

GARP-expressing Tregs as a source of immunosuppressive TGF- β 1 in human tumors

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Abbreviation list

ABBREVIATION LIST

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α-SMA	Alpha smooth muscle actin
2D	Two dimensions
AEC	3-amino-9-ethylcarbazole
BEC	Blood endothelial cell
CAF	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
cDNA	Complementary DNA
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DNA	Desoxyribonucleic acid
dT	Deoxythymidine
ECM	Extracellular matrix
Em.	Emission
EMT	Epithelial mesenchymal transition
EndoMT	Endothelial mesenchymal transition
ER	Endoplasmic reticulum
EV	Extracellular vesicle
Ex.	Excitation
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
FOXP3	Forkhead box P3
H&E	Hematoxylin and eosin
HDST	High-definition spatial transcriptomics
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
IF	Immunofluorescence
IFN-α	Interferon-alpha
IHC	Immunohistochemistry
IL	Interleukin
IMC	Imaging mass cytometry
ISH	In situ hybridization
ISS	In situ sequencing
LAP	Latency associated peptide
LEC	Lymphatic endothelial cell
LH3	Lysyl hydroxylase 3
LRRC33	Leucine-rich repeat containing protein 33
LTBP	Latent TGF- β binding protein
MERFISH	Multiplexed error-robust FISH
MIBI	Multiplexed ion beam imaging
mIF	Multiplexed immunofluorescence
MMP	Matrix metalloproteinase

ABBREVIATION LIST

mRNA	Messenger ribonucleic acid
NGS	New generation sequencing
NSCLC	Non-small cell lung carcinoma
OCT	Optimal cutting compound
osmFISH	Ouroboros single molecule FISH
pTreg	Peripheral Treg
RCA	Rolling circle amplification
RNA	ribonucleic acid
scRNAseq	Single cell RNA sequencing
SeqFISH+	Sequential FISH +
smFISH	Single molecule FISH
TGF-β	Transforming growth factor-beta
TGFR	TGF- β receptor
TIL	Tumor-infiltrating lymphocyte
TOF	Time of flight
Tregs	Regulatory T cells
TSA	Tyramide signal amplification
TSP-1	Thrombospondin-1
tTreg	Thymic Treg
PDGF	platelet-derived growth factor
PDGFRβ	PDGF receptor beta
FAP	Fibroblast activation protein
NRP1	Neuropilin-1
BMP-9	Bone morphogenic protein 9
ANG1	Angiopoietin1
VEGF	Vascular endothelial cell growth factor
FGF	Fibroblast growth factor
AREG	Amphiregulin
EGF	Epidermal growth factor
PMA	Phorbol 12 myristate 13-acetate

ABSTRACT

Abstract

ABSTRACT

Abstract

TGF- β 1 is produced as a latent cytokine that requires activation to exert its pleiotropic activities. All cells produce latent TGF- β 1 but only very few can activate the cytokine via cell type-specific mechanisms. In cancer, TGF- β 1 favors disease progression by suppressing anti-tumor T cell responses. Which cells produce immunosuppressive TGF- β 1 in human tumors is not known. Putative sources include GARP-expressing cells, which comprise mostly activated Tregs and blood endothelial cells. GARP is a transmembrane protein that forms a covalent complex with latent TGF- β 1 (GARP:TGF- β 1) on the cell surface and which enables activation of TGF- β 1 by integrins. Our laboratory developed monoclonal antibodies that block the activation of TGF- β 1 on the surface of GARP-expressing Tregs and, when combined with anti-PD-1 therapy, restore anti-tumor responses in mouse tumor models that were otherwise resistant to anti-PD-1 monotherapy. Phase 1 and 2 trials currently assess the clinical impact of one of these anti-GARP:TGF- β 1 antibodies in patients suffering from various solid tumors. However, the prevalence of GARP-expressing Tregs in human tumors is still unclear. While GARP-expressing Tregs have been shown to activate TGF- β 1 in mouse models, it remains uncertain whether the same applies to human tumors. Moreover, the role of GARP expressed on blood endothelial cells is poorly understood.

We developed an experimental approach combining multiplexed immunofluorescence and computerized image analysis to study the spatial relationships between sources and targets of TGF- β 1 signaling in human tissues. We used this approach in the analysis of more than 100 tumor samples from 5 cancer types (colorectal, urothelial, lung and breast primary carcinomas and cutaneous melanoma metastases), compared to patient-matched adjacent non-cancerous tissues and tonsils. We conclude that, in human tumors, GARP-expressing Tregs rather than blood endothelial cells appear to represent a source of TGF- β 1 acting on nearby tumor-infiltrating T cells. This might contribute to a form of local immunosuppression that could be reversed with anti-GARP antibodies. Our approach could help distinguish patients with tumors heavily or poorly infiltrated by GARP-expressing Tregs in order to select those that are more likely to respond.

Introduction

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1 Cytokine-mediated communications between cytokine producing cells and responder cells in tissues

Tissue homeostasis requires the coordinated interactions between various types of cells. Cellular interactions are initiated by various stimuli and result in the activation of cellular signaling pathways. Stimuli include environmental factors, such as temperature or pressure, external factors such as pathogens, or signals from upstream cellular interactions. Among cellular interactions, cell-to-cell communication plays a significant role. It is mediated by a cell producing a signaling molecule, or ligand, and a target responder cell that expresses the receptor for the ligand [2, 3]. Binding of the ligand to its receptor induces a coordinated reaction in the responder cell.

Soluble factors are often used for intercellular communication. They are secreted by producer cells and act as ligands to specific receptors expressed by the target cells, leading to the activation of downstream signaling pathways in these cells. Soluble factors can be divided in two main families, even though overlaps exist between both. Growth factors group soluble factors capable of stimulating cell proliferation, wound healing and cellular differentiation. Cytokines are important immunomodulators, whose functions include the control of cell survival, growth and differentiation, as well as effector functions [4]. Dysregulation in cytokine-mediated communication can cause or favor pathologies such as auto-immune, inflammatory and neoplastic diseases. As my work focuses on the tumor immune-suppression, I'll limit my introduction to cytokines.

Cytokine signals can be classified according to the nature and respective distance between producer and responder cells.

1.1 Autocrine communication

In autocrine signaling, the cell that encounters a stimulus is both the producer of the cytokine and its cellular target (**Figure 1A**). This type of communication is common among leukocytes. Autocrine signaling is important for cellular and tissue homeostasis and during an immune response. For example, activated CD4⁺ T cells secrete interleukin-2 (IL-2) molecules which bind, among others, the IL-2 receptors on their own surface [5]. Similarly, regulatory T lymphocytes (Tregs) release active transforming growth factor-beta 1 (TGF- β 1), which acts, among others, on the Tregs themselves. TGF- β 1 signaling in Tregs is essential for the initiation and maintenance of the expression of the *forkhead box P3* (*FOXP3*) gene, a key transcription factor in Treg development and function [6].

1.2 Juxtacrine communication

Juxtacrine signaling involves a direct contact between the signal inducer and responder cells (**Figure 1B**). In the context of cytokine-mediated communication, the cytokine is anchored to the membrane of the producer cell and binds to the receptor of the responder cell during a cell-to-cell contact. For example, the precursor of the TGF- α (pro-TGF- α) is expressed as a transmembrane protein [7]. Pro-TGF- α expressed by bone marrow stromal cells (BMSCs) interacts with the

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epidermal growth factor receptor expressed on adjacent BMSCs and induces calcium uptake [7]. Another example is IL-15, often presented in complex with IL-15R α on the cell membrane of human monocytes, which is able to interact with the IL-15 receptor expressed on T and B cells and to induce their proliferation [8].

1.3 Paracrine communication

In paracrine signaling, the cytokine diffuses from the inducing cell to nearby responding cells within the same tissue (**Figure 1C**). These paracrine factors act over short distances, typically limited to a few microns [9, 10]. Paracrine communication is the most common cytokine-mediated way of communication. For example, type 1 interferon-alpha (IFN- α), secreted by immune cells, fibroblasts and endothelial cells upon viral infection, induces an anti-viral response in dendritic cells and natural killer cells [11]. Another example of a paracrine factor is IL-2. While it acts in an autocrine manner, it also functions in paracrine signaling by inducing the expansion and differentiation of adjacent T cells [12]. Chemokines are a subfamily of cytokines that attract leukocytes via paracrine communication. They participate in the homing of T cells to inflamed organs [13]. At the onset of an inflammatory response, various cell types such as immune cells, endothelial cells, epithelial cells and fibroblasts secrete proinflammatory chemokines that spread in the surrounding tissue, thus forming a gradient that attracts leukocytes towards the site of inflammation [14].

It has long been thought that cytokines diffuse only in a soluble form. However, it is now acknowledged that cytokines can also transit in extracellular vesicles (EV) [15]. Beside some cytokines, EVs can also transport various molecules that are not efficiently transported in a soluble form, such as lipids or messenger ribonucleic acid (mRNA).

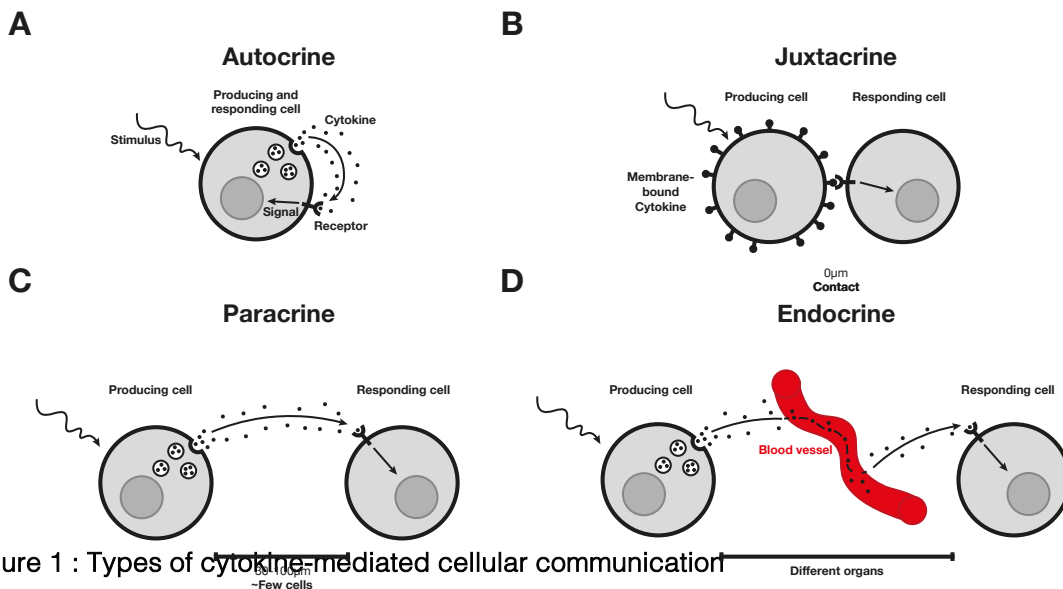


Figure 1 : Types of cytokine-mediated cellular communication

All types of cytokine-mediated cellular communications are initiated by an upstream stimulus and result in the activation of cellular signaling pathways. A. Autocrine signaling involves cells that both produce and respond to a secreted cytokine. B. Juxtacrine signaling involves cell pairs that communicate through a physical contact via a membrane-bound cytokine and a cognate receptor. C. In paracrine signaling, the cell that secretes a cytokine is located close to the responder cell, which expresses the cognate receptor. D. In endocrine signaling, the cytokine is secreted by producer cells in an organ, transported via the blood stream and is recognized by the cognate receptor on responder cells situated in a distant organ.

1.4 Endocrine communication

In endocrine signaling, the signaling molecules, called endocrine factors, reach responder cells present in distant organs via the blood stream (**Figure 1D**). Major endocrine factors are hormones, which are produced by the endocrine system to regulate systemic processes in physiology and behavior. In certain conditions, cytokines can also exert systemic effects. This includes pro-inflammatory cytokines, such as IL-1 β and IL-6, which are released by leukocytes at sites of acute inflammation, reach the hypothalamus via the blood stream and induce fever [16]. Massive systemic and uncontrolled secretion of pro-inflammatory cytokines in hypercytokinemia, also known as cytokine storm, is an example of pathogenic cytokine-mediated endocrine communication [4]. One example of cytokine storm is the toxic shock syndrome, a rare but fatal disease that is caused by *Staphylococcus* or *Streptococcus* infection [17]. During the infection, the pathogens produce toxins that trigger a massive systemic release of inflammatory cytokines, leading to respiratory failure and vascular leakage and can be fatal. Another example is the cytokine release syndrome caused by inflammatory cytokines resulting from the infusion of chimeric antigen receptor T cells (CAR T cells) for the treatment of B cell lymphoma [18].

1.5 Methods to study cytokine-mediated cell-to-cell communications in tissues

The study of cytokine-mediated communications in tissues can be challenging for two main reasons.

First, intercellular signaling may involve a multiplicity of producer cells, cytokines and responder cells. Inflammation is an example of such complex interactions. All the types of signaling described in the previous section can take place simultaneously in tissues (**Figure 2**). For example, IL-2 produced by a lymphocyte can target both the cell producing it (autocrine) and neighboring lymphocytes (paracrine). Tissues consist of multiple cell types, which may include different types of cells producing and responding to the same cytokine, a phenomenon known as pleiotropy. Patterns of cytokine production and cytokine response may differ from one cell type to another. For example, level of expression of the receptor, or expression of co-receptors may differ from one cell type to another, thereby influencing the cytokine response. Endoglin, a co-receptor of the TGF- β receptor highly expressed by proliferating endothelial cells, can influence downstream SMAD signaling upon TGF- β stimulus [19]. Thus, in the presence of endoglin, the TGF- β receptor activates the SMAD1/5/8 pathway rather than the SMAD2/3 pathway, which is turned on in its absence. Finally, multiple cytokines may circulate within the same tissue. Redundancy in the context of cytokine signaling consists in activation of the same signaling pathway by two or more cytokines. For example, during inflammation, the NF- κ B signaling pathway is activated among others by IL-1 β , by TNF- α or by damage- or pathogen-associated molecular patterns (DAMPs and PAMPs) [20]. Another example is that of the type I receptor- γ chain subunit common to the receptors of the IL-2, -4, -7, -9, -15 and -21 cytokines, which all activate the same JAK-STAT-mediated signaling pathway via this common subunit [21]. Redundancy is also found among the TGF- β 1, - β 2 and - β 3 cytokines, all of which bind to the same TGF- β receptor and activate the same SMAD pathway.

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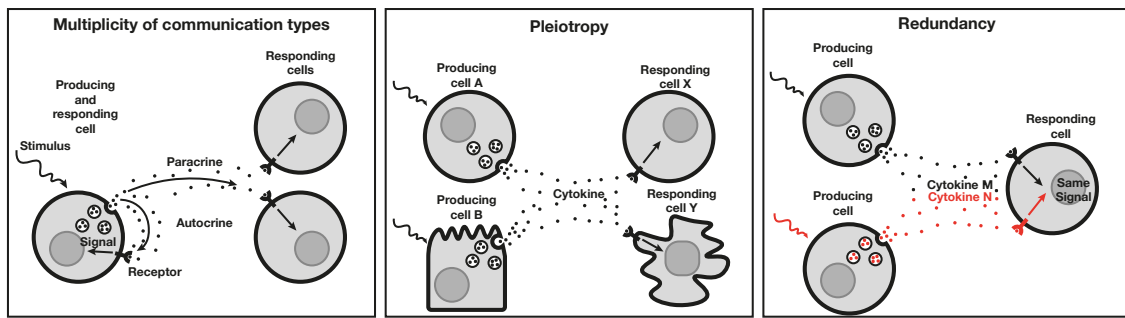


Figure 2 : Complexity in cytokine-mediated cell-to-cell interactions

All types of cell communications can take place simultaneously (left panel). The same cytokine can be involved in different cell to cell communications, a property known as pleiotropy (middle panel). Different cytokines can cause the activation of the same signaling pathway, a property referred as redundancy (right panel).

A second difficulty in studying cytokine-mediated signaling in tissues arises from spatial and temporal changes. Paracrine factors diffuse locally, forming high-concentration gradients, called niches [9]. Heterogeneity in cytokine exposure causes variability in cell responses. It is assumed that in the context of paracrine communications, cytokines can travel $\sim 100\mu\text{m}$, but distances longer than $800\mu\text{m}$ were observed for $\text{IFN-}\gamma$ diffusion in a mouse tumor model [10]. How far cytokines can diffuse in tissues depends on several parameters: the density of cells that take up the cytokine around the producer cells, the rate of cytokine consumption per cell, the molecular diffusion coefficient and other variables. In 2017, a study was conducted to determine and model the cytokine niche in co-cultures of IL-2 producing cells, IL-2 consumer cells and IL-2 non-responsive cells [9]. The greater the density of consumer cells around producer cells, the shorter the distance travelled by the cytokine before getting consumed. Co-cultures of IL-2 producer cells with pure responder cells gave $30\mu\text{m}$ -wide niches, while co-cultures of IL-2 producer cells with 10% consumer cells and 90% of non-responsive cells gave $100\mu\text{m}$ -wide niches. Moreover, the size of the niche is also defined by the rate of consumption of the cytokine per consumer cell. This measurement is influenced by parameters including the amount and avidity of the receptor. The size of the niche also depends on the molecular diffusion coefficient of the cytokine in the extracellular space. This parameter reflects the capacity of a given substance to diffuse in a given milieu at a given temperature. This coefficient is unique to each cytokine and broadly depends on the weight and on the shape of the cytokine [22]. Cytokine-mediated communication is also limited in time because of the high degradation rates of cytokines *in vivo*. Kinetics of cellular response after cytokine-receptor binding may also have to be considered in the study of cytokine-mediated communications. How fast and how long the signal may be induced in responder cells upon cytokine exposure is critical and could compromise a study. Finally, cell motility in tissues can complexify the observation of cytokine-mediated communications.

Recent technological advances improve our possibilities to study cytokine-mediated cell-cell communications in tissues at the cellular level. New labelling technologies, high-resolution imaging techniques, high-throughput sequencing techniques and advanced computational tools have led to

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the availability of tools for the study of spatially resolved (sub)cellular expression of multiple genes in tissues in 2 dimensions (2D), 3D and, upon addition of time variable, also in 4D.

1.5.1 Analysis of tissue sections (2D analyses)

Histology and staining

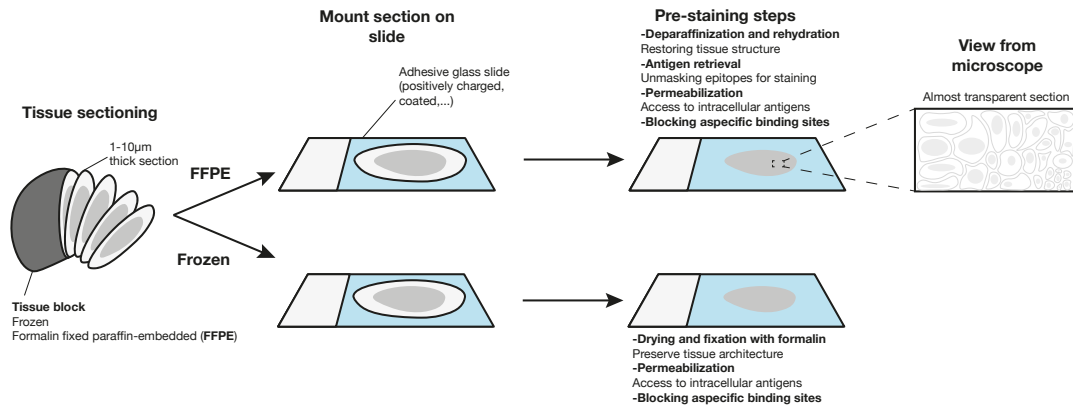
Histology is the study of the morphology of cells and structures within their natural tissue environment. Still today, histology is an essential tool used by anatomopathologists to identify disease state and guide diagnosis. It is also frequently used in many aspects of biomedical research to visualize cellular processes and to study tissue architecture.

