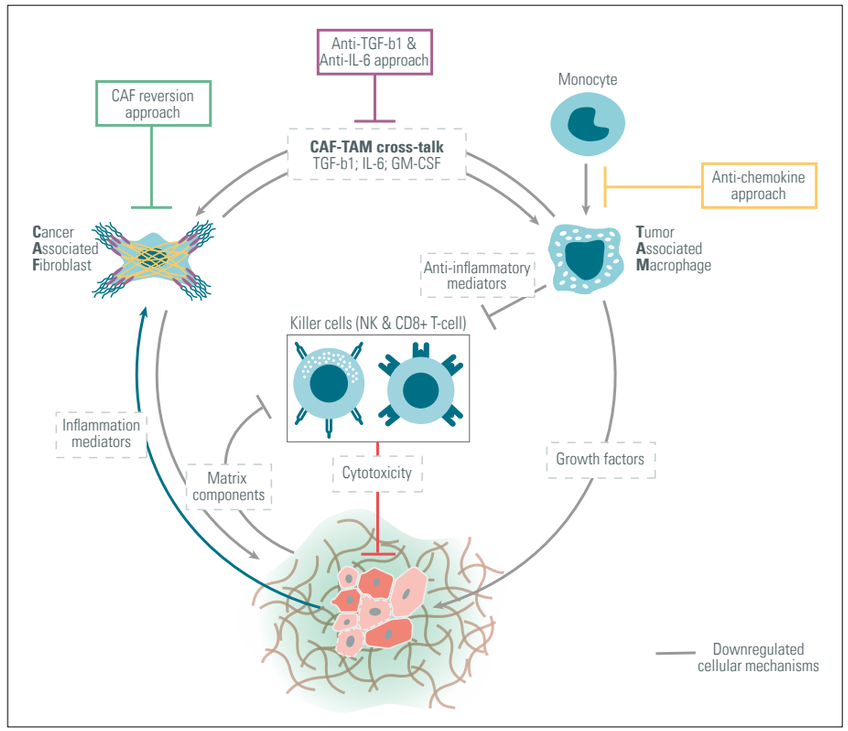
Therapeutic research

However, there is evidence that CAFs and TAMs are contributing to immune checkpoint inhibitor resistance in at least two ways: the physical exclusion of tumors from cytotoxic killer cells (NK and CD8+ T-cells) and the neutralization of immune checkpoint inhibitors in the tumor microenvironment by expressing immune checkpoint ligands[28]. Attempts at restoring the power of immune checkpoint inhibitors in such contexts have already been investigated.

Blocking TGF-β1, which is the main driver of CAF activation and matrix depositing functions as well as a key mediator of the CAF-TAM crosstalk, results in improved efficacy of anti-PD-L1 treatment and promotes CD8+ T-cell infiltration into solid tumors[33].

Attempts at neutralizing CAF chemokine signaling seems promising too. CAFs are the main chemokine source in tumor stroma and draw monocytes from the blood to differentiate into macrophages on site. Disrupting that recruitment could be a strategy to limit CAF-TAM collaboration and experiments focusing on the inhibition of CXCL12 in pancreatic cancer models resulted in better T-cell infiltration and accumulation into tumors when associated with immune checkpoint therapy (anti-PD-L1)[34].

Some investigations are bolder and aim not to mitigate CAF activity but to restore CAFs to a normal fibroblast phenotype altogether. Experiments on pancreatic CAFs showed that inhibiting the somatostatin receptor SST1 reduced the expression of inflammatory mediators by CAFs, specifically of CSF-1 which is a macrophage growth factor. This effectively disrupted the CAF-TAM crosstalk and resulted in less aggressive tumors[35]. Using Eribulin (an approved neoplasia drug) to selectively inhibit TGF-β1 signaling pathways in CAFs yielded a similar effect and hinted at the potential return of the tumor microenvironment of pancreatic cancer models to a normalized state[36].