Freshly resected or biopsied tissues intended for long-term storage are typically snap-frozen and cryopreserved, or are immersed in formaldehyde, dehydrated and embedded in paraffin. The latter is the preferred technique in histology and anatomopathology, because it offers practical archiving possibilities, such as room temperature storage and longer shelf life compared to frozen tissues. It also offers better preservation of tissue architecture. Moreover, formalin-fixed paraffin-embedded (FFPE) tissue sections can undergo aggressive chemical and physical treatments without damaging the tissue structure. Despite these advantages, cryopreservation can be preferred over FFPE for two main reasons. First, formaldehyde breaks proteins, DNA and RNA present in the tissue and forms crosslinking bridges between the fragments, thus reducing the quality and quantity of macromolecules extracted from the tissue to run analyses such as RNA sequencing. Second, FFPE treatment can alter the structure of epitopes recognized by antibodies used in immunostaining. Consequently, antibodies that bind conformational antigens in a frozen section may not recognize them in a FFPE section. For example, despite testing various conditions, none of the many monoclonal antibodies either commercially available or developed in our laboratory targeting GARP, the protein on which the current work is focused, were effective on human FFPE tissues, although some did work on frozen sections [23].

A tissue section undergoes several steps before being analyzed under a microscope (**Figure 3**). First, tissues are cut in 1-10 μ m thick sections and placed on an adhesive glass slide. Slides undergo several pre-staining steps that depend on their embedding. FFPE slides are first deparaffinized and rehydrated to restore tissue structure. Additionally, FFPE sections undergo an antigen retrieval step, which consists in heating at nearby boiling point in a buffered solution, in order to unmask and restore the 3D structure of tissue antigens. Frozen sections usually undergo a short fixation in formaldehyde after thawing. Both FFPE and frozen sections are permeabilized with a detergent to allow the access of the reagents within cells and nuclei. Finally, in the case of immunostaining, molecules that bind the antibodies nonspecifically are blocked with serum or with milk to avoid false positive staining. Unstained sections are almost totally transparent under the microscope. They are treated with chemical dyes to visualize cells and tissue structures. A combination of hematoxylin and eosin (H&E) is the standard staining used to highlight nuclei in blue and cytoplasm in red, respectively.

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Preparation of the tissue section



Histologic staining

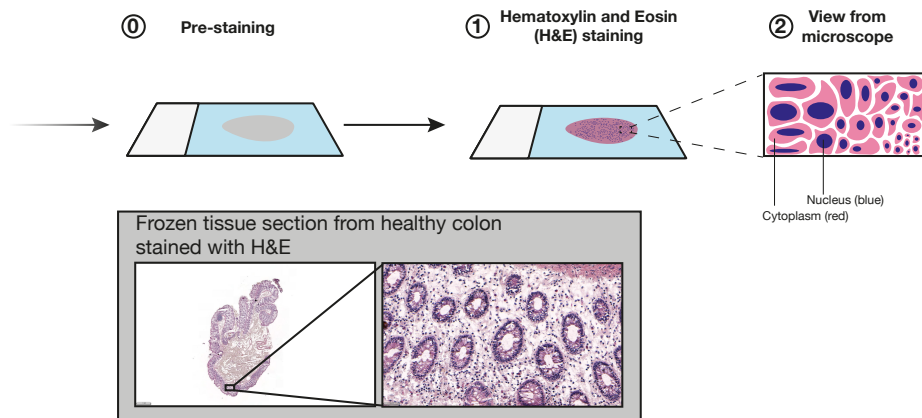


Figure 3 : Histological tissue preparation and staining

Tissues are preserved either by freezing at -80°C or after fixation in formaldehyde and embedding in paraffin.

Unstained tissues are almost transparent under the microscope. Chemical dyes, such as hematoxylin and eosin (H&E) allow cell visualization by staining the nuclei in blue and the cytoplasm in red, respectively.

Immunostaining

Antibodies can be used to stain specific antigens in tissues. Antibodies used in immunostaining are often coupled to reagents that allow their detection in tissue sections. In **immunohistochemistry** (IHC), antibodies are coupled to an enzyme that, upon further incubation with a chromogenic dye, catalyze its local precipitation or binding. In **immunofluorescence** (IF), antibodies are coupled to fluorochromes that can be visualized with a fluorescence microscope. The fluorescent signal can be amplified with secondary antibodies and enzymatic reactions with fluorescent substrates. In **multiplexed immunofluorescence** (mIF), several antigens of interest are stained on the same tissue section using distinct fluorochromes. Antibodies can also be tagged with specific heavy metals or oligonucleotides, which allow to multiply the number of different antigens that can be visualized. These techniques are further detailed below.

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Immunohistochemistry (IHC)

In IHC, chromogenic dyes precipitated by enzyme-coupled antibodies are used to stain an antigen of interest (**Figure 4**). After pre-staining, the tissue section is incubated with an antibody targeting the antigen. The antibody is coupled to an enzyme, often horseradish peroxidase (HRP), which catalyzes the precipitation of a chromogenic substrate, commonly 3,3'-diaminobenzidine (DAB, brown color) or 3-amino-9-ethylcarbazole (AEC, red). Before being examined under a microscope, the slide is counterstained with hematoxylin to stain the nuclei. IHC can be used in many applications, for example to identify the cancer cells in melanoma samples after staining with an antibody directed at a protein present exclusively in melanoma cells.

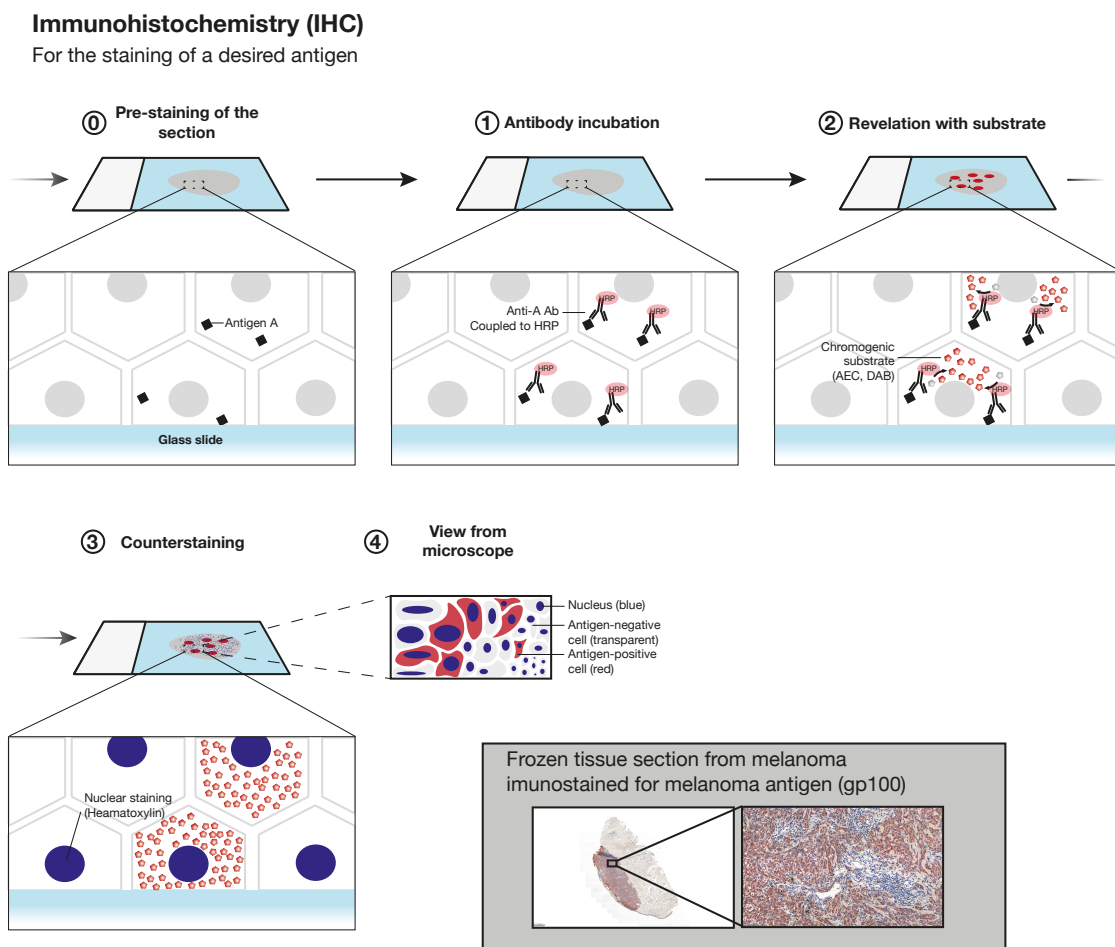


Figure 4 : Immunohistochemistry (IHC)

In immunohistochemistry (IHC), enzyme-coupled antibodies are used to stain desired antigens in tissue sections with chromogenic dyes.

For example, IHC can be used to detect a melanoma antigen

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Immunofluorescence (IF)

In IF, antibodies are coupled to fluorochromes, and the fluorescence is detected using a fluorescence microscope (**Figure 5**). Fluorochromes are molecules that, upon excitation with light at a given wavelength range that differs from one fluorochrome to the other, absorb the light, become excited, and return to baseline after emission of light at higher wavelength than the excitation light. The emitted light is very weak but can be detected by sensitive cameras after filtering to distinguish it from the excitation light.

After staining with the fluorochrome-bound antibody, the sections are counterstained with a fluorescent DNA-intercalating dye, usually 4',6-diamidino-2-phenylindole (DAPI) or Hoechst, to stain the nuclei. More than with IHC, an amplification of the signal is often necessary in IF. Several molecules present in the tissue such as hemoglobin, elastin and collagen have autofluorescent properties and cause unwanted fluorescent signals.

The signal to noise ratio is often poor when using directly coupled fluorochrome antibodies. Several methods can be applied to amplify the signal. First, instead of using a fluorochrome coupled antibody that targets the antigen, one can use the antibody uncoupled, referred as the primary antibody, then follow with a secondary antibody that binds the Fc-fragment of the primary antibody. This secondary antibody, which is coupled to the fluorochrome, is polyclonal, which allows it to bind multiple sites on the primary antibody, thus amplifying the signal. Primary and secondary antibodies are usually derived from different animal species (e.g. a mouse 1° and a goat 2° antibody, the latter obtained by immunization of goats with mouse immunoglobulins). A second way to increase the signal is to use tyramide signal amplification (TSA). TSA uses secondary antibodies coupled to HRP, which reacts with fluorochrome-bound tyramide molecules. Tyramide, the reactive form of tyramine, similar to the tyrosine amino acid, is highly reactive and binds covalently to neighboring proteins containing aromatic amino acids. In addition to an amplified signal, the strong binding of the fluorochrome at the place of the antigen ensures that the signal will resist subsequent aggressive treatments of the tissue sections such as antibody denaturation or elution, which allows to repeat the process with another antibody and fluorophore (see multiplexed immunofluorescence below). Finally, an additional step of signal amplification can be obtained by using a secondary antibody that is coupled to a dextran polymer to which multiple HRP molecules are bound. It is noteworthy that these signal amplification methods can be combined with each other, thus achieving particularly high signal to noise ratios, easily distinguishable from background and autofluorescence. They can also be used in IHC staining, alone or in combination.

Multiplexed immunofluorescence (mIF)

In multiplexed immunofluorescence (mIF), several antigens can be detected and visualized on the same tissue section by using, for each target antigen, its cognate antibody associated with a distinct fluorochrome (**Figure 6**). The main limitation in multiplexing fluorochromes is determined by the number of fluorescence channels available on the slide scanner (see below). In the absence of

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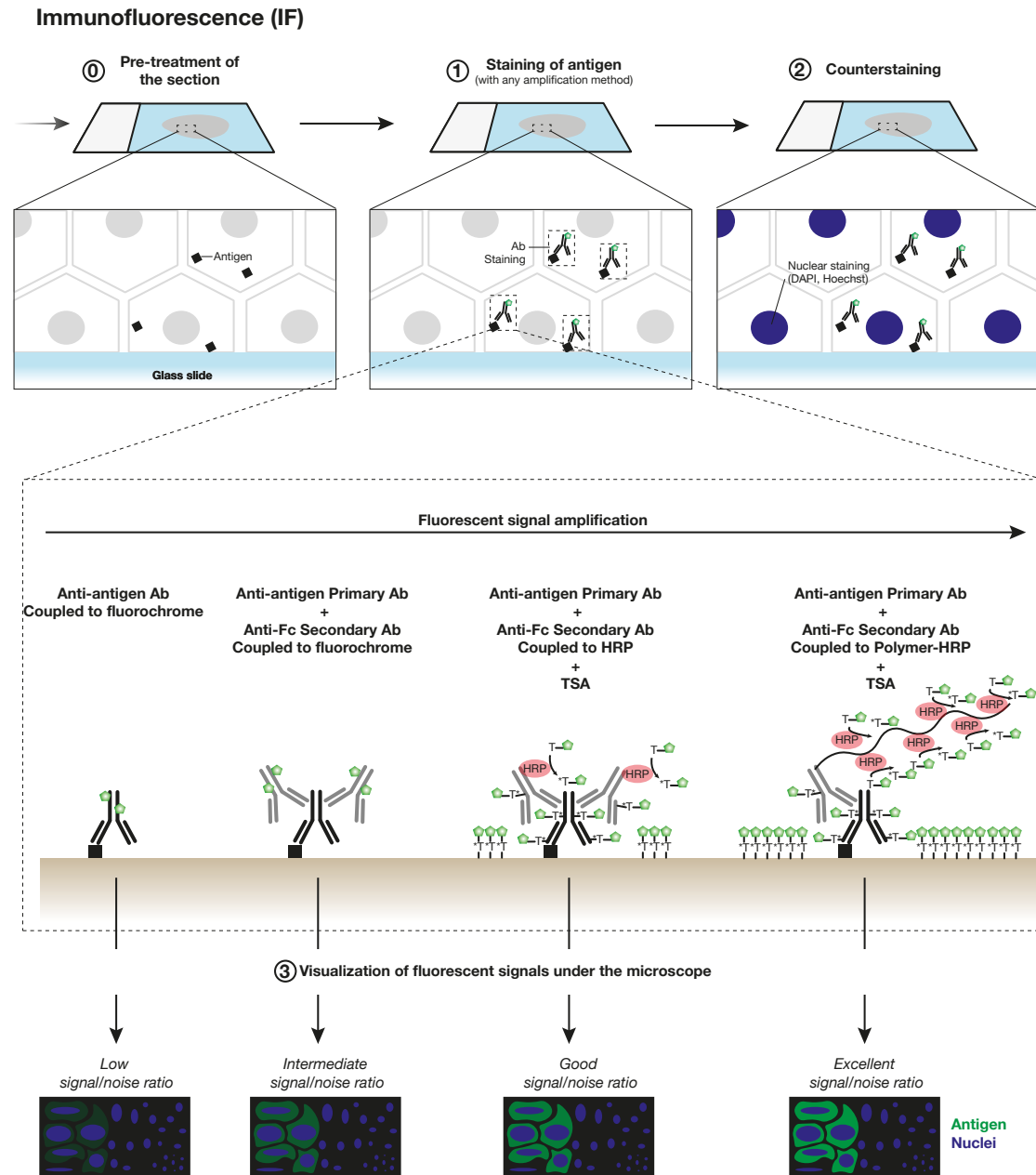


Figure 5 : Immunofluorescence (IF)

In immunofluorescence, antibodies are coupled to fluorochromes, and the fluorescence is visualized through a fluorescence microscope. Signal amplification is often necessary to improve signal/noise ratio.

amplification, directly coupled fluorochrome antibodies can be incubated simultaneously on the tissue section. When using a dual antibody technique, the primary antibodies must have distinct isotype and/or species origin, and the fluorochrome-bound secondary antibodies must match these isotypes and species. With TSA, the staining steps must be repeated for each antigen. Briefly, the section is incubated with the first primary antibody, followed by the HRP-coupled secondary antibody and finally by the tyramide-fluorochrome reaction. The section is then treated by heating under controlled pH to inactivate the HRP and to denature or elute the antibodies, in order to avoid cross-reactivity issues. This process is then repeated sequentially for each additional antigen. A

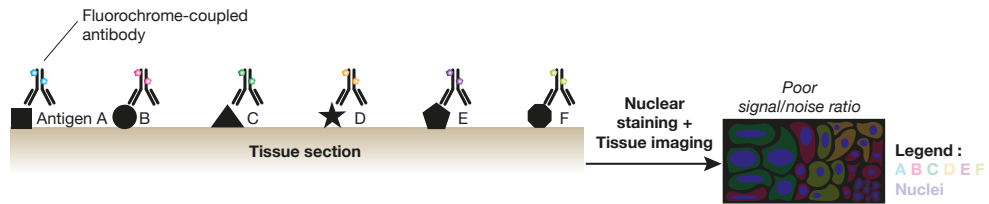
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major advantage of sequential TSA staining, apart from amplified signal, is the fact that it is no longer necessary to use primary antibodies with distinct isotype or species origin. However, the sequential staining steps make this technique much more labor intensive.

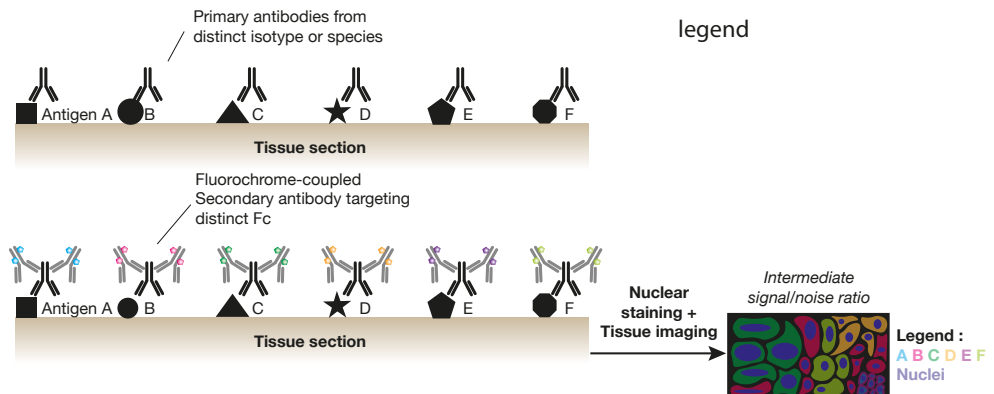
To increase the number of different antigens that can be visualized on mIF images, an alternative method, known as cyclic immunofluorescence, was invented [24] (**Figure 7**). This method consists in staining 1 to 4 different antigens either with fluorochrome-conjugated antibodies or with combinations of primary antibodies and fluorochrome-conjugated secondary antibodies. After the staining, the slide is imaged. Then the slide is treated to inactivate the fluorochromes and/or elute the antibodies. The same tissue section can undergo the same cycle of staining with different antibodies, imaging and inactivation for multiple iterations. The iterative 1 to 4-plex mIF images can be superimposed together and result in a single mIF image that can combine up to >100 antigens [25]. This method is now used in automated slide staining-scanning systems.

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Antibody directly coupled to fluorochrome



Primary antibody + secondary antibody coupled to fluorochrome



Tyramide (T) Signal amplification with Horse radish peroxidase (HRP) -coupled secondary antibodies

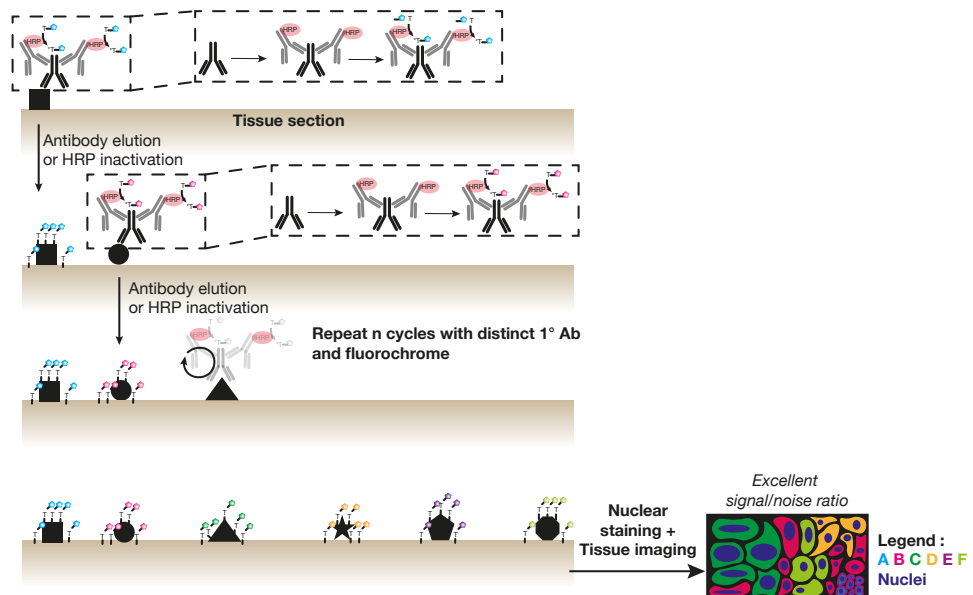


Figure 6 : Multiplexed immunofluorescence (mIF)

In multiplexed immunofluorescence (mIF), multiple antigens are visualized on the same tissue section using antibodies labeled with distinct fluorochromes. As in IF, signal amplification is required to improve the signal/noise ratio.

Cyclic immunofluorescence

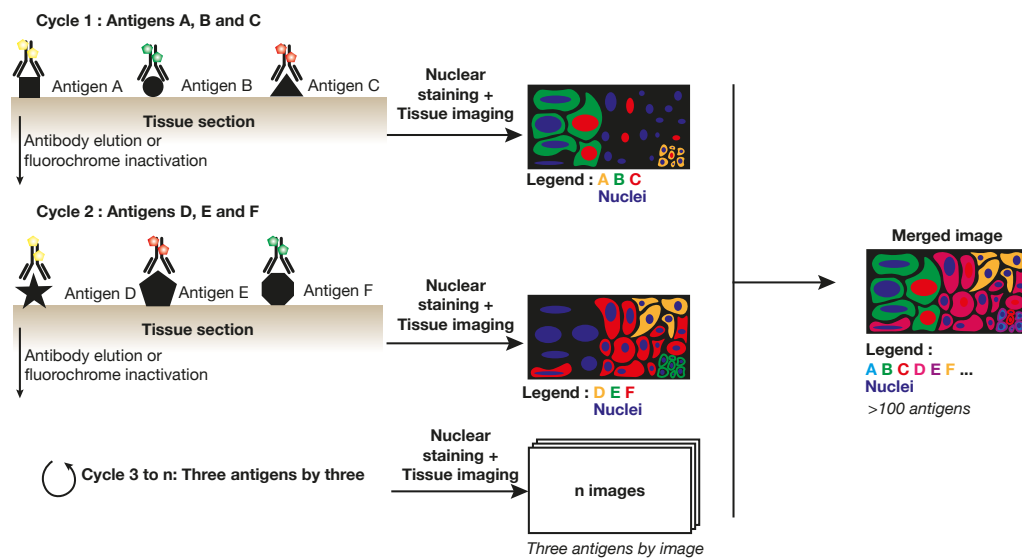


Figure 7 : Cyclic immunofluorescence (Cyclic IF)

Cyclic immunofluorescence allows to stain multiple antigens by using repeated sequential staining cycles. In each staining cycle, one to four antigens are stained, either with fluorochrome-coupled antibodies or with a combination of primary antibodies with fluorochrome-coupled secondary antibodies.

Fluorescence microscope

The fluorescent microscope is used to visualize the fluorescence signals from IF and mIF stained slides (**Figure 8**). Each fluorescence microscope is equipped with a light source, either mercury lamp or LEDs, which excites fluorochromes. LEDs tend to be favored over mercury lights because they have narrower emission spectra and are more easily operated through the microscope software. The excitation light source passes through an excitation filter, which narrows the spectrum to wavelengths that can excite the fluorochrome. Then, the excitation light is reflected by a dichroic mirror. This mirror reflects light under a certain wavelength and transmits higher wavelengths. The objective lens focuses the light onto the sample. The fluorochrome absorbs the excitation light and returns emission light, which is weaker and has a higher wavelength range. This light passes through the objective lenses in the opposite direction and passes through the dichroic mirror. The fluorescence is then filtered by an emission filter whose role is to remove unwanted light signals from the excitation light or from autofluorescent structures in the sample. Finally, the filtered emission light is captured by a highly sensitive black-and-white camera and transformed into a digital image.

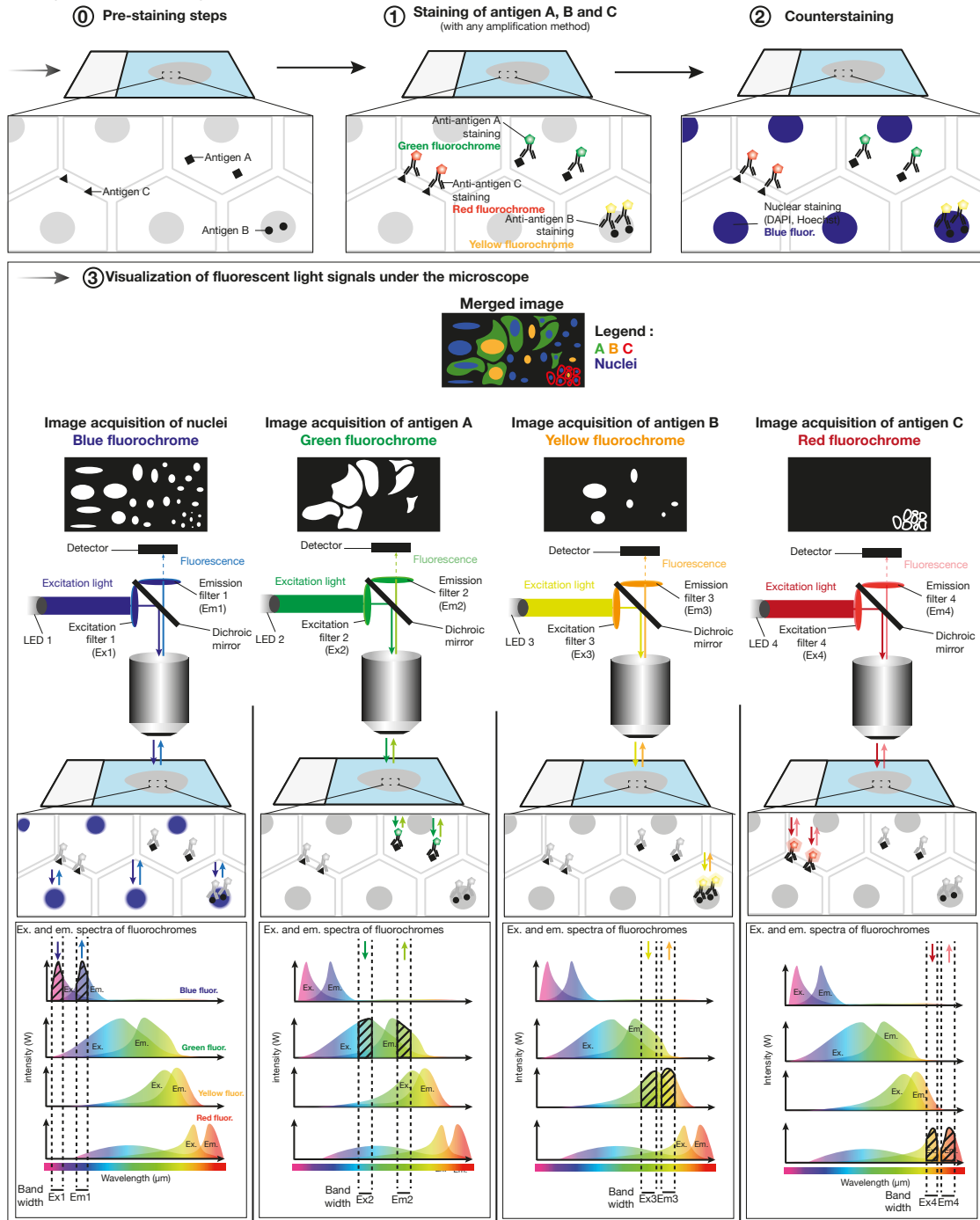
In mIF, each fluorochrome possesses a unique excitation spectrum and emission spectrum. Fluorescence microscopes are fitted with several LED lamps and independently switchable filter sets (each containing an excitation filter, a dichroic mirror and an emission filter) to separately excite the fluorochromes and collect their emitted light. Each staining is acquired sequentially. After the acquisition of all fluorochromes, the images are superimposed to generate a multichannel fluorescence image, in which each fluorochrome can be 'recolorized' digitally by the operator (i.e. nuclei in blue, antigen A in green,...). A fluorescence slide scanner is a fluorescence microscope equipped with a motor stage that moves the tissue section from top to bottom and left to right across the whole tissue section or selected area. It rasters the section and captures digital images at each position. Its associated computer then stitches the images together in order to reconstitute a high-resolution digital fluorescence image of the entire tissue section. **Figure 9** shows a section of frozen melanoma metastasis stained with mIF.

mIF has often been used to study cytokine-mediated cell-to-cell communication. In most publications, cytokines, which are mostly soluble proteins, are not a suitable target for mIF antibodies. Indeed, cytokines are produced at very low concentration (pM range) and, in the case of inflammatory cytokines, are released for only short periods, making them difficult to detect. Besides, the staining of cytokine is expected to be very diffuse, making it challenging to identify the producer and the target cells. Instead, antibodies used in mIF panels are selected to identify markers unique to the phenotype of producer cells or target cells.

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Multiplexed IF (mIF)

Example with three antigens



Antibodies coupled to heavy metals

Mass-based protein imaging uses distinct metal-tagged antibodies to identify specific protein targets within tissues. Antibodies are revealed with a time-of-flight (TOF) mass spectrometer which can capture differences in metal tag masses. Two main competing platforms exist: the multiplexed

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Figure 8 : Fluorescence microscope setup

Each fluorochrome has a unique excitation and emission spectrum. In mIF, the role of the fluorescence microscope is to excite and capture the light emitted by each fluorochrome separately with no spillover. To achieve this task, microscopes are equipped with multiple switchable LED lights and filter sets (i.e. dichroic filter, excitation and emission filters). The light of the LED passes through an excitation filter, which filters the light of wavelengths within a defined bandwidth. Then, the filtered light is reflected on a dichroic mirror and is directed on the tissue by objective lenses. Once excited, the fluorochrome emits fluorescence, which goes through the objective lenses. The fluorescence is transmitted by the dichroic mirror and filtered by the emission filter to remove background fluorescence out of a desired bandwidth. The fluorescence is then captured by a detector and converted into an image. Each fluorochrome is acquired sequentially, and the images of each fluorescence are superimposed to create a multichannel fluorescence image which can be artificially recolored (i.e. nuclei in blue, antigen A in green, ...).

ion beam imaging (MIBI; IonPath) and the Hyperion imaging mass cytometry (IMC; Standard BioTools). Both platforms can image up to 40-plex in one single image, limited by the number of stable metal isotopes available on Earth [26]. The main advantage of mass-based protein imaging over mIF is the limited channel spillover. However, mass-based protein imaging is impractical for high-throughput imaging because of high acquisition times. 1 mm²-wide scan can take up to 2 hours [27].

Antibodies coupled to oligonucleotides

Antibodies used to detect antigens can be conjugated with antigen-specific oligonucleotides of deoxyribonucleic acid (DNA). Two methods of staining of the oligonucleotide exist, either using fluorescent probes or sequencing techniques. The major advantage of oligonucleotide barcoded antibodies is the easiness of combination with spatial transcriptomics technologies, as both can share the same readout technology.

Spatial transcriptomics

Unlike traditional RNA sequencing which provides bulk measurements of gene expression without spatial dimension, spatial transcriptomics enables researchers to map and quantify the expression of multiple genes at cellular or even subcellular resolution in a tissue section while preserving the spatial information. The spatial gene expression values are combined with a high-resolution digital image of the tissue section obtained by slide scanning, after immunohistochemical or immunofluorescent staining [28].

FISH-based method

In 1969, in situ hybridization (ISH) was first used to localize specific sequences of DNA and RNA in tissues. Initially, isotope-labelled probes revealed the targets with autoradiography [29]. In 1980, the technique evolved to fluorescent ISH (FISH), in which probes are coupled to a fluorochrome (**Figure 10**) [30]. FISH was originally used to detect and localize the presence or absence of genes in chromosomes. Almost 20 years later, in 1998, single molecule FISH (smFISH) was introduced, enhancing the sensitivity of FISH and enabling to detect single transcripts. smFISH uses shorter probes that bind multiple sites of a transcript, amplifying the fluorescent signal.

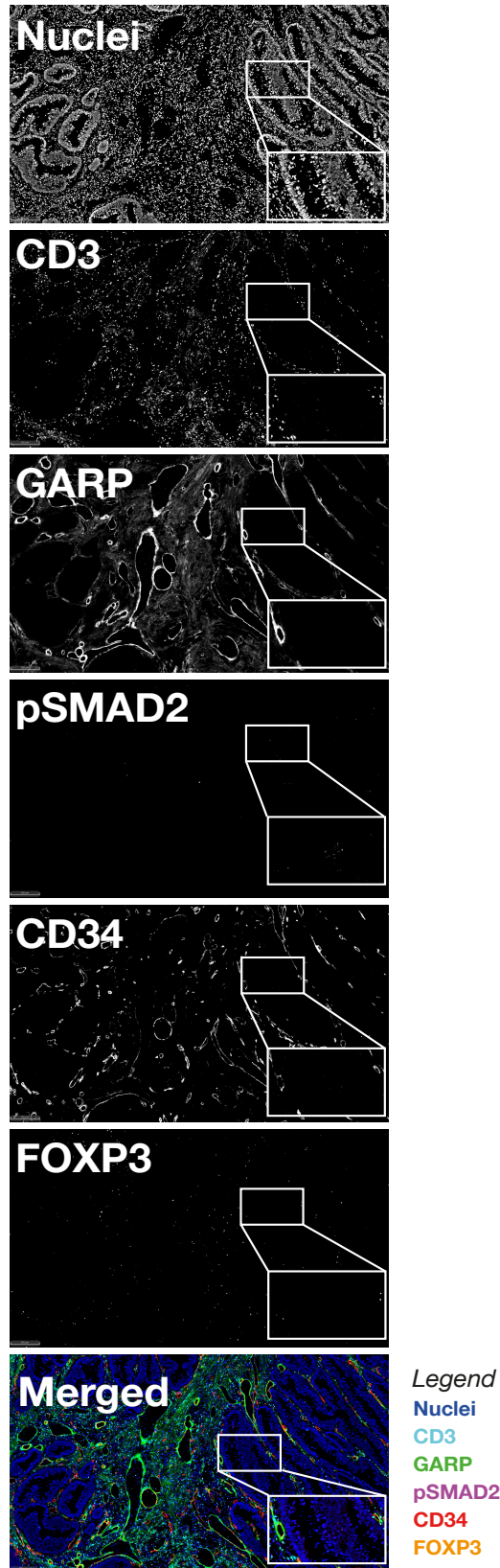


Figure 9 : Section of a frozen melanoma metastasis stained with mIF with antibodies targeting CD3, GARP, phosphSMAD2 (pSMAD2), CD34 and FOXP3.

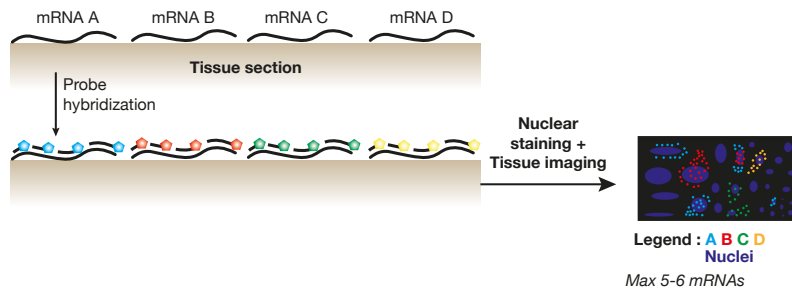
Similarly to cyclic IF, cyclic FISH has allowed to increase the capacity of multiplexing. Ouroboros smFISH (osmFISH) is an example of cyclic smFISH [31]. In each round, two or three transcripts are hybridized with distinct fluorochrome-coupled probes. The section is then imaged, and the probes are detached with formamide. OsmFISH was used to localize 33 transcripts in a mouse cortex section [31].

Another group of smFISH derived techniques uses transcript barcoding, which drastically increases the achievable multiplex number (>10 000-plex) [28]. Barcoded FISH is a technique used to identify transcripts by assigning to each a combination of fluorescent labels that is assessed by sequential rounds of probe hybridization. It starts with the hybridization of 'encoding probes' which contain a region that binds a specific transcript and one or several transcript-specific 'readout sequences'. In sequential hybridization rounds, the readout sequences are labeled with fluorochrome-coupled probes. Between each round, the section is imaged, and the fluorochrome-coupled probes are annealed. The images are then superimposed all together to align the fluorescent readouts of each transcript. The presence or absence of signal, along with the type of fluorochrome in each round, recreates the unique barcode for each transcript. Multiplexed error-robust FISH (MERFISH) and sequential FISH + (seqFISH+) are two examples of barcoded FISH-based methods [32, 33]. MERFISH works with one fluorochrome, while seqFISH+ uses three. MERFISH includes redundancy in the barcodes which renders them more robust to errors. Commercial

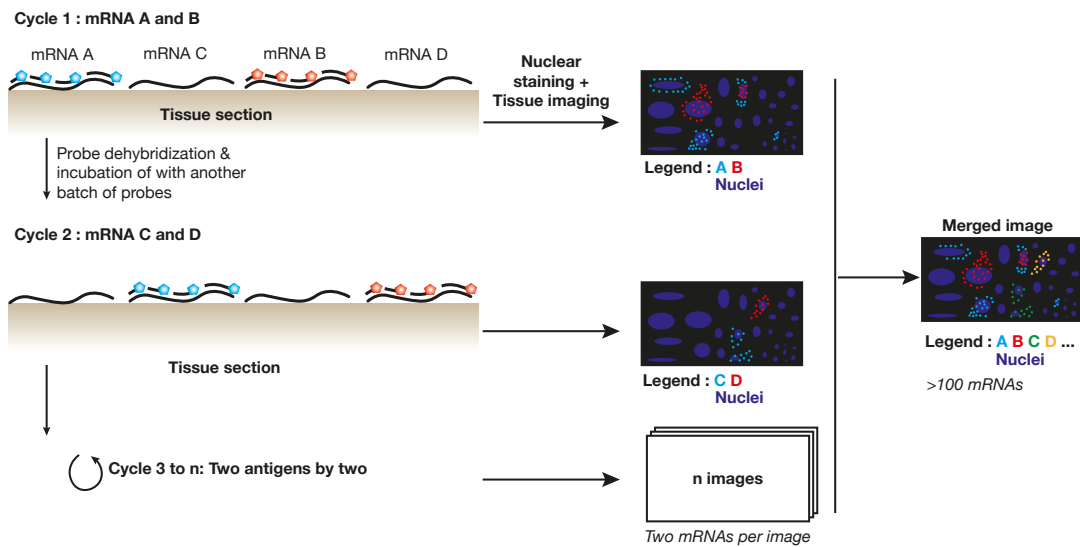
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barcode FISH based tools include Molecular Cartography (Resolve Bioscience) and CosMX (Nanostring).