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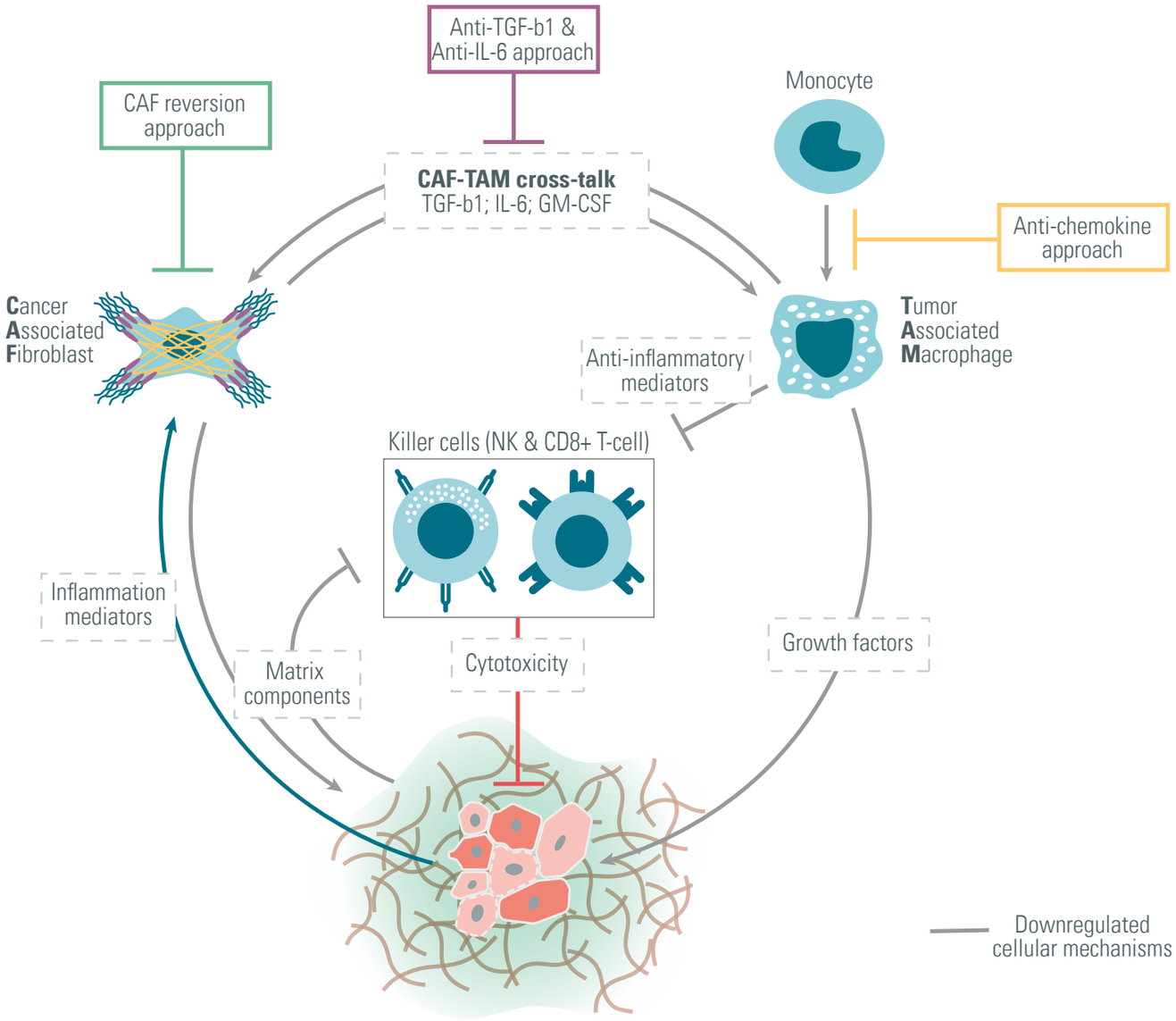

Figure 5: Therapeutic approaches targeting immune checkpoint inhibitor resistance driven by CAF-TAM phenotypes and crosstalk. Disruption of the CAF-TAM crosstalk by CAF normalization, TGF-β1/IL-6 inhibition or chemokine inhibition (inhibition of monocyte supply) result in improved immune checkpoint therapy effectiveness.