Single molecule fluorescent in situ hybridization (smFISH)



Ouroboros smFISH (osmFISH)



Barcoded smFISH (SeqFISH+)

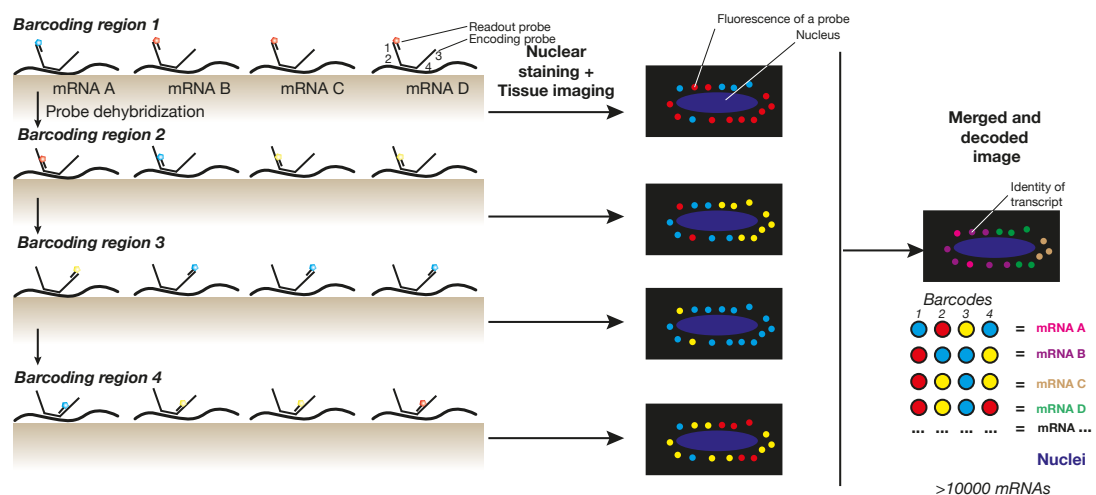


Figure 10: Fluorescent in situ hybridization (FISH) derived techniques

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High-throughput sequencing-based method

Spatial barcoding of transcripts is a recent approach in which the transcripts from a tissue section are captured by probes which possess a spatial identifier sequence [34] (**Figure 11**). Probes are either bound directly to the chip in spots or attached to beads that are deposited on the chip prior to tissue section application. Each spot of the chip or to each bead possesses a unique spatial identifier. Transcripts are captured by hybridization of their poly-A tail to an oligo-dT sequence on the probe. Following the capture, complementary DNA (cDNA) is synthesized *in situ*, incorporating the spatial barcode. cDNA's are retrieved from the chip and sequenced by high-throughput sequencing (HTS). The gene expression can be linked to tissue locations using the spatial barcode. Visium (10x Genomics), Slide-seq (ref [35]) and High-definition Spatial Transcriptomic (HDST, ref [36]) are tools available to perform transcript spatial barcoding. In Visium, probes are bound to the slide in 55 μ m-wide spots, a spatial resolution not small enough to study gene expression at cellular resolution. However, the latest release of Visium, Visium HD, now enables single-cell resolution with spots of 2 μ m. In Slide-seq and HDST, probes are bound to 10 μ m and 2 μ m-wide beads, respectively [28].

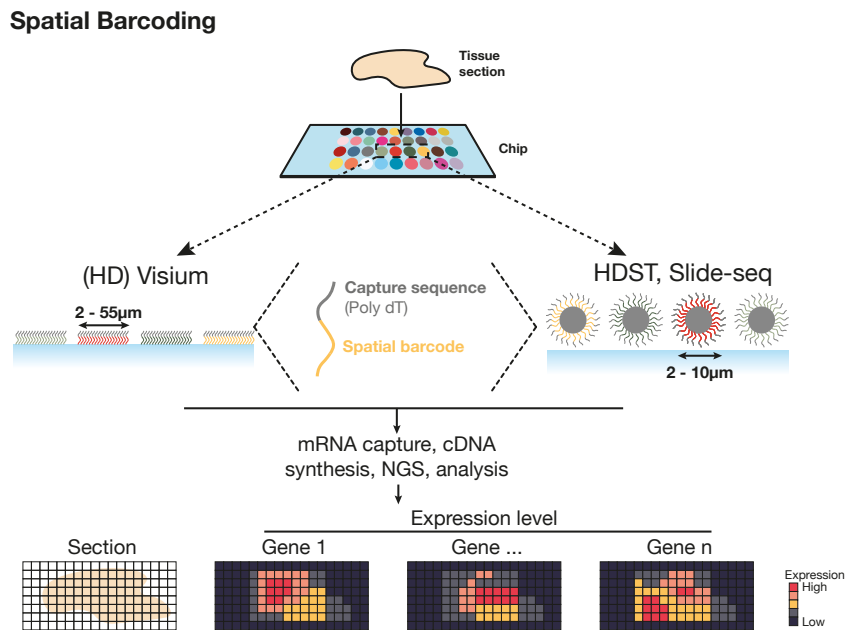


Figure 11 : High-throughput sequencing-derived spatial transcriptomics

Spatial barcoding of transcripts allows the capture of all transcripts in a tissue section using probes with unique spatial identifier sequences. In Visium, the probes are directly tagged on the chip in spots of 55 μ m (2 μ m for HD-Visium). In high-definition spatial transcriptomics (HDST) and in Slide-seq, beads tagged with the oligonucleotides are placed on the chip. The tissue section is placed on top of the chip. Then, the transcripts are captured by a poly-deoxythymidine (dT) tail present on the probes, followed by a cDNA synthesis, high-throughput sequencing (HTS) and analysis of the results. The output consists in a spatially resolved expression map of each gene.

In situ sequencing

In situ sequencing (ISS) yields spatial transcriptome information by sequencing transcript barcodes directly within the tissue [37] (**Figure 12**). First, transcripts are reverse transcribed in

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complementary DNA (cDNA). A pool of padlock probes then hybridizes to the cDNAs of interest. The extremities of the padlock probes are then ligated, followed by a rolling circle amplification (RCA), creating localized linear amplification of the padlocks. Then, the amplicons are interrogated by sequencing. Sequencing is done by ligation, where each cycle identifies a single nucleotide using fluorescently labelled probes which are degenerated except for one query nucleotide. Multiple rounds of imaging are used to build the full sequence. Similar to what was previously explained, the images of all sequencing rounds are aligned together, and the barcodes are decoded to retrieve the transcript identities. Xenium (10x Genomics) is a commercial example of ISS. Signal amplification provided by the RCA increases the sensitivity of the technique and allows to map weakly expressed genes.

In situ sequencing (ISS)

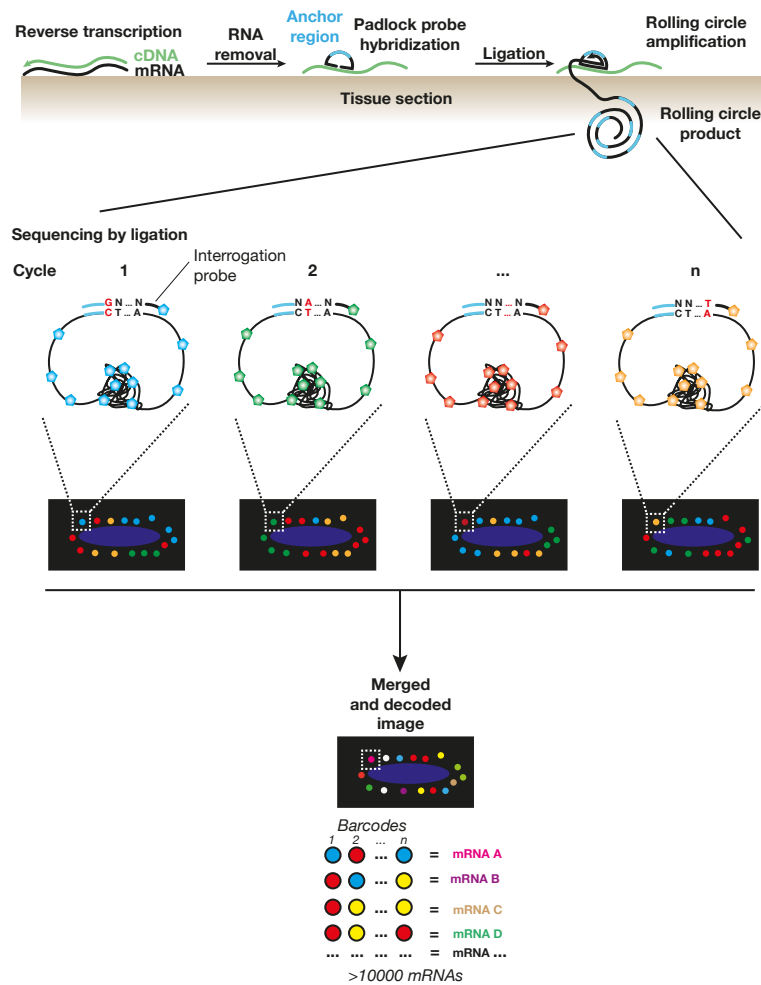


Figure 12 : *In situ* sequencing

Inspired from [1]

Multi-omics

Individually, spatial transcriptomics and immunostaining do not allow to capture the full cellular information. Spatial transcriptomics generally allows a higher plex number than immunostaining but does not capture protein expression, emphasizing the need for a multi-omics approach. Spatially resolved (single cell) multi-omics is a new trend in sciences, ranked as one of the seven most awaited technologies in 2022 [38]. It groups all the technologies that allow to capture several spatial -omics data in a tissue section. Notably, it allows to combine, on the same section, the analysis of the transcriptome with the analysis of proteins. An easily applicable approach consists in combining smFISH with antibodies tagged with fluorochromes. Commercial technologies include Molecular Cartography (Resolve Biosciences) and GeoMX (Nanostring). Oligo-coupled antibodies can be used in combination with barcoded probes, with revelation methods including fluorescence or NGS. Visium (10x Genomics) uses spatial barcodes to label transcripts and oligonucleotide-tagged antibodies. Xenium (10x Genomics) does not provide the possibility to include antibodies to the set of probes.

Multi-omics also includes recent technologies that allow to capture the genome or epigenome [38]. DNA-SeqFISH+ and DNA-MERFISH are two examples of combined spatial genomics and transcriptomics [39, 40]. ATAC&RNA-seq and CUT&Tag-RNA-seq are two recent technologies that allow to combine spatial transcriptomics with a spatial analysis of chromatin accessibility, a major genomic determinant of gene expression, to near single cell resolution [41].

1.5.2 3D imaging, with or without the time component

Recent advancements in organoid culture unleashed the need for 3-dimensional (3D) imaging technologies. While traditional 2D imaging is valuable for most applications, 3D imaging is required for specific studies. Tissue structures such as blood vessels or neuronal networks can be difficultly appreciated in 2D. Complex spatial distribution of cells or rare cell populations can necessitate the need for a 3D analysis of a tissue. A time consuming but basic approach to record 3D images consists in stacking 2D images of serial tissue sections (**Figure 13**). Progresses in image registration now allow to align several hundreds to thousands 2D images, acquiring data of >1cm³ tissues [27]. In 2022, the team of Denis Wirtz achieved to register over 4000 H&E images of 13 normal and tumoral pancreatic tissues up to 3.5cm³ with CODA, a deep learning-based algorithm [42]. To a larger extent, all the 2D imaging techniques reviewed previously can be used for 3D serial imaging. In 2023, 3D cyclic mIF was performed on serial slides of a colorectal tumor sample [43]. 3D spatial transcriptomics with spatial barcodes was used in serial sections of biopsies of inflamed synovia from chronic rheumatoid arthritis patients [44].

In some applications such as intravital imaging, non-destructive imaging techniques are required [45]. Initiated by confocal microscopy, 3D tissue microscopy allows to capture the 3D information

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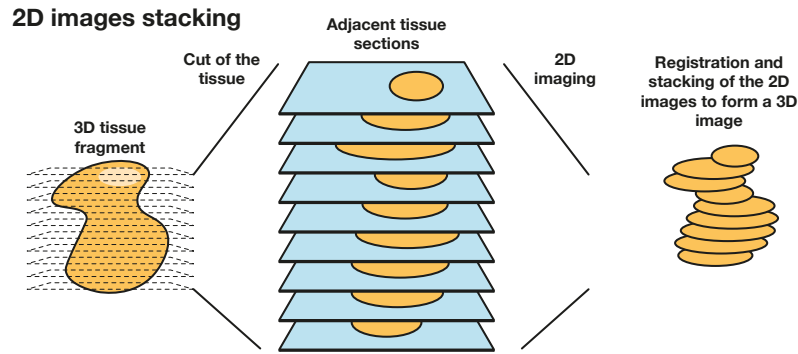


Figure 13 : Stacking of 2D images

2D-images of serial sections can be aligned to create a stacked 3D image of a tissue.

of a tissue, mainly by fluorescence, without having to section it [46]. It includes confocal microscopy, laser multiphoton microscopy and light sheet microscopy. The main limitation of conventional widefield fluorescence microscopy is that it detects the light emitted from the entire thickness of a tissue sample, resulting in poor resolution. Confocal microscopy possesses a pinhole filter situated before the detector and whose role is to filter the light emitted from a particular depth of the tissue, also known as focal plane. With a confocal microscope scanner, tissues can be scanned at serial focal planes, resulting in a 3D image. The maximal scanning depth is limited to a few hundred micrometers, explained by the low penetrance of excitation light into the sample. Tissue clearing techniques can help to increase the optical transparency of tissues, thereby improving the resolution as well as increasing the maximal scanning depth [47]. Laser multiphoton microscopy uses lights of longer wavelengths, which are more penetrant (up to 1mm depth) than those used in confocal microscopy. As photons of longer wavelengths are less energetic than those of shorter wavelength, multiple photons are required to excite a fluorochrome. In multiphoton microscopy, no pinhole filter is required, as only the fluorochromes of a known focal point are sufficiently excited to emit fluorescence. The focal point corresponds to the point where all photons of the laser light source converge and supply sufficient energy for fluorescence emission. Both confocal and multiphoton microscope scanners require long scanning time, as they must raster each focal plane of a 3D image. The light-sheet microscope uses a thin plane of light, rather than a light beam, to image the tissue. All fluorescence signals emanating from the excitation plane are captured at once by a highly sensitive and high-capacity detector placed perpendicularly to the excitation plane. The light-sheet microscope scanner requires much lower scanning times to get a 3D image of a tissue, as it does not require to raster each focal plane. Light-sheet microscopy unravels the possibility to track high-speed 3D biological phenomenon, such as interactions of cells and viruses before infection [48]. In 2022, the team of Liberali used a light sheet microscope to study the 3D live growth of healthy intestinal organoids by capturing time-lapsed images [49]. Development of entire zebrafish embryo was captured with a light-sheet scanner at subcellular resolution [50].

1.6 Publications studying cytokine-mediated cell-to-cell communications in tumors

Below, the reader will find a short summary of recent publications that studied cytokine-mediated cell-to-cell communications in tumors, highlighting the techniques that were employed.

The team of Raue performed scRNAseq on cell suspensions of 6 untreated syngeneic mouse tumor models (CT26, MC38, B16-F10, EMT6, LL2 and Sa1N) to calculate receptor-ligand 'interaction scores' between various cell types [51]. The expression levels of cytokines and receptors were used to infer the presence of a communication mediated by a cytokine between cells in the tumor. Interaction scores were calculated as the product of the average expression of the ligand and the average expression of the matched receptor for each cell type. This work demonstrates the presence of cytokine-mediated communications by inference [52]. Studies of inferred interactions from scRNAseq data lack the spatial dimension, which is crucial to study cytokine-mediated cell-to-cell communications.

A second publication studied cytokine-mediated communication in a highly modified mouse tumor model, designed to simplify the study of a single cytokine (IFN- γ) between one producer cell type (CD8⁺ T cells) and one target cell type (tumor cells) [10]. The tumors were composed of two OVCAR5 tumor cell lines embedded in Matrigel, which were either engineered to express an IFN- γ -inducible fluorescent reporter, or transduced to express a CDK4 tumor neoantigen. The tumors were transplanted in immunodeficient mice (NGS mice) and were injected with CD8⁺ T cells directed against the neoantigen two weeks thereafter. In the tumors, CD8⁺ T cells killed the antigen-expressing tumor cells and released IFN- γ , which diffused in the tumor and bound to the receptor of fluorescent reporter cells. Using time-lapsed intravital imaging with a multiphoton microscope, the team identified that IFN- γ produced by CD8⁺ T cells could travel up to 800 μ m in the tumor 40h after T cell injection. This publication is useful to study the IFN- γ cytokine niche. However, this highly controlled and simplified tumor model only partially reflects the complexity of cytokine communications taking place *in vivo*.

In cancer patients, the majority of publications I found used biopsy sections stained with mIF to study the proximity of cell types as a biomarker of disease outcome or therapy response [53-58]. In these publications, distances between various immune cell types or between immune cells and tumor cells were measured, with no attention given to cytokine communications. Nonetheless, in one publication, the phosphorylation of SMAD3, used as a marker to identify cells undergoing TGF- β signaling, was included in the mIF panel [59]. pSMAD3 expression in immune cells was correlated with the survival of patients with early-stage non-small cell lung carcinoma (NSCLC). More precisely, the team identified that a high frequency of FOXP3⁺ Tregs positive for pSMAD3⁺ in close vicinity (<30 μ m) of CD8⁺ T cells within the tumor identified a rapidly progressing group of patients with NSCLC. Concerns may arise from this study, notably because the high local density of Tregs in close proximity to CD8⁺ T cells can be explained by a general increase in Treg density in tumors, irrespective of their proximity to T cells.

Together, these studies highlight the need for new approaches that encompass the complexity of cytokine-mediated cell-to-cell communication in tumors.

2 TGF- β 1 biology from the perspective of cellular sources and responder cells

The TGF- β superfamily consists in a large, conserved group of diverse growth factors that regulate vital cellular processes, such as cell proliferation, differentiation, communication, apoptosis and tissue remodeling. It is composed of more than 40 members divided in several subfamilies, including bone morphogenetic proteins, growth differentiation factors, activins, inhibins, nodal and the TGF- β family [60]. In mammals, the TGF- β family is composed of three isoforms, TGF- β 1, TGF- β 2 and TGF- β 3. The three isoforms are encoded by three distinct genes, share a high sequence homology and bind to the same receptor, the TGF- β receptor (TGFBR). TGF- β 2 and TGF- β 3 are involved in embryogenesis and development, while TGF- β 1 plays an important role in immune system regulation. As TGF- β 1 is the main isoform involved in the regulation of immune responses and has many implications in cancer, the present work focuses on this isoform.

2.1 Cells producing latent TGF- β 1

2.1.1 Latent TGF- β 1 synthesis and secretion

The TGF- β 1 protein is synthesized as a precursor that comprises, from the N-terminal to the C-terminal end of the sequence, a signal peptide sequence that directs the nascent TGF- β 1 to the endoplasmic reticulum (ER), the latency associated peptide (LAP) and the mature TGF- β 1 (mTGF- β 1) polypeptide (**Figure 14**). The signal peptide is removed once the precursor enters the ER. In the ER, the precursor forms a homodimer covalently bound by three interchain disulfide bonds. A furin endoprotease cleaves the dimer between the LAP and mTGF- β 1 sequences. The resulting two dimeric fragments form a non-covalent complex in which mTGF- β 1 is enclosed in LAP. “Latent TGF- β 1” refers to this non-covalent complex. Latent TGF- β 1 is biologically inactive, as LAP masks the binding sites of mTGF- β 1 to the TGFBR. To exert its activity, mTGF- β 1 must be released from LAP in a process referred to as “TGF- β 1 activation” [61]. The LAP comprises a rigid “arm” domain and a flexible “straitjacket” domain. The mTGF- β 1 is held fastened to the LAP by a region of the straitjacket referred to as the “latency lasso”.

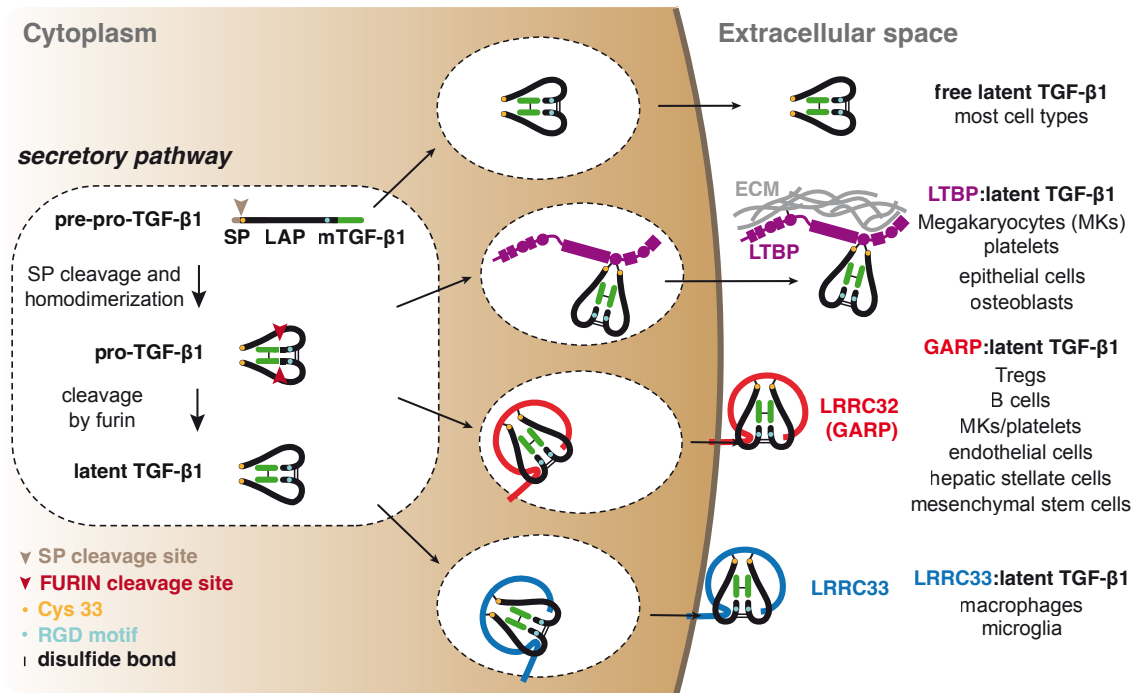


Figure 14 : Schematic representation of TGF-β1 processing and trafficking

TGF-β1 is first produced as a precursor protein called pre-pro-TGF-β1. Following the cleavage of its signal peptide (SP) and homodimerization through disulfide bond formation, the pro-TGF-β1 is then cleaved by the Furin enzyme. The cleaved homodimer is called "latent TGF-β1". Its N-terminal fragment, or latency associated peptide (LAP), remains non-covalently bound to the C-terminal fragment, named mature TGF-β1 (mTGF-β1). Latent TGF-β1 is secreted by many cell types in a soluble form. Specific cell types can produce latent TGF-β1 in covalent association to tethering proteins such as LTBP, GARP or LRRC33. To bind to its receptor, mTGF-β1 must first be released from LAP in a process referred to as "TGF-β1 activation".

2.1.2 Latent TGF-β1 is associated with LTBPs or LRR presenting proteins that control its activation

Latent TGF-β1 can be secreted by virtually all cells in a free soluble form. It can also be complexed by disulfide bonds to another protein, either latent TGF-β1 binding protein (LTBP), glycoprotein A repetition predominant (GARP), or leucine-rich-repeat-containing protein 33 (LRRC33). LTBP is bound to the extracellular matrix (ECM), whereas GARP and LRRC33 are transmembrane proteins.

LTBP:latent TGF-β1 complexes (LTBP:TGF-β1)

The LTBP family comprises four extracellular matrix proteins, LTBP-1, -2, -3 and -4 [62]. LTBPs are large multi-domain proteins produced by many cell types, including mesenchymal cells. They were initially discovered during the purification of TGF-β1 from platelets [63]. All LTBP members, except LTBP-2, can form a covalent complex with latent TGF-β1. LTBPs possess a binding motif that enables them to bind to extracellular matrix compounds such as fibronectin or fibrillin.

GARP:latent TGF- β 1 complexes (GARP:TGF- β 1)

GARP, a transmembrane protein encoded by the LRRC32 gene, is a member of the leucine-rich repeats-containing (LRR) protein family. LRR proteins contain a structural α/β horseshoe fold motif. This motif is composed of 20-30 amino acids enriched in the hydrophobic amino acid leucine. In GARP, a long horseshoe motif is associated with a transmembrane domain and a short cytoplasmic tail. GARP expression has been widely documented on the surface of activated Tregs [64-66]. GARP is also expressed by activated B lymphocytes, activated platelets and megakaryocytes, blood and lymphatic endothelial cells (BECs and LECs, respectively), perivascular cells, hepatic stellate cells, fibroblasts and mesenchymal stromal cells [67-73]. GARP has two cysteine residues in its extracellular domain that covalently bind LAP:latent TGF- β 1 complexes and thus present the cytokine on the cellular membrane. Soluble GARP:TGF- β 1 complexes were observed in cultured activated Tregs and may result from the shedding of membrane-bound GARP:TGF- β 1 complexes by proteases [66]. The 3D structure of GARP:TGF- β 1 complexes was solved via analyses of X-ray diffraction by GARP:TGF- β 1 crystals [74].

LRRC33:latent TGF- β 1 complexes (LRRC33:TGF- β 1)

LRRC33, another member of the LRR family, has 34% sequence homology with GARP. Like GARP, it can present LAP:TGF- β 1 complexes extracellularly. It was first identified on microglial cells [75]. Its expression is limited to cells of hematopoietic origin, particularly in the myeloid lineage. Mice with a deletion of the *Lrrc33* gene (*Lrrc33*^{-/-} mice) die within 5 months from ascending paraparesis, which is characterized by a demyelination and a loss of axons and neurons in the cortex and in the spinal cord. Gene Set Enrichment Analysis of brain microglia isolated from wild-type and *Lrrc33*^{-/-} mice revealed a decreased TGF- β signaling in *Lrrc33*^{-/-} microglia.

2.2 Activation of latent TGF- β 1

To exert its biological activity, mTGF- β 1 must be released from the LAP in order to be able to bind to the TGFBR on target cells. *In vitro*, TGF- β 1 activation can occur in acidic conditions or at high temperatures. In biological conditions, TGF- β 1 activation relies on highly regulated molecular mechanisms restricted to a few specialized cell types. These mechanisms involve at least one additional partner, notably thrombospondin-1 (TSP-1) or RGD-binding integrins.

2.2.1 Proteins involved in latent TGF- β 1 activation

Thrombospondin-1

TSP-1 is a large, secreted glycoprotein known to interact with several ECM components, receptors and proteases involved in cell-to-cell interactions, platelet aggregation, angiogenesis and tumorigenesis. TSP-1 possesses a KRFLK motif that binds to the LSKL motif of the LAP and disrupts the non-covalent interactions between LAP and the mature TGF- β 1 [76]. TSP-1 null mice share some but not all phenotypical similarities with TGF- β 1 null mice, acknowledging the fact that TSP-1 is responsible for part but not all TGF- β 1 activation *in vivo* and that other mechanisms may exist [77].

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RGD-binding integrins

Integrins are non-covalent heterodimeric transmembrane proteins and are main contributors of cell-to-cell interactions and cell-to-ECM adhesion. Integrins are composed of an α and a β subunit. In humans, integrins can be assembled from eighteen α and eight β subunits. A subset of integrins can bind an RGD sequence present on ligands. The RGD motif is present on the LAP of latent TGF- β 1. In humans, RGD-binding integrins include $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_8\beta_1$. Only $\alpha_v\beta_1$, $\alpha_v\beta_6$, $\alpha_v\beta_8$ were shown to be involved in TGF- β 1 activation [78].

$\alpha_v\beta_1$ integrin

$\alpha_v\beta_1$, highly expressed on activated fibroblasts, was shown to activate TGF- β 1 *in vitro* [79]. *In vivo*, an $\alpha_v\beta_1$ integrin-specific inhibitor reduced bleomycin-induced lung fibrosis in mice, an effect attributed to an inhibition of TGF- β 1 activation mediated by $\alpha_v\beta_1$ integrin, as lower frequencies of pSMAD3⁺ cells (i.e. cells undergoing TGF- β signaling) were observed [79]. The mechanism of activation has still to be characterized.

$\alpha_v\beta_6$ integrin

$\alpha_v\beta_6$ is mainly expressed by epithelial cells. It is overexpressed in inflammation and in various cancers. In the late '90s, it was shown that $\beta_6^{-/-}$ mice were protected against bleomycin-induced pulmonary fibrosis, an effect probably mediated by a reduced activation of TGF- β 1 by lung epithelial cells [80]. It was discovered that activation of latent TGF- β 1 mediated by $\alpha_v\beta_6$ integrin is driven by deformation of the LAP through mechanical pulling forces [80, 81].

$\alpha_v\beta_8$ integrin

$\alpha_v\beta_8$ integrin is expressed by many cell types including fibroblasts, epithelial cells, dendritic cells, monocytes, Tregs and endothelial cells. Genetic ablation of β_8 integrin results in embryonic death caused by profound defects in blood vessel formation [82]. Conditional deletion of $\alpha_v\beta_8$ integrin expression on dendritic cells (DC) caused autoimmunity and colitis in mice. This phenotype could be explained by a failure of DC to induce and maintain Tregs in tissues, which is dependent on TGF- β 1 [83]. The mechanism of TGF- β 1 activation mediated by $\alpha_v\beta_8$ integrin is not fully elucidated yet. It was suggested that the binding with $\alpha_v\beta_8$ integrin allows a sufficient deformation of the latent-TGF- β 1 to exhibit mTGF- β 1 binding sites to TGFBR without the release of the mTGF- β 1 from the LAP [83]. We and others propose an alternative model in which the $\alpha_v\beta_8$ integrin leads to the unfastening of the straitjacket of the LAP with a release of the mTGF- β 1 [74, 84].

2.2.2 Stimuli inducing TGF- β 1 activation

Given the pleiotropic and biologically important effects of TGF- β 1, its activation must be tightly regulated. This activation is controlled by the specific cell types that express latent TGF- β 1 complexed with its presenting molecules, either LTBP, GARP and LRRC33, and by the partner activators including TSP-1 and RGD-binding integrins. In the next sections, the molecular mechanisms of TGF- β 1 activation by cell type are described.

Activation of TGF- β 1 by Tregs

Tregs are CD4⁺ lymphocytes characterized by a stable and constitutive expression of the transcription factor FOXP3 and the α -chain of the IL-2 receptor (CD25). Tregs play an important role in immune tolerance by promoting unresponsiveness to self-antigens and by suppressing excessive immune responses [85]. In cancer, Tregs contribute to an immunosuppressive environment by inhibiting effector T lymphocytes, either by direct contact or by the production of cytokines such as TGF- β 1 or interleukin-10. Importantly, immunosuppression by Tregs requires antigen-induced Treg activation.

We and others discovered that active TGF- β 1 was produced by Tregs after TCR stimulation which enabled the expression of GARP and integrin $\alpha_v\beta_8$ [23, 64, 71, 86, 87]. We showed that TGF- β 1 activation by Tregs is blocked with antibodies targeting α_v or β_8 subunits *in vitro* or *in vivo* (**Figure 15**). It is still unclear whether TGF- β 1 is activated in *cis* (activation by one Treg that co-expresses GARP:TGF- β 1 and $\alpha_v\beta_8$), in *trans* (activation by a Treg expressing GARP-TGF- β 1 and another Treg, or other cell, expressing $\alpha_v\beta_8$), or both.

Our laboratory developed monoclonal antibodies that target human or mouse GARP:TGF- β 1 complexes and block the activation of TGF- β 1 on the surface of Tregs *in vitro*. Our team solved the crystal structure of the human GARP:TGF- β 1 complex bound to a Fab fragment of a blocking anti-

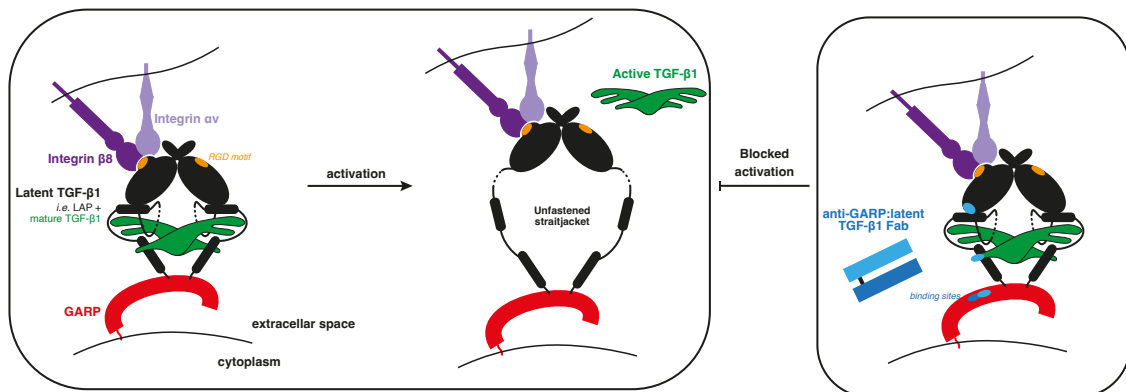


Figure 15 : Proposed mechanism of TGF- β 1 activation by GARP and $\alpha_v\beta_8$.

Integrin $\alpha_v\beta_8$ binds to the RGD-binding motif located on the LAP of the latent TGF- β 1. On the other side, the latent TGF- β 1 is covalently linked to GARP. The binding may induce the unfolding of the latency lasso of the LAP, which allows the release of mature TGF- β 1. It is hypothesized that opposite tensile forces are responsible for the unfolding of the lasso, such as in the TGF- β 1 mediated by $\alpha_v\beta_6$. Anti-GARP:TGF- β 1 antibodies bind the complex at multiple sites of GARP, LAP, and mature TGF- β 1 (blue circles) and prevent the activation of TGF- β 1.

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GARP:TGF- β 1 (anti-GARP) antibody [74]. In a mouse model of xenogenic graft-versus-host-disease induced by the transfer of human peripheral blood mononuclear cells (PBMC) into immunodeficient mice, anti-human GARP:TGF- β 1 antibodies abrogated the protective effect provided by co-transferred Tregs [74]. Blocking anti-mouse GARP:TGF- β 1 antibodies were also tested in murine models of solid tumors and hematologic malignancy. The combination of anti-GARP:TGF- β 1 and anti-programmed cell death-1 (PD-1) antibodies reduced the progression of the experimental tumors MC38 and CT26 and increased survival compared to anti-PD-1 monotherapy [71, 88]. The anti-tumor effect provided by the anti-GARP:TGF- β 1 antibody was dependent on the expression of GARP by Tregs, as the anti-tumor effect was not observed with mice carrying a Treg-specific deletion of the GARP gene. In the CT26 model, the anti-tumor effect was mediated by a restoration of the cytolytic activity of anti-tumor CD8⁺ T cells. In MC38, the anti-tumor effect was mediated by an increased density of functional blood vessels which allowed more extravasation of tumor-infiltrating T cells. In a model of myeloproliferative neoplasm characterized by clonal expansion of malignant myeloid cells and bone marrow fibrosis, the anti-GARP:TGF- β 1 antibody used as a monotherapy (without anti-PD1) reduced not only the fibrosis but also the clonal expansion of tumor cells [69]. Mice carrying a Treg-specific deletion of the GARP gene showed a reduced tumor burden. Whereas mice with megakaryocyte/platelet specific GARP deletion did not. Altogether, results suggested that anti-GARP:TGF- β 1 blocked TGF- β 1 activation on Tregs and restored anti-tumor CD8⁺ T cells responses in murine models of myeloproliferative neoplasms.

Activation of TGF- β 1 by endothelial cells

Endothelial cells can activate TGF- β 1 in hypoxic condition *in vitro* and *in vivo* [89]. In the context of inflammation with release of tumor necrosis factor-alpha (TNF- α) and IL-1 β , endothelial cells are stimulated and activate TGF- β 1 [90]. Interactions between BECs and mural cells, which are either smooth muscles cells in veins and arteries or pericytes in capillaries, are highly complex and are essential in angiogenesis and vasculature maturation [91]. Co-culture of mural cells with BECs, but not monocultures, activate TGF- β 1, which otherwise remains latent [92]. In co-cultures, mural cells promoted cytotaxis of BECs, an effect that was abolished with an anti-TGF- β 1 antibody. In a 3D culture system, the addition of aortic adventitial fibroblasts, a specialized mural cell of the aorta, to endothelial cells increased microvessel density formation and enhanced ECM deposition around blood vessels. These effects were abolished with an inhibitor targeting ALK5, a subunit of the TGFBR [93]. These findings suggest that signals exchanged between BECs and mural cells contribute to the activation of TGF- β 1 by one or both cell types. Signal exchanges include paracrine factors such as platelet-derived growth factor (PDGF) produced by BECs, angiopoietin 1 (ANG1) and sphingosine-1-phosphate (SP1) produced by pericytes, as well as TGF- β 1, produced by both cells. Other signals depend on contact between these cells, including NOTCH1 and NOTCH3, which bind DLL and JAG1, respectively [94]. However, the mechanism underlying TGF- β 1 activation caused by the cooperation between endothelial cells and mural cells remains unknown.

GARP expression on endothelial cell was shown to have no influence on the development of thrombosis, despite evidence about the implication of GARP in the formation of thrombosis in GARP KO zebrafishes [95]. To date, the role of GARP in TGF- β 1 activation by BECs remains to

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be discovered. Our group exposed co-cultures of either GARP-expressing or non-expressing BECs and TGF- β 1 reporter cells to various stimuli in order to investigate possible TGF- β 1 activation by BECs. Among the stimuli tested were proangiogenic factors, such as amphiregulin (AREG), vascular endothelial cell growth factor (VEGF) or epidermal growth factor (EGF), or pro-inflammatory cytokines, such as IL-6, TNF- α and IL-1 β . No TGF- β 1 activation by human or mouse BECs was observed when exposing the cells to individual proangiogenic factors (Charlotte Bertrand, unpublished results). As angiogenesis is a multistep process, administering a combination of pro-angiogenic factors may have a synergistic effect and may activate BECs more effectively than if administered separately. In this sense, coadministration of VEGF and ANG1 synergistically increased the number of angiogenic sprouts in 3D monocultures of BECs, more than doubling the predicted additive effect of both factors individually [96]. Combination of VEGF, PDGF and fibroblast growth factor (FGF) has synergic effects on sprouting of BECs in 3D spheroid co-cultures with mesenchymal stem cells [97]. As far as we know, the effect of exposing BECs to combinations of pro-angiogenic factors on their ability to activate TGF- β 1 has not been tested. TGF- β 1 activation by BECs could be mediated by RGD-binding integrins, as $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrins were detected on the membrane of unstimulated HUVEC, primary endothelial cells grown from cord blood [70, 98]. Apart from $\alpha_v\beta_6$ and $\alpha_v\beta_8$, BECs express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ under angiogenic conditions. These integrins play a role in adhering to various ECM components, facilitating cell migration [99]. Despite the fact that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ do not bind to latent TGF- β 1 with high affinity and cannot activate TGF- β 1 on transfected HEK293 cells [78], it was shown that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ participate in TGF- β 1 activation by human cardiac myofibroblasts [100]. Exposure of cardiac myofibroblasts to anti- β_3 and anti- β_5 antibodies reduced TGF- β 1 activation by 50% in luciferase assays. The mechanism by which these integrins promote TGF- β 1 activation is unelucidated.

Other publications in the literature mention that TGF- β 1 could be activated by BECs via proteolysis as it was shown that ECM-bound latent TGF- β 1 can be activated by MMPs [101-103]. During angiogenesis, the role of MMPs produced by BECs is to degrade the ECM around vessels and remodel the basement membrane to allow the formation of new vessels. Among MMPs, transmembrane MT1-MMP was the most studied as candidate for triggering TGF- β 1 activation. It can be expressed by mural cells and BECs, notably by sprouting BECs during angiogenesis [104]. *In vitro*, monocultures of HUVEC stimulated with phorbol 12 myristate 13-acetate (PMA), a compound promoting vascular remodeling, promoted the degradation of ECM and allowed the release of latent TGF- β 1 from proteolysis of LTBP-1 by MT1-MMP [101]. However, no active TGF- β 1 nor pSMAD2 could be detected in PMA-stimulated HUVEC cultures. In another publication, a MT1-MMP- and $\alpha_v\beta_8$ -dependent activation of TGF- β 1 was observed on transfected cancer cell lines. It was suggested that $\alpha_v\beta_8$ offers an anchorage to latent TGF- β 1 that is subsequently degraded by MT1-MMP [103]. *In vivo*, inhibition of MMPs caused leakiness of blood vessels, a phenotype that was similar when mice were treated with an ALK5 inhibitor or an anti-TGF- β antibody [102]. One publication mentions that MMP-2, another TGF- β 1 activating MMP, interacts with $\alpha_v\beta_3$ on the surface of melanoma cells [105]. Hypothetically, $\alpha_v\beta_3$ could act as a docking protein for MMP-2 and allow BECs to activate TGF- β 1 by proteolysis.