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Conclusion

As of today, CAF research is gathering speed in the immunotherapy field and there are multiple investigations going on in relation to CAFs, looking for strategies to interfere with their activation and/or functions, or attempting to revert them to a normal healthy phenotype with the hope that it would result in a normalized tumor stroma less favorable to tumor development. So far, CAF diversity and the multiplicity of functions they fulfill have prevented non-specific therapeutic attempts from yielding the desired tumor control. These findings suggest CAF-targeted immune-therapies have the potential to become efficient and a significant boost to immunotherapies, but will need to be aimed at specific CAF subtypes or functions[20,29].

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The right technology choice for your next scientific breakthrough

When it comes to choosing the right assay for your research, traditional technology almost always gives you the choice between going with ELISA (enzyme-linked immunosorbent assays) or Western Blot.

While these techniques are well known and widely used, others like AlphaLISA, HTRF (Homogeneous Time-Resolved Fluorescence), and LANCE (Figure 6) can also be implemented, providing you with additional benefits like ease of use, scalability, or the absence of washing, without compromising on sensitivity and specificity.

To facilitate the study of tissue fibrosis and accelerate the identification of effective drugs, Revvity has developed a platform of cell-based immunoassays based on highly proven technologies dedicated to the analysis of key intracellular and secreted profibrotic proteins and fibroblast activity biomarkers.

The following section provides you with key examples of data obtained using a selection of Revvity immunoassays.

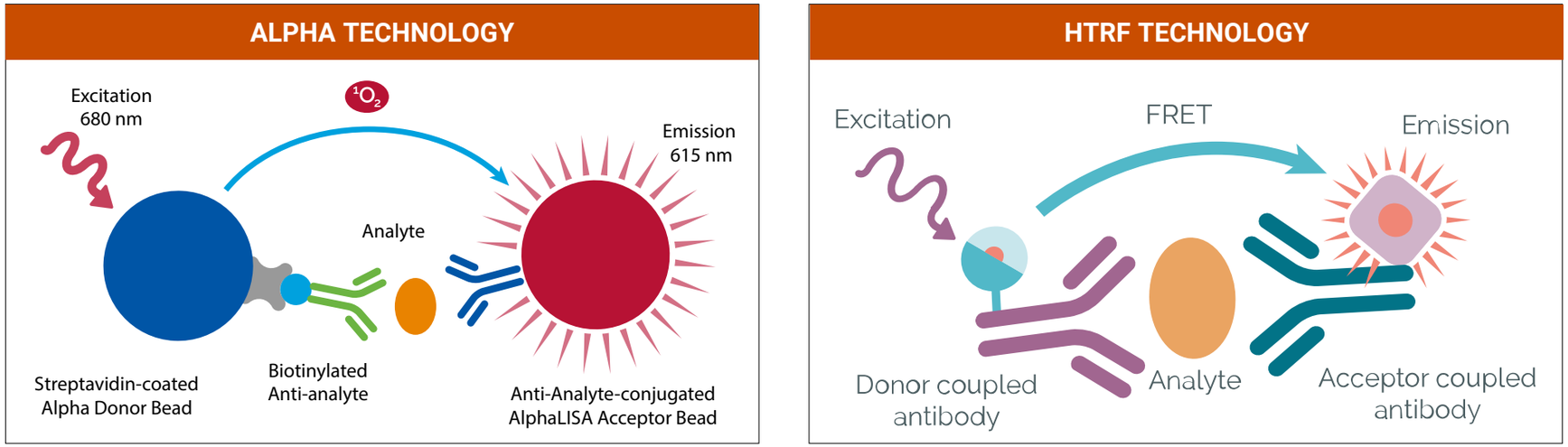

Figure 6: Alpha is a versatile, bead-based platform that enables you to assay the most complex samples in one well and with no wash steps. HTRF and LANCE are fast, sensitive, homogeneous, and ready to use assay platforms with no wash steps. Note that, the example illustrated here takes the example of an HTRF assay.