Activation of the TGF- β 1 by other cells

Megakaryocytes and platelets

Both megakaryocytes and platelets produce large amounts of latent TGF- β 1. In platelets, a large proportion of this latent TGF- β 1 is stored in complex with LTBP-1 in α -granules, which also contain TSP-1 [106]. Blood coagulation induces platelet activation, a process which causes a rapid degranulation and a production of active TGF- β 1. TSP-1 does not appear to be involved in TGF- β 1 activation, as platelets of *Tsp*^{-/-} mice produce as much active TGF- β 1 as wildtype mice [106]. Another pool of latent TGF- β 1 is found in complex with GARP at the surface of activated platelets [69, 107, 108]. We and others identified that GARP is necessary for TGF- β 1 activation by platelets, as platelets from mice carrying a specific deletion of GARP on the megakaryocytic lineage fail to activate TGF- β 1 during coagulation [69, 107]. In addition, an anti-GARP:TGF- β 1 antibody blocks TGF- β 1 activation by platelets *in vitro*. The complete mechanism of TGF- β 1 activation by platelets is not elucidated yet. The team of Zihai Li suggested that thrombin, a protease involved in blood coagulation used to activate platelets *in vitro*, could be involved in a GARP-dependent TGF- β 1 activation mechanism by proteolysis of GARP [109].

Fibroblasts

TGF- β 1 is a well-established pro-fibrotic cytokine, both involved in tissue repair as well as pathological-associated fibrosis [110]. Latent TGF- β 1 expression by fibroblasts is increased under oxidative stress, toll-like receptor signaling or proinflammatory cytokines [111-113]. Latent TGF- β 1 was found in complex with LTBP1 in the extracellular matrix of cultured fibroblasts and HT-1080 fibrosarcoma cells [114]. GARP is expressed on the membrane of cultured human lymph node fibroblasts as well as in association with the extracellular matrix of normal and metastatic lymph nodes [70].

The mechanism by which fibroblasts activate TGF- β 1 is not elucidated yet. TGF- β 1 activation by $\alpha_v\beta_1$ expressed by activated fibroblasts was reduced with an $\alpha_v\beta_1$ competitive inhibitor in a mouse model of lung and liver fibrosis [79]. Matrix metalloproteinase-9 (MMP-9) is a protease produced by fibroblasts and involved in extracellular matrix degradation. It was shown that MMP-9 is recruited on the cell surface of fibroblasts by lysyl hydroxylase 3 (LH3) docking protein and activates TGF- β 1 by cleavage, most probably of the LAP [115, 116]. Competitive inhibition of MMP-9 docking to LH3 decreases TGF- β 1 activation by fibroblasts.

B lymphocytes

Active TGF- β 1 is released from the GARP:TGF- β 1 complex expressed by human BCR-stimulated B lymphocytes. This induces an isotype switch towards immunoglobulin A (IgA) production [68]. However, B cell-specific GARP deletion in mice did not cause a reduced IgA production in response to bacterial infection or protein immunization [117].

Hepatic stellate cells and liver sinusoidal endothelial cells

Hepatic stellate cells (HSC) are mesenchymal cells which are involved in vitamin A storage, tissue repair and in modulation of immune responses. GARP expressed by HSC was found to be essential for the development of liver fibrosis via TGF- β signaling [72]. Sinusoidal endothelial cells are highly specialized endothelial cells found in the liver, with vital physiological and immunological functions. GARP expressed by sinusoidal endothelial cells was found to be involved in the TGF- β 1 dependent induction of tissue-resident Tregs [118]. The mechanisms of TGF- β 1 activation in both cell types are still undetermined.

2.3 Cellular responses to TGF- β 1 signaling

2.3.1 TGF- β receptors and SMAD or non-SMAD dependent signaling

TGFBR is a transmembrane hetero-tetramer receptor composed of two serine/threonine kinase dimers known as receptor type I and receptor type II [119]. TGFBR is ubiquitously expressed by all cells. Mammalian genomes possess 7 versions of the type I receptor and 5 versions of the type II receptor which together can form various combinations, some of which can bind specific TGF- β family members. In the case of TGF- β 1, the receptor is composed of ALK5 (or TGFBR1) and TGFBR2. Active TGF- β 1 binds with high-affinity to TGFBR2, which then recruits and phosphorylates ALK5 (**Figure 16**). ALK5 in turn activates the SMAD pathway by phosphorylation of the SMAD2 and SMAD3 proteins (pSMAD2 and pSMAD3). pSMAD2 and pSMAD3 then form heterotrimers with SMAD4 that translocate into the nucleus to induce or repress gene expression. BECs can also express ALK1 on top of ALK5, which can phosphorylate SMAD1, 5 and 8 rather than SMAD2 and 3 [120].

TGF- β signaling via the SMAD pathway is referred to as the canonical TGF- β signaling pathway. Other SMAD-independent, non-canonical TGF- β -induced signaling pathways exist, including the MAPK, PI3K-Akt, Rho GTPase pathways.

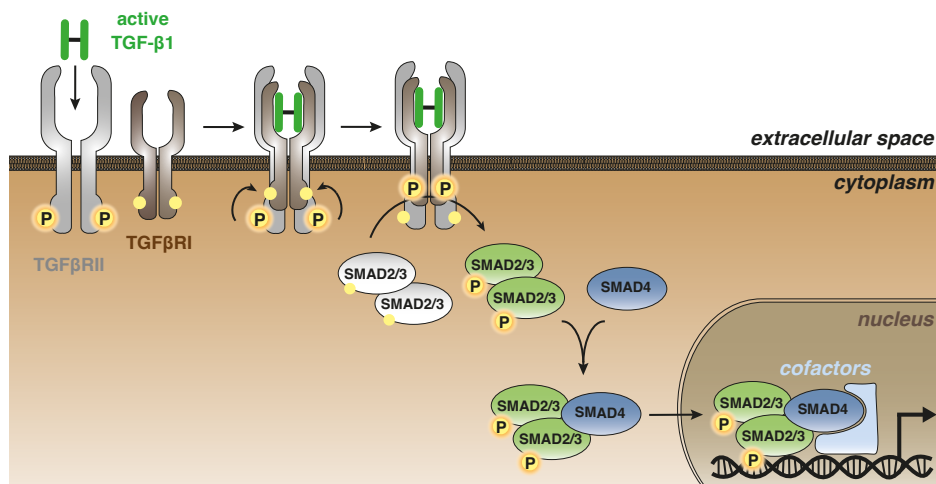


Figure 16 : SMAD-dependent TGF- β 1 signaling

Active TGF- β 1 binds to the TGF β RII homodimer which then recruits and activates the TGF β RI homodimer. Once activated, TGF β RI phosphorylates SMAD2/3 which forms an heterotrimer with SMAD4. The trimer SMAD complex translocates into the nucleus where it regulates gene expression through association with other cofactors.

2.3.2 Effects of the TGF- β 1 signaling on the cells of the tumor microenvironment

Figure 17 summarizes the effects of TGF- β 1 on the main cell types present in the tumor environment.

Effects on immune cells

DCs are professional antigen-presenting cells which are pivotal in the priming and activation of anti-tumor responses. TGF- β 1 was shown to dampen antigen presentation in lymphoid organs and in tumors by reducing MHC class II expression on DC and by inhibiting endocytic and phagocytic functions of DC [121-123]. TGF- β 1 also reduces the migration of DCs by modulating the expression of chemokine receptors [124].

CD4⁺ T helper lymphocytes (T_H) play pivotal roles in the coordination of immune responses, notably by producing cytokines. TGF- β 1 is an inhibitor of the differentiation of T cells to T_H1 and T_H2 via inhibition of the expression of the key transcription factors T-bet and GATA-3, respectively [125, 126]. Conversely, mice with T-cell specific deletion of the *Tgfb2* gene display enhanced T_H responses [127]. TGF- β 1 also inhibits T cell proliferation by silencing IL-2 production which elicits subsequent T_H proliferation [128]. TGF- β 1 activated SMAD cooperate with ROR γ t and induce T_H17 cell differentiation. These cells are involved in anti-bacterial and anti-fungal immunity as well as in the development of autoimmunity [119].

CD8⁺ cytotoxic T cells (CTL) eliminate cancer cells and pathogen-infected cells through the release of cytolytic factors. TGF- β 1 represses the differentiation and the proliferation of CTL through activation of the SMAD pathway through interaction with the FOXP1 transcription factor [129, 130]. Expression and/or secretion of CTL effector molecules such as IFN- γ , Fas ligand, perforin, granzyme A and granzyme B, is dampened by SMAD2 and SMAD3 [131]. It was also shown that TGF- β 1 enhances the expression of PD-1 on CTL in a SMAD3 dependent manner [132]. PD-1 is an immune checkpoint which tampers signaling pathways that are crucial for T cell survival, proliferation, activation and effector functions.

Tregs are indispensable to restrain T cell activation and proliferation. In tumors, Tregs contribute to tolerance of immune cells to tumor antigens. TGF- β 1 production is one of the mechanisms that Tregs use to exert immunosuppression. Tregs originate either from T cell progenitors in the thymus (tTregs) or from TCR-stimulated naïve CD4⁺ T cells in secondary lymphoid organs in the periphery (pTregs). Implication of TGF- β 1 in tTregs is unclear. Conditional deletion of *Tgfb1* in the T cell lineage blocked the appearance of tTreg at postnatal days 3-5 [133]. However, starting from week 1 after birth, *Tgfb1*-mutant mice showed a rapid expansion of the tTreg population, which was attributable to an overproduction of IL-2. TGF- β 1 plays a central role in the generation of pTregs by upregulating FOXP3 in a SMAD-dependent manner and in the presence of IL-2 [134]. TGF- β 1-mediated FOXP3 expression is essential for the maintenance of Treg functions. The presence of Tregs in tumors is associated with a dismal prognosis in several types of cancer.

INTRODUCTION

TGF- β 1 can act as an indirect tumor suppressor by preventing chronic inflammation, which predisposes pre-malignant cells to progress to tumor formation [119]. This is particularly the case in the gastrointestinal tract where inflammatory reactions to commensal microbiota by epithelial cells and immune cells are prevented by TGF- β 1 [135].

Effects on epithelial and endothelial cells

TGF- β 1 strongly influences the phenotypic plasticity of epithelial cell progenitors. It regulates the differentiation and the phenotypic transition in tissue development and repair. TGF- β 1 also contributes to the expression of profibrogenic factors in normal and malignant epithelial cells in lung metastases model [136]. TGF- β 1 is also the most widespread and potent signaling molecule of epithelial to mesenchymal transition (EMT). EMT is a process during which epithelial cells lose apicobasal polarity and intercellular adhesive contacts, while they gain in motility. While EMT is essential in development and in tissue repair, it contributes to cancer invasiveness and metastasis in mammary, pulmonary, renal and hepatic carcinoma [119].

TGF- β 1 is crucial for the formation of blood vessels during embryogenesis. Mice with deletion of TGF- β 1 or TGF- β 1 signaling elements (ALK1 KO, ALK5 KO, TGFBR2 KO) die at the embryonic development stage from vascular abnormalities. The tumor vasculature is often characterized by a disorganized network, with vessels missing perivascular cells, causing vessel collapsing and leakiness [137]. It consequently leads to poor blood tissue perfusion causing hypoxia, promotes metastasis and impairs diapedesis of immune cells in the tumor. Endothelial cells can also undergo endothelial to mesenchymal transition (EndoMT), which is associated with several pathologies such as lung fibrosis. Recent evidence highlights a putative role of TGF- β 1 in this process [138]. In cancer, the effect of TGF- β 1 on blood vessels is contextual, both promoting angiogenesis via stimulation of pro-angiogenic factors such as VEGF or inhibiting angiogenesis by cytostasis of endothelial cells [139]. The balance between proangiogenic and cytostatic properties of TGF- β 1 is proposed to be regulated via a balance in ALK1 and ALK5 signaling, respectively [140]. In the MC38 tumor model, combination of anti-PD1 and anti-GARP antibodies increased the density and promoted the normalization of intratumoral blood vessels by increasing pericyte coverage [88]. We hypothesized that this increase was attributable to the anti-GARP antibody which could block a TGF- β 1 cytostatic effect on endothelial cells.

Effects on fibroblasts

TGF- β 1 is a major profibrotic cytokine in the tumor context [141]. It stimulates the production of ECM components. TGF- β 1 also inhibits ECM degradation by increasing the expression of protease inhibitors. It promotes the dedifferentiation of several cell types (i.e epithelial cells, endothelial cells or mesenchymal cells) to cancer associated fibroblasts (CAF). The rest of the CAF pool is composed of resident fibroblasts which are activated by TGF- β 1. CAF are different from normal fibroblasts based on their morphology, increased proliferation and different expression profile. CAF support tumor growth, metastasis and immune evasion.

INTRODUCTION

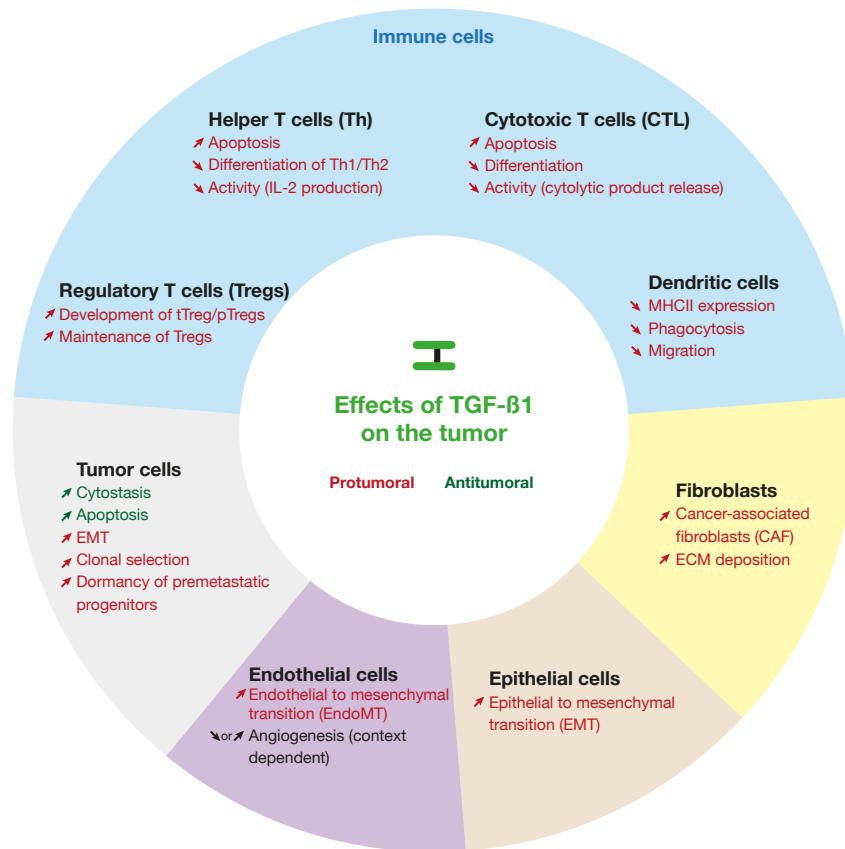


Figure 17 : Summary of the effects of active TGF-β1 on the tumor environment

Summary of pro-tumoral (red) and anti-tumoral (green) effects induced by TGF-β1 in each major cell type present in the tumor environment.

Effects on tumor cells

TGF-β1 can promote or suppress carcinoma progression depending on the stage of the disease. At an early stage, TGF-β1 induces cell apoptosis of epithelial progenitors harboring RAS mutations [119]. During cancer progression, a clonal selection of cells harboring a mutation in the TGF-β signaling pathway may occur, allowing them to elicit apoptosis and leading to tumor growth. Mutations of *TGFBR2*, *SMAD4* or *SMAD2* were detected in human colorectal or pancreatic adenocarcinomas [142-144]. .

Objectives

OBJECTIVES

Our laboratory developed monoclonal antibodies directed against the GARP:(latent)TGF- β 1 complex that block TGF- β 1 activation and immunosuppression by Tregs *in vitro* and *in vivo*. Anti-GARP:TGF- β 1, alone or in combination with anti-PD1, exerted anti-tumor effects in models of blood cancer or solid tumors in mice. The anti-tumor effect provided by the anti-GARP:TGF- β 1 antibody depended on the expression of GARP on Tregs, as mice carrying a Treg-specific *Garp* knock out did not experience solid tumor regression after co-administration of anti-GARP:TGF- β 1 and anti-PD-1. Currently, anti-GARP:TGF- β 1 antibodies are being tested in clinical trials in several cancer types (colorectal, urothelial, lung, pancreatic, ovarian, liver and head and neck squamous cell carcinoma).

The prevalence of GARP-expressing Tregs in patient tumors is unknown. While it has been demonstrated that GARP-expressing Tregs are a source of immunosuppressive TGF- β 1 in mouse models, it remains uncertain whether the same holds true in human tumors. Which are the cell types targeted by the TGF- β 1 activated by GARP-expressing Tregs in tumors also remains to be identified. In tumors, GARP is expressed by other cell types than Tregs, including BECs. The role of GARP expressed on BECs, and whether GARP-expressing BECs can activate TGF- β 1 and contribute to tumor progression is unknown.

Addressing these questions could enhance our understanding of the role of GARP in TGF- β 1-mediated cell-to-cell communication in tumors. In the future, this knowledge could help identify which patients would be most likely to benefit from anti-GARP:TGF- β 1 immunotherapy.

The objectives of my doctoral thesis work were to :

- Develop an experimental approach to study TGF- β 1 mediated cell-to-cell communication in human tissues, with a focus on GARP-expressing Tregs and GARP-expressing BECs. Our experimental approach includes :
 - Multiplexed immunofluorescence (mIF), combined with
 - Computerized distance calculation
- Utilize this approach to analyze TGF- β 1 mediated cell-to-cell communication in over 100 human tumor samples of various histological origin.

The results of this work are presented in two articles in the Results section. A first method article describes the experimental approach that we developed. In a second research article, we succeeded in applying the experimental approach to study >100 archived tissues, including colorectal, lung, urothelial, breast primary carcinoma, melanoma metastases and patient-matched tumor-associated normal tissues.

Results

RESULTS

mIF analyses to quantify spatial relationships between sources and targets of TGF- β 1 signaling in human tissues

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Abstract

TGF- β 1 is a cytokine that can exert pro-tumoral effects, by favoring tumor progression, promoting fibrosis and inhibiting anti-tumor immune responses. GARP-expressing cells, which include activated regulatory T cells (Tregs) and blood endothelial cells (BECs), may represent a source of active TGF- β 1 in tumors. In this study, we developed a novel multiplexed immunofluorescence (mIF) workflow combined with digital image analysis to investigate the role of GARP-expressing cells as a source of TGF- β 1 *in situ*. Our workflow is suitable for frozen tissue sections. It comprises five sequential staining cycles followed by fluorescent image acquisition. Each cycle includes staining with a primary monoclonal antibody against either GARP, phospho-SMAD2 (pSMAD2), FOXP3, CD3 and CD34, followed by a species or isotype-specific secondary polyclonal antibody coupled to polymer-bound horseradish peroxidase, followed by a tyramide-coupled fluorochrome. Between cycles, the enzyme is neutralized with a peroxidase inhibitor. Digital image analysis allows to identify, localize and quantify cells, including putative TGF- β 1 producer cells, comprising T cells, GARP+FOXP3+ activated Tregs and GARP+CD34+ endothelial cells, and TGF- β 1-stimulated cells (pSMAD2+) as well as blood vessels. A computer program developed in house then allows to measure the distances between cell types. To determine whether two cell types (e.g. TGF- β 1 producer and responder cells) are located closer to each other than they would be by chance, the program randomly relocates one of the cell types, measures the new distances again and compares these virtual distances with the original ones. This workflow can be used to study the role and the effects of GARP-expressing cells in TGF- β 1-mediated immunosuppression in tumoral and healthy tissues.

Introduction

Recent advances in tissue imaging, coupled with enhanced multiplexing capacities in histological staining and powerful image analysis tools, have made possible the study of complex intercellular communications *in situ* [1]. Multiplexed immunofluorescence (mIF) enables to simultaneously image several markers on the same tissue section. Tyramide signal amplification (TSA) and horseradish peroxidase (HRP)-grafted polymer coupled antibodies increase light signals emitted by fluorescent molecules and increase the signal/noise ratio. High-resolution digital slide scanners allow to distinguish up to 7 or 8 different fluorescence channels, expanding the number of antigens that can be imaged on the same tissue section. Image analysis softwares facilitate the quantitative analysis of histological and multiplexed immunofluorescence images. The addition of artificial intelligence has boosted the precision and accuracy of computerized image analysis [2].