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Key fibroblast biomarkers

Myofibroblasts are characterized by de novo expression of **alpha-SMA**, which is incorporated into actin stress fibers and confers a high contractile activity to the cells. Chronic tissue injury (as tumors are considered to be) leads to persistent de novo formation of myofibroblasts (alpha-SMA+), excessive contraction, and deposition of ECM.

Fibronectin is a large glycoprotein of the ECM whose functions are connected to cell adhesion, growth, migration, and differentiation. Fibronectin level regulation is strongly related to inflammation and fibrotic disorders, as well as to the matrix re-organization that occurs in the environment of some cancers. Tumor containment, migration, and expansion is critically dependent on a tumor's surrounding matrix and key adhesive and/or fibrous proteins such as fibronectin. The EDA domain of fibronectin is an alternatively spliced version of fibronectin that is almost entirely absent from healthy tissues, and mostly expressed in active fibroblasts at injury or inflammation sites. Because of this special feature, **EDA-fibronectin** is a marker specific to fibroblast-induced fibrotic disorders, as well as being a readout of pro-fibrotic activity in a tumor matrix environment.

Matrix components such as **collagens** and **MMPs** are also direct markers of fibroblast activity as their deposition in the vicinity of wounds and inflammation sites is a hallmark of fibroblast-driven ECM remodeling.

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Human fibronectin EDA expression in cancer cell line after TGFβ1 treatment

The Adenocarcinomic human alveolar basal epithelial cells, A549, were plated in a culture-treated 96-well plate (50,000 cells/well) in complete culture medium and incubated at 37°C - 5% CO2. The next day, the cells were treated with 10 ng/mL of TGF-ß1 for 48 hours in serum-free culture medium supplemented with 1% BSA. The supernatant was collected, and cells were lysed. Lysate or supernatant were transferred into a low volume white microplate before the addition of the HTRF Human Fibronectin EDA or HTRF Human/Mouse Fibronectin detection antibodies. The HTRF signal was recorded after an overnight incubation. TGF-ß1 treatment results in an increase of Fibronectin EDA and Fibronectin expression level in lysate and supernatant, which demonstrates an upregulation of an epithelial–mesenchymal transition (EMT) marker, as expected.