Cytokine-mediated intercellular communications play an important role in tissue homeostasis and disease. TGF- β 1 is a cytokine with pleiotropic effects. It is involved in embryogenesis, blood vessel development, tissue repair and immune regulation [3]. Dysregulation of TGF- β 1 signaling can lead to auto-immune disease or favor cancer. In tumors, TGF- β 1 has anti-tumoral and pro-tumoral effects, depending on the stage of the disease. At early stages, it favors apoptosis of tumor cells. At later stages, it promotes angiogenesis, fibrosis and immunosuppression. The activity of TGF- β 1 is controlled at the post-translational level. It is produced by many cells in an inactive form, complexed with the latency associated peptide (LAP), further referred to as latent TGF- β 1. Latent TGF- β 1 must undergo an activation step to become functional, a process that is tightly regulated and only achievable by few types of cells, such as myeloid cells, fibroblasts, endothelial cells and activated regulatory T cells (Tregs). The latter express a transmembrane protein, GARP, which binds latent TGF- β 1 [4-8].

Here, we aimed at studying GARP-expressing cells as a potential source of TGF- β 1 in sections of human tissues using mIF and digital image analysis. To enhance the quality of the image analysis, we amplified the fluorescence signals using TSA. We previously derived monoclonal antibodies directed against GARP [9]. Despite the many tested conditions, none of these antibodies stained GARP on formalin-fixed paraffin-embedded (FFPE) tissue sections, while some did work on snap-frozen tissues [5]. This restricted our study to frozen tissues, for which the possibilities of multiplexing are more limited than for FFPE tissues, mainly due to their fragility under the conditions used to denature antibodies between staining cycles. Therefore, we developed a mIF staining protocol suitable for frozen tissue sections which uses peroxidase inhibitors to inactivate the HRP between staining cycles, and distinct isotypes or species for each pair of primary and secondary antibodies in each cycle. The mIF was followed by digital imaging and computerized image analysis to count and localize cells and blood vessels, and completed by in house developed computer scripts that calculate the distances between two cell types. In order to determine whether these distances are shorter than if the two cell types were spatially distributed by chance, without interaction, the latter cells are digitally removed and randomly repositioned, after which the distances are measured again and compared with the original distances. The workflow can be

RESULTS

used, for example, to assess the proximity of cells producing TGF- β 1 to the nearest cell responding to TGF- β 1.

Results

Design of a sequential tyramide signal-amplified 5-plex mIF staining protocol suitable for fresh-frozen tissue sections.

A 7 μ m-thick cryosection of cutaneous melanoma sample was mounted on a microscope slide, fixed in formaldehyde 4% during 5 min, and washed in Tris-buffered saline (TBS) supplemented with 0.005% Tween20 (TBS-T). The section underwent multiplexed immunofluorescence staining (mIF), consisting of 5 sequential staining cycles, one per target antigen. During each cycle, the tissue section was incubated with a primary monoclonal antibody of distinct species or isotype, then with a matched secondary polyclonal antibody coupled to either one horseradish peroxidase (HRP) molecule or to a polymer backbone covered with several HRP molecules, and finally with a tyramide-coupled fluorophore, the latter different for each antigen, ensuring distinct light excitation and emission wavelength ranges (**Figure 1A**). Between each cycle, the enzyme was inactivated with a peroxidase blocking reagent. The target antigens were, in staining order, GARP, phospho-SMAD2 (pSMAD2), FOXP3, CD3 and CD34 (**Figure 1B**). The primary antibodies were all monoclonal and were obtained commercially, except the anti-GARP antibody, which was produced in house [9, 10]. All the antibodies and reagents used in the mIF staining are listed in **Supplemental Table 1**. After completing the 5 staining cycles, the slides were counterstained with Hoechst33342 (ThermoFisher Scientific), mounted with Fluorescent Mounting Medium (Agilent) and covered. A high-resolution digital microscopy image of the stained tissue section was obtained with a multichannel fluorescence-compatible slide scanner (**Figure 1C**).

RESULTS

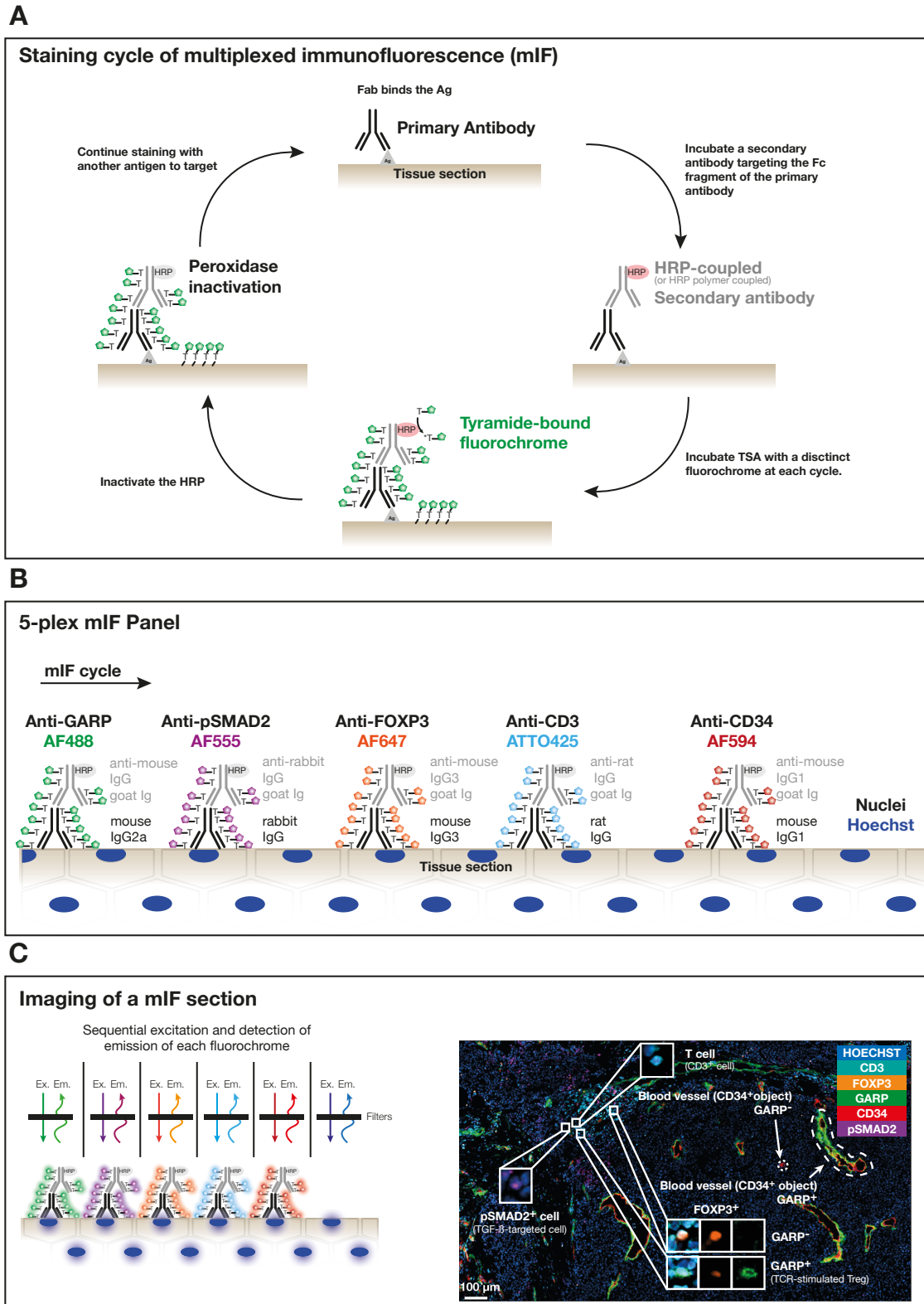


Figure 1 : Sequential multiplexed immunofluorescence (mIF) staining protocol and imaging

A. Schematic representation of a cycle of the sequential mIF protocol. B. Illustration of the five target antigens stained in the mIF panel. C. High-resolution fluorescence scan of the tissue section. Each fluorochrome is imaged sequentially, using a dedicated channel that is composed of a distinct LED light source and filter set (left panel). A cutaneous melanoma metastasis was stained with the mIF panel

RESULTS

and imaged (right panel). The multiplex staining allows to visualize T cells (CD3⁺), FOXP3⁺ cells, GARP⁺FOXP3⁺ activated Tregs, GARP⁺ or GARP⁻ CD34⁺ blood endothelial cells (BECs) forming blood vessels and TGF- β 1-stimulated cells (pSMAD2⁺).

Image analysis

The scanned digital image was analyzed with the HALO software (v3.3, IndicaLabs) (**Figure 2**). The region of interest (ROI), corresponding to the tumoral region, was drawn on the mIF image based on an adjacent section of the melanoma sample stained by immunohistochemistry using antibodies targeting melanoma antigens (**Figure S1**). The HighPlex v4.1.3 module of the software was used to identify individual cells. Their nuclei were segmented based on their Hoechst staining using the default AI segmentation tool of the module. For each segmented cell, the mean fluorescent intensity of each marker (GARP, pSMAD2, CD3, FOXP3, CD34) was calculated by the software. Then, for each marker, a fluorescence intensity threshold was set visually to distinguish the positively and negatively stained cell populations. These values were saved in a data table, including the X and Y spatial coordinates of each cell. In parallel, the Object Colocalization v1.0 module of the software was used to identify CD34⁺ objects (blood vessels) and GARP⁺ objects (extracellular GARP signal associated with perivascular or fibrotic areas). The objects were identified by the software based on criteria including size, roundness, intensity and contrast of the fluorescent staining. The resulting data table contained the X and Y spatial coordinates, the type (GARP⁺ or CD34⁺) and the size of each object. It also included a column indicating, for each CD34⁺ object corresponding to a blood vessel, whether or not it showed an overlap with a GARP⁺ object.

When a FOXP3⁺GARP⁺ cell is detected within a GARP⁺ object, it is impossible to determine whether the GARP immunoreactivity is due to the FOXP3⁺ cell expressing GARP or to overlap with the GARP signal associated with the perivascular or fibrotic area. Therefore, FOXP3⁺GARP⁺ cells were quantified only in areas that do not contain GARP⁺ objects. For this purpose, each FOXP3⁺GARP⁺ cell was classified in either of two categories: those included in the smallest vertical rectangle enclosing a GARP⁺ object and those located outside these rectangles.

RESULTS

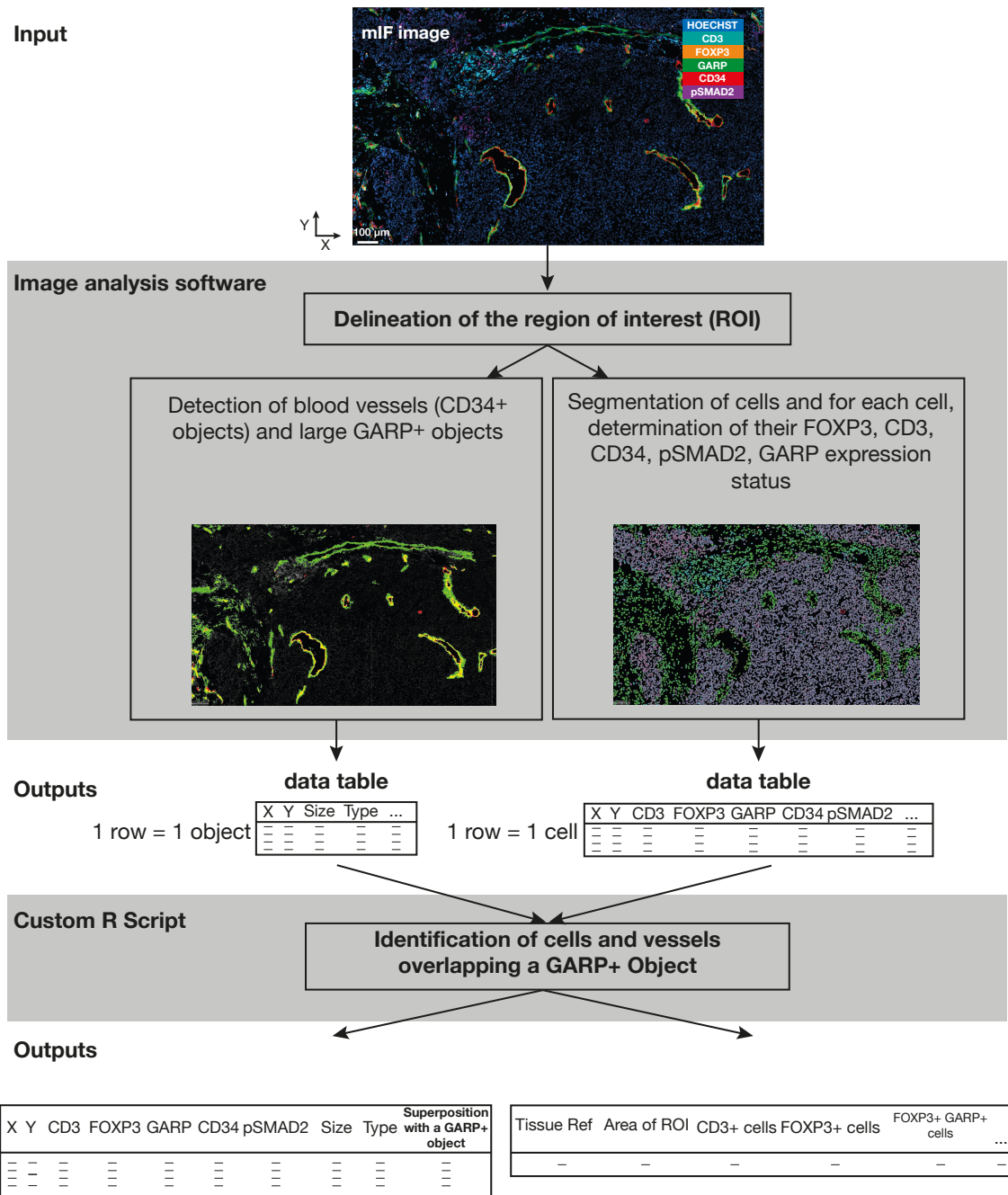
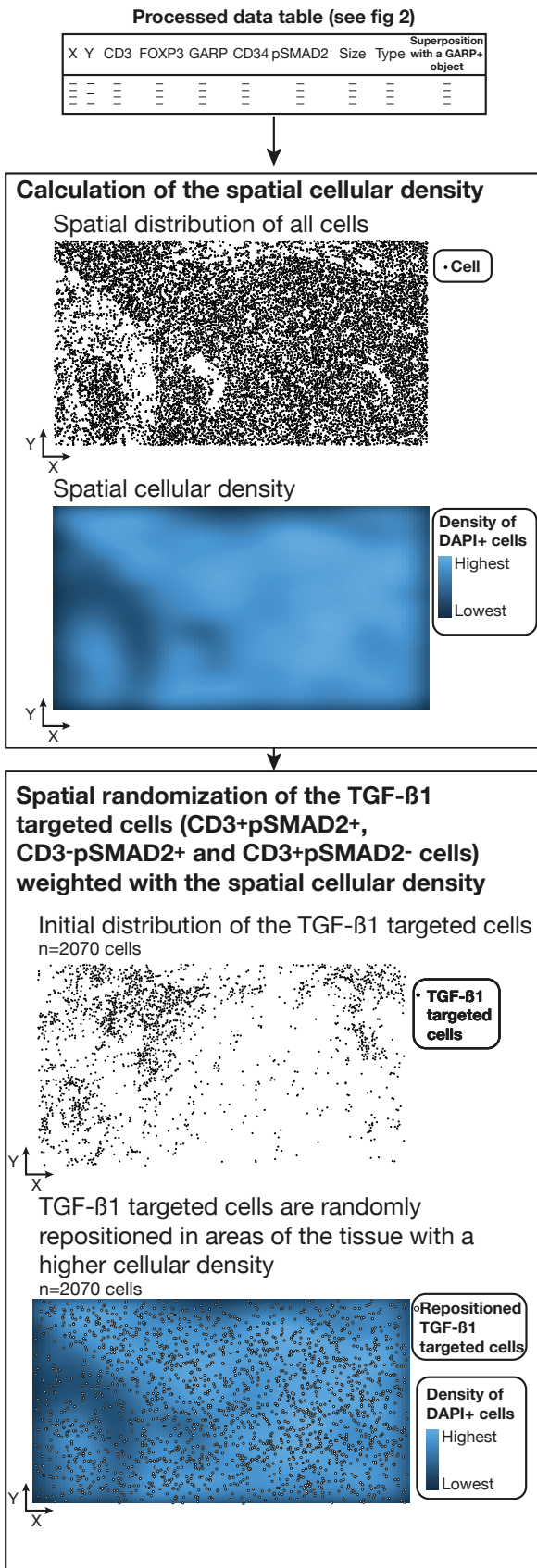


Figure 2 : mIF image processing

Design of an R workflow to quantify spatial relationships between cells.



We developed a workflow in the R programming language that calculates the euclidean distance between each cell of a given type 'A' and the nearest cell of a type 'B', to study cell-to-cell spatial interactions. In order to determine whether the 'A' cells were closer to the 'B' cells than would occur by chance, these distances were compared to those obtained after randomizing the 'A' cell positions. Practically, 'A' cells were digitally removed and randomly repositioned within the ROI. This process was repeated five times, and the mean of the real distances was compared to the mean of the distances obtained from the five randomizations. The random relocation process was weighted against the local density of nuclei that was estimated thanks to the Kernel density function. Thus, cells had a greater probability of being relocated in regions of the ROI with a higher density of nuclei.

We applied our workflow to the characterization of immunosuppressive TGF-β1 signaling in human tumor samples. To do so, we calculated the distances between either CD3+pSMAD2+, CD3-pSMAD2+ or CD3+pSMAD2- cells ('A' cells) and FOXP3+ cells or CD34+ cells ('B' cells). **Figure 3** illustrates the spatial randomization of CD3+pSMAD2+, CD3-pSMAD2+ and CD3+pSMAD2- cells. **Figure 4** illustrates the mean distance measurements between CD3+pSMAD2+ cells and FOXP3+ cells before and after cell repositioning.

Figure 3 : Illustrative portion from the mIF staining of a melanoma metastasis showing the weighted random repositioning of TGF-β1-stimulated cells based on cellular density

The data table generated in the figure 2 was used as input of a dedicated R script. First, the spatial distribution of all cells within the region of interest (ROI) was visualized. It was then used to calculate the local cellular density with a Kernel density function, producing a heatmap of the density. Then, the CD3+pSMAD2+ cells were randomly repositioned within the ROI. This repositioning was weighted according to the heatmap of the cellular density. Cells had a greater chance to be repositioned in areas of the ROI with a high cellular density compared to the areas with a lower density. The randomly repositioned cells are stored in a data table that was used for the distance calculations. The process was repeated for CD3-pSMAD2+ and CD3+pSMAD2- cells.