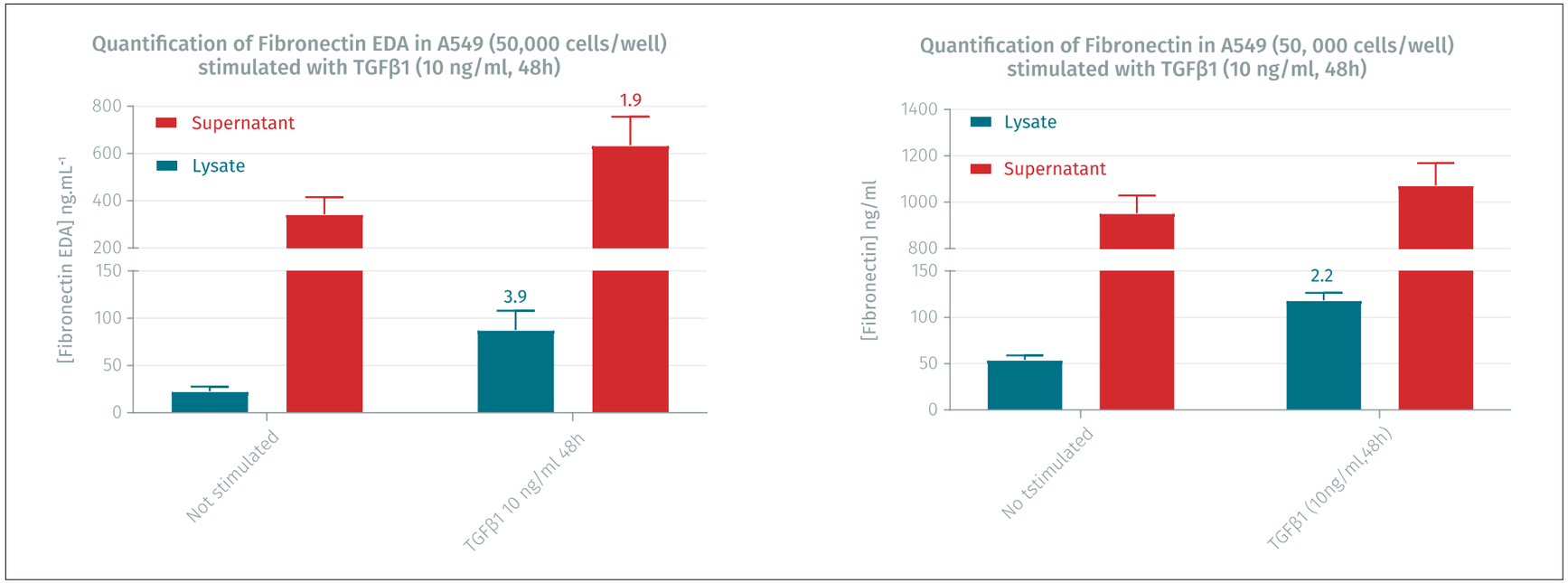

Figure 7: Human Fibronectin EDA expression in cancer cell line after TGFβ1 treatment.

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Alpha-SMA expression upon differentiation of fibroblasts into myofibroblasts

The mouse fibroblast cell line NIH/3T3 was plated in a culture-treated 96-well plate (6,250 and 12,500 cells/well) in complete culture medium and incubated at 37°C - 5% CO2. The next day, the cells were treated with increasing concentrations of TGF-ß1 for 48 hours in serum-free and antibiotic-free culture medium supplemented with 0.2% BSA. After medium removal, the cells were lysed with 200 µL of supplemented lysis buffer #3 for 30 minutes at RT under gentle shaking, and 16 µL of lysate were transferred into a low volume white microplate before the addition of 4 µL of the HTRF® Alpha-SMA detection antibodies. The HTRF signal was recorded after an overnight incubation. TGF-β1 long-term treatment results in a three-fold increase of Alpha-SMA expression level, which demonstrates the differentiation of fibroblasts into myofibroblasts.

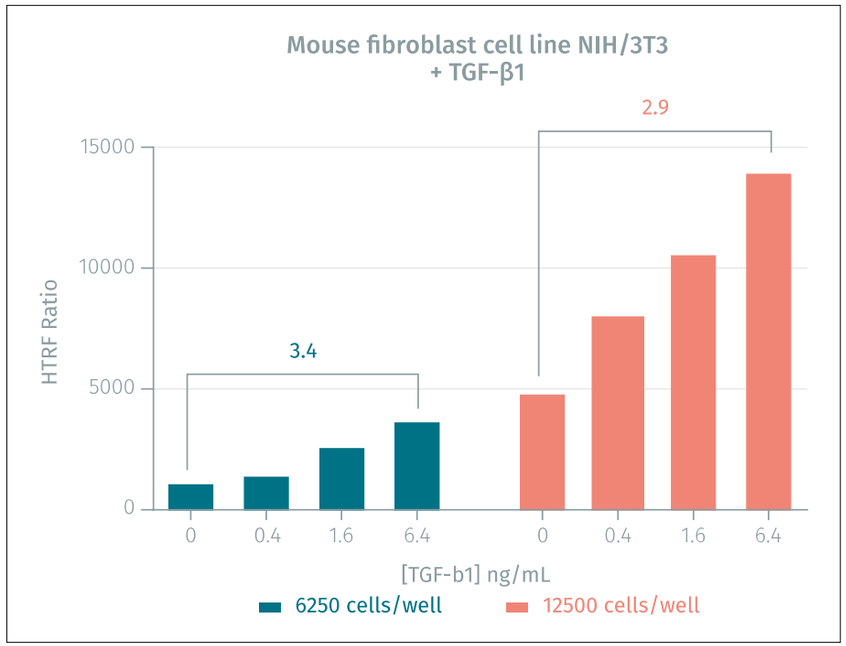

Figure 8: Alpha-SMA expression upon differentiation of fibroblasts into myofibroblasts

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Quantification of ECM molecules secreted by myofibroblasts

The LX-2 cell line (EMD Millipore, #SCC064) was plated in 96-well plates (50K or 100K cells/well) in complete medium. The next day, cells were treated with increasing doses of TGF-β1 or LPS diluted in serum-free medium with 0.2% BSA. After different incubation time periods (overnight to 48h), cell supernatants were collected and their concentrations of pro-collagen type 1 (HTRF assay 63ADK014PEG), TIMP1, MMP2, and MMP9 were quantified.

TGF-B1 Treatment

The profibrotic cytokine TGF-β1 induces the activation of the transcription factor SMAD3, responsible for the expression and secretion of pro-collagen type 1. This ECM molecule is then processed into mature collagen which accumulates in the extracellular space and participates in ECM deposition.

In hepatic fibrosis, the ECM-regulators MMP2 and TIMP1 are also key fibrogenic markers upregulated by TGF-β1 signaling.

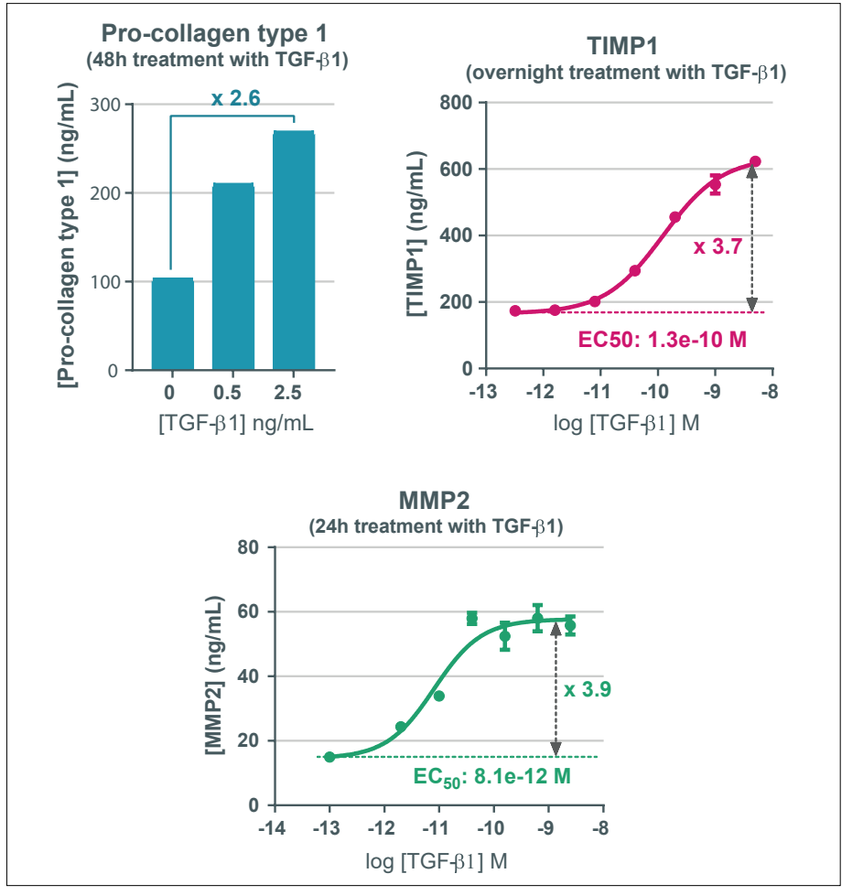

Figure 9: Pro-collagen type 1, TIMP1, and MMP2 analysis for myofibroblast activity in hepatic fibrosis model

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LPS Treatment

Cell treatment with LPS for 24h induces a 2.7-fold increase in the secretion of MMP9. This MMP is involved in the proteolytic activation of TGF-β1, and therefore represents another important fibrogenic marker.

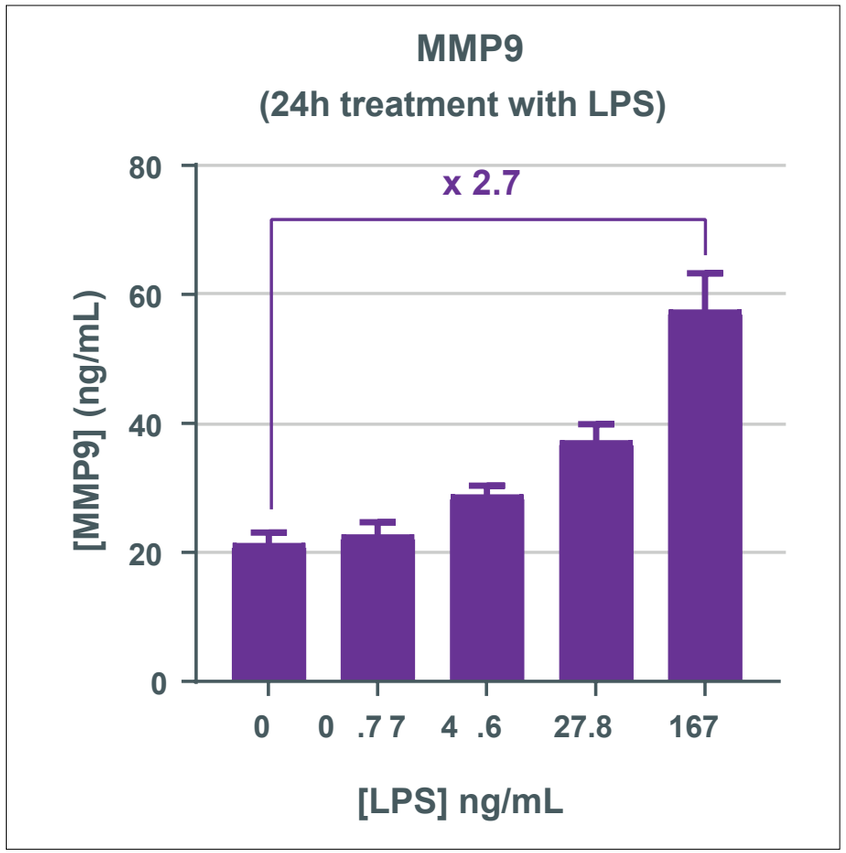

Figure 10: MMP9 analysis for myofibroblast activity in hepatic fibrosis model

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REVVITY OFFERING FOR TISSUE FIBROSIS STUDIES

Key fibroblast biomarkers

Target	AlphaLISA (part – 500 tests)	HTRF (part – 500 tests)	LANCE (part – 500 tests)
Alpha-SMA	AL3143C	62ASMAPEG	
Fibronectin (Human)	AL3056C		TRF1351C
Fibronectin (Human Plasma)	AL351C		
Fibronectin (Human/Mouse)	AL3174C	64HMFNPEG	
Fibronectin EDA	AL3175C	64HFNEDAPEG	

ECM components

Target	AlphaLISA (part - 500 tests)	HTRF (part - 500 tests)	LANCE (part - 500 tests)
Pro-collagen Type 1 (Human)		63ADK014PEG	
hPIP Collagen	AL353C		TRF1353C
Human COL1A1	AL371C		TRF1371C
Hyaluronic acid	AL354C	63ADK089PEG	

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Fibrogenesis pathway

Target	AlphaLISA (part - 500 tests)	HTRF (part - 500 tests)	LANCE (part - 500 tests)
TGF beta 1		62HTGFBPEG	
hTGF-#1	AL336C*		
hTGF-β1 BF	AL361C*		
SMAD1 phospho-S463/465	ALSU-PSM1-A500** / TBSU-PSM1-A500***/ TGRSM1S500	63ADK062PEG	TRF4011C
SMAD1 total	ALSU-TSM1-A500**	63ADK063PEG	
SMAD2 phospho - S465/467	ALSU-PSM2-A500**/TGRSM2S500	64SMAD2S5PEG	TRF4012C
SMAD2 total	ALSU-TSM2-A500**	64SMAD2TPEG	
Multiplex SMAD2-phospho/total	MPSU-PTSM2-K500		
SMAD3 phospho-S423/425	ALSU-PSM3-A500** / TBSU-PSM3-A500*** / TGRSM3S500[a]	63ADK025PEG	TRF4014C
SMAD3 total	ALSU-TSM3-A500**	64ND3PEG	

* AlphaLISA

** AlphaLISA *SureFire® Ultra™*

*** Terbium AlphaLISA *SureFire® Ultra™*

[a] Alpha Surefire®

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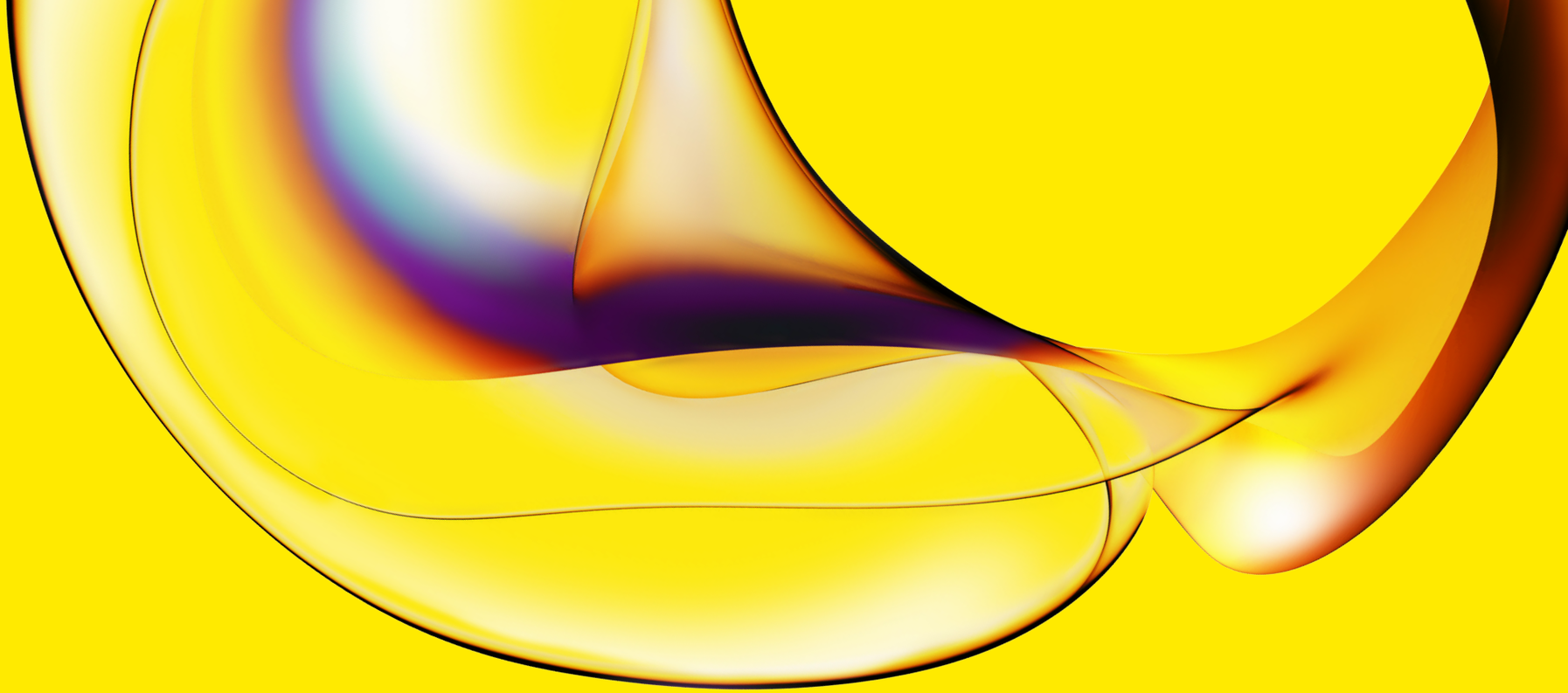

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