RESULTS

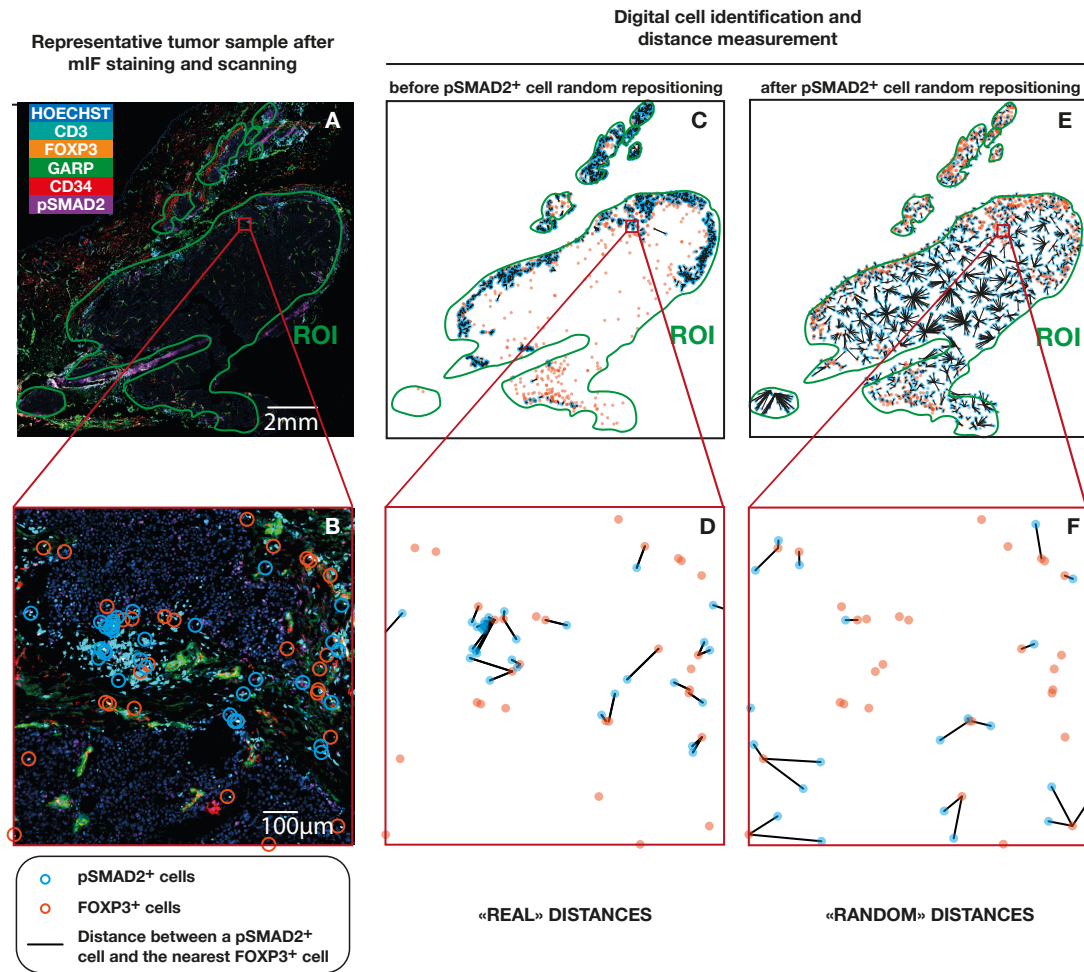


Figure 4: Assessment of the proximity between TGF- β 1-stimulated cells and either FOXP3+ cells or BECs.

Illustrative example of a melanoma analyzed by mIF and image analysis. A & B mIF image and digital image analysis of a melanoma sample. C&D The distance between each pSMAD2+CD3+ T cell (shown as pSMAD2+ cells in this figure) and the nearest FOXP3+ cell were calculated. E&F The pSMAD2+ T cells were digitally removed and randomly repositioned within the same ROI. The distances were recalculated as above. This process was repeated a total of five times, and the mean of the real distance was compared to the mean of the distances obtained from the five randomizations. The same approach was applied with either pSMAD2+CD3- cells or CD3+pSMAD2- cells instead of CD3+pSMAD2+ cells, and also with BECs instead of FOXP3+ cells.

“Real” and “random” distance measurements indicate whether the calculation was performed before or after pSMAD2+ cell repositioning. A high “random” to “real” distance ratio indicates that there is a greater proximity between the cell types than expected by chance. In this melanoma sample, the greatest ratio was between the CD3+pSMAD2+ cells and FOXP3+ cells (ratio = 4.5), indicating that pSMAD2+ T cells are on the average about 5 times closer to FOXP3+ cells than expected by chance (**Figure 5**). In contrast, the ratio of the distances between CD3-pSMAD2+ cells and FOXP3+ cells was much smaller (1.6), suggesting that CD3-pSMAD2+ cells are not in close proximity with FOXP3+ cells. CD3+pSMAD2- were found near FOXP3+ cells, but with a ratio (3.6) smaller than that of the distance between CD3+pSMAD2+ cells and FOXP3+ cells. Together, the results suggest that, in this sample, FOXP3+ cells can be a source of immunosuppressive TGF- β 1 in tumors, as these cells are more preferentially located near T cells than non-T cells. The ratios

RESULTS

obtained between CD3⁺pSMAD2⁺, CD3⁻pSMAD2⁺ or CD3⁺pSMAD2⁻ cells and the nearest CD34⁺ cell were all below 2, suggesting that BECs are a less likely source of active TGF- β 1 in that sample.

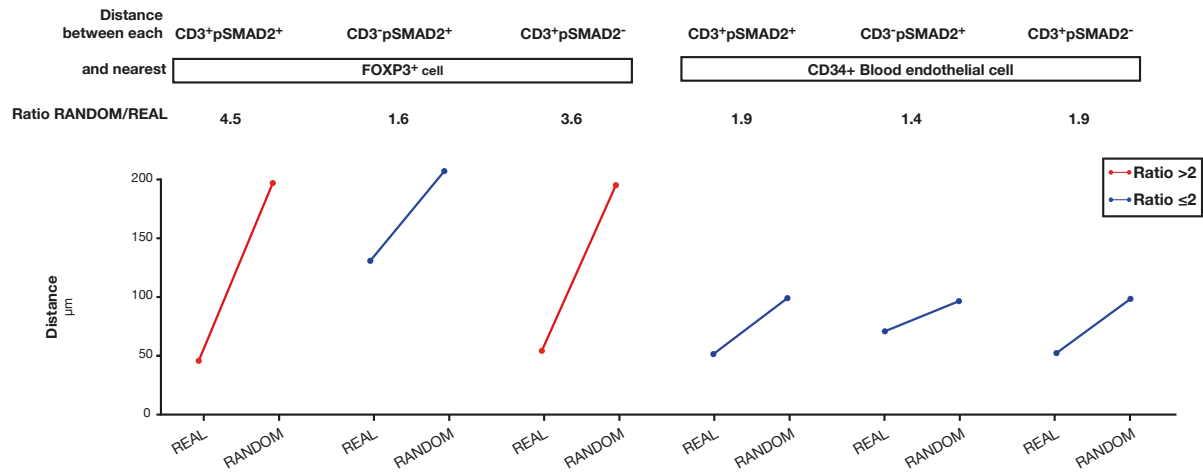


Figure 5 : Spatial Relationship Between TGF- β 1-Targeted Cells and Potential TGF- β 1 GARP-expressing sources in a particular melanoma metastasis

Each line segment represents the mean calculated distances between the two cell types indicated above, with the left (REAL) and right (RANDOM) dots standing for the distances obtained before and after random repositioning, respectively. RANDOM and REAL mean distances are connected by a blue or red line segment if their ratio is ≤ 2 or >2 , respectively. The ratios are indicated on top of the plot.

Discussion

In this study, we aimed at developing a novel multiplexed immunofluorescence (mIF) workflow combined with image analysis and dedicated R scripts for the study of GARP-expressing cells as a possible source of TGF- β 1 in human tumors. mIF offers the advantage of enabling the study of cellular communications with spatial context, which is unassessable when using classic bulk techniques or single cell techniques on dissociated tissues.

Our workflow includes a 5-plex mIF staining protocol that uses a three-step fluorescent signal amplification with TSA and that is suitable for frozen sections. Aggressive chemical treatments are often used to denature antibodies between staining cycles, but these methods are not suitable for frozen sections because of potential damage to tissue architecture. Instead, we used a peroxidase inhibitor to inactivate HRPs between staining cycles, which preserves tissue integrity. The antigens targeted in the panel are GARP, pSMAD2, FOXP3, CD3, and CD34. This panel was chosen to enable the quantification of putative TGF- β 1 producing cells including GARP-expressing Tregs and GARP-expressing BECs, as well as the TGF- β 1 targeted cells (i.e. pSMAD2⁺ cells), which possibly include CD3⁺ T cells. A limitation of our mIF staining approach is the challenge of finding different isotypes or species for each pair of primary and secondary antibodies in each staining cycle.

Image analyses were performed to localize and count cells and objects. CD34⁺ objects corresponded to blood vessels and GARP⁺ objects were defined as GARP fluorescence associated to perivascular or fibrotic areas. A difficulty of this study was to distinguish activated GARP⁺FOXP3⁺ Tregs from FOXP3⁺ co-localized with a GARP⁺ object. For this purpose, we wrote a dedicated R script to identify FOXP3⁺GARP⁺ cells that are outside GARP⁺ objects and that can unambiguously be considered as GARP-expressing Tregs. To avoid this step, other markers of activated Tregs, such as CD25, could have been useful to identify activated Tregs but were not included in the panel due to the limited number of antigens that can be stained on the same section. Unfortunately, this method worked in most cancer types (cutaneous melanoma metastasis, colorectal carcinoma, urothelial carcinoma and breast carcinoma) but not in non-small cell lung carcinoma (NSCLC). In the latter cancer type, pulmonary capillary architecture, where small capillaries resemble single cells and are not detected as GARP⁺ objects, leads to an overestimation of activated Tregs. We replaced this method by visual inspection of all or a random fraction of at least 10% of the FOXP3⁺GARP⁺ cells identified by the image analysis software in all the samples. For each sample, the number of validated FOXP3⁺GARP⁺ cells was calculated as the total number of FOXP3⁺GARP⁺ cells obtained by automated counting times the proportion of validated cells. The proportion of FOXP3⁺GARP⁺ cells was obtained by dividing the respective validated counts by the total number of cells.

A second script was developed to study TGF- β 1-mediated intercellular communication in tissues. More specifically, the code computes the distance between each TGF- β 1-targeted cells (pSMAD2⁺ cells) to their nearest putative source of TGF- β 1, which can either be a FOXP3⁺ cell or a CD34⁺ BEC. These distances are compared to a situation in which the TGF- β 1 targeted cells are randomly relocated on the tissue section.

This workflow could be easily adapted for the study of other cytokine-mediated cell-to-cell communications using mIF.

Materials and Methods

Samples

The tissue sample shown here was obtained by surgery as surgical discard, after informed consent and under approval of the Commission d’Ethique Biomédicale Hospitalo-Facultaire, Brussels, Belgium (reference 2021/03JUI/264). Tissue fragment was snap-frozen, embedded in Optimal Cutting Temperature Compound (Sakura) and stored at -80°C.

Staining and immunostaining of tissue sections

A 7µm-thick tissue cryosection underwent multiplexed immunofluorescence (mIF) consisting of 5 sequential cycles of staining, one per target antigen (**Suppl. Table 1**). For practical reasons, the staining was split over three days. GARP was stained on the day of the fixation, pSMAD2 and FOXP3 on the following day and CD3 and CD34 on the last day.

An adjacent section underwent immunohistochemistry (IHC) with an antibody cocktail specific for melanocytes and melanoma cells. It was fixed for 5 min in 4% formaldehyde, treated with Bloxall (Vector Laboratories) for 15 min, rinsed with TBS-T, permeabilized and blocked with Normal Goat Serum Blocking Solution 2.5% (Vector Laboratories) for 30 min, incubated for 1h with a cocktail of 4 antibodies (Biocare, #165), washed in TBS-T, incubated with EnVision+ System-HRP Anti-mouse (Agilent), washed twice in TBS-T, treated with 3-amino-9-ethylcarbazole substrate (ThermoFisher), washed in TBS-T, counterstained with hematoxylin (Roth), mounted with IHC Mounting Medium (Enzo) and covered.

Chemical synthesis of ATTO425-tyramide

ATTO425-tyramide was synthesized according to a modified procedure of Hopman et al [11]. Briefly, a solution of tyramine hydrochloride (Sigma-Aldrich) (1.6 mg/ml) in dimethyl sulfoxide (DMSO) and a small excess of N-methylmorpholine (Sigma-Aldrich) (2 µl) was added in equimolar amounts to a solution of ATTO425 NHS ester (ATTO-TEC) (5 mg/200µl) in DMSO under stirring. The reaction was allowed to proceed in the dark at room temperature for two hours. Finally, ATTO425-tyramide was purified by reverse phase high pressure liquid chromatography, lyophilized and dissolved in DMSO to prepare a 3 mg/ml stock solution.

Digital Scanning

The mIF stained slide was scanned with an Axioscan.z1 slide scanner, equipped with a Colibri 7 light source, a 20x/0.8x Plan-Apochromat objective (all from Zeiss), an Orca Flash 4.0 V3 camera (Hamamatsu) and Dylight405-C, SpAqua-C, Cy5-A filter sets (all from Semrock), Green3, GoldFish and Red2 filter sets (all from Chroma). The IHC slide was scanned with a Panoramic P250 Flash III slide scanner (3DHistech), equipped with a 20x/0.8x Plan-Apochromat objective (Zeiss) and a Quartz Q-12A180 camera (Adimec)

Data Analysis

The digital images were analyzed with the HALO v3.3 software and its HighPlex v4.1.3 and Object Colocalization v1.0 modules (Indicalabs). Quantitative analyses were carried out with in-house computer scripts written in the R programming language (v.4.3.1). Due to rare misidentification of GARP⁺ objects close to excluded portions of the ROI, an additional detection of the GARP⁺ objects was conducted on the entire scan image. The GARP-expressing objects identified in both analyses were used for the detection of GARP⁺FOXP3⁺ cells outside of these areas.

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RESULTS

Supplementary material

Cycle	Peroxidase Block	Protein Block	Primary Antibody	Secondary Antibody	HRP Substrate
1	Peroxidase-Blocking Solution ¹	Home-made blocking solution ³	Anti-GARP antibody ⁴ (MHG-6, 5µg/mL), 90 min	EnVision+ System-HRP Anti-mouse secondary antibody ¹ , 1 h	Tyramide AF-488 Congugate ⁶ in Borate Buffer, 10 min
2	Bloxall ²	Normal Goat Serum 2.5% ²	Anti-phospho SMAD2 antibody ⁵ (138D4, 4.5 µg/mL), 90 min	EnVision+ System-HRP Anti-rabbit secondary antibody ¹ , 1h	Tyramide AF-555 Congugate ⁶ in Borate Buffer, 10 min
3	Bloxall ²	Normal Goat Serum 2.5% ²	Anti-FOXP3 antibody ⁶ (450, 5µg/mL), 15 min	Peroxidase AffiniPure Goat Anti-Mouse subclass 3 specific secondary antibody ⁸ , 1 h	Tyramide AF-647 Congugate ⁶ in Borate Buffer, 20 min
4	Bloxall ²	Normal Goat Serum 2.5% ²	Anti-CD3ε antibody ⁷ (CD3-12, 5µg/mL), 90 min	ImmPRESS HRP Goat Anti-Rat ² , 1 h	Tyramide ATTO-425 Congugate (in-house), 10min
5	Bloxall ²	Normal Goat Serum 2.5% ²	Anti-CD34 ⁶ antibody (QBEnd-10, 0.25µg/mL), 90 min	Peroxidase AffiniPure Goat Anti-Mouse subclass 1 specific secondary antibody ⁸ , 1 h	Tyramide AF-594 Congugate ⁶ in Borate Buffer, 20 min

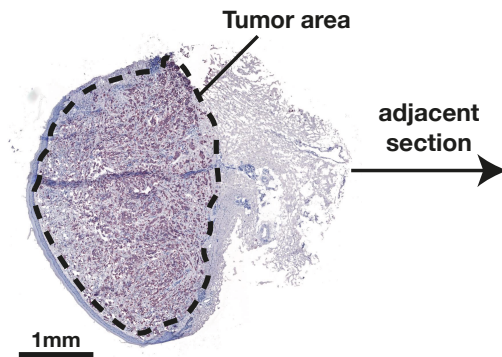
¹ Agilent, ² Vector Laboratories, ³ TBS-T 2% milk, 5% bovine serum albumin and 1% human Ig (IgIV, CSL Behring), ⁴ ref [10], ⁵ Cell Signaling Technology, ⁶ ThermoFisher, ⁷ LSBio, ⁸ Jackson ImmunoResearch, ⁹ Borate Buffer containing 0.1M boric acid, 3M NaCl, 0.1% Tween 20, pH7.8, supplemented with 0.003% H₂O₂.

Supplementary table 1: The reagents, amounts and incubation times used in multiplexed immunofluorescent staining.

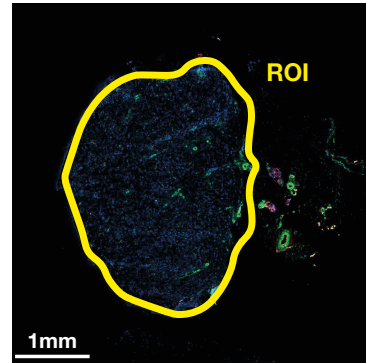
RESULTS

Region of Interest (ROI)

Hematoxylin + immunohistochemistry



5-plex mIF



Supplemental Figure 1 : The region of interest on the mIF image, corresponding to the tumor area was drawn based on an adjacent slide stained with IHC, using antibodies targeting melanoma antigens.

GARP-expressing Tregs as a source of immunosuppressive TGF- β 1 in human tumors

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Abstract

TGF- β 1 is produced as a latent cytokine that requires activation to exert its pleiotropic activities. All cells produce latent TGF- β 1 but only very few can activate the cytokine via cell type-specific mechanisms. In cancer, TGF- β 1 favors disease progression by suppressing anti-tumor T cell responses. Which cells produce and activate immunosuppressive TGF- β 1 in human tumors is not known. Putative sources include GARP-expressing cells, comprising mostly activated regulatory T cells (Tregs) and blood endothelial cells (BECs). We performed multiplexed immunohistofluorescence and computerized image analyses on 186 tumor samples from 5 cancer types (colorectal, urothelial, lung and breast primary carcinomas and cutaneous melanoma metastases), compared to patient-matched adjacent non-cancerous tissues and to tonsils. The proportion of activated Tregs (GARP⁺FOXP3⁺ cells) was higher in tumors than in matched non-cancerous tissues. GARP⁺ Tregs were present in 29 to 75% of the various types of tumor samples and in the majority of the tonsils. Unexpectedly, the proportion of GARP⁺ Tregs did not correlate with that of CD3⁺ tumor-infiltrating T lymphocytes (TILs). The density of blood vessels was similar across samples, with more than half of the vessels expressing GARP. The proportion of cells undergoing TGF- β 1 signaling (pSMAD2⁺ cells) was approximately twice as high in tumors compared to matched non-cancerous tissues and correlated with the proportion of GARP⁺ Tregs in urothelial carcinomas but not in other tumor types. In most tumor types, pSMAD2⁺ TILs were also twice closer to the nearest FOXP3⁺ cell than after random repositioning, at a distance ($\sim 70 \mu\text{m}$) consistent with short-range paracrine TGF- β 1 signaling. In contrast, pSMAD2⁺ non-T cells and pSMAD2⁻ TILs were not closer to FOXP3⁺ cells, neither were pSMAD2⁺ cells (TILs and others) to BECs. We conclude that, in human tumors, GARP-expressing Tregs rather than BECs appear to represent a source of TGF- β 1 acting on nearby TILs. This could contribute to local immunosuppression and may be blocked with anti-GARP:TGF- β 1 antibodies, particularly to treat patients with tumors heavily infiltrated by GARP-expressing Tregs.

Introduction

Cytokines of the transforming growth factor-beta (TGF- β) family control multiple biological processes including embryonic development, angiogenesis, tissue repair and immune responses [1]. The family comprises the TGF- β 1, β 2 and β 3 isoforms, which signal through a common TGF- β receptor (T β R). While TGF- β 2 and β 3 play non-redundant roles in development, TGF- β 1 is the most important isoform regulating immune responses after birth. Defects in TGF- β 1 production or signaling lead to autoimmunity and inflammation [2, 3]. In cancer, TGF- β 1 signaling can suppress tumor growth by inducing apoptosis in pre-malignant cells or inhibiting the proliferation of cancer cells, but it also promotes tumor progression by inducing EMT, stimulating neo-angiogenesis, and suppressing anti-tumor T cell responses [4]. The cellular sources and activation mechanisms of immunosuppressive TGF- β 1 in tumors are not known.

Virtually all cell types produce TGF- β 1 in a latent form, comprising the mature TGF- β 1 homodimer non-covalently associated with the latency associated peptide (LAP) homodimer. LAP masks the binding sites of mature TGF- β 1 to the T β R, precluding biological activity [5, 6]. Most cells produce latent TGF- β 1 in association with a partner protein that is covalently linked to LAP via two disulfide bonds. Depending on the cell type, the partner protein can be either one of four secreted Latent TGF- β 1 Binding Proteins (LTBP-1 to 4), or one of two transmembrane proteins containing leucine-rich repeats known as GARP and LRRC33. Secretion of LTPB:TGF- β 1 complexes results in the deposition of a latent TGF- β 1 reservoir in the extracellular matrix, whereas production of GARP:TGF- β 1 or LRRC33:TGF- β 1 complexes results in presentation of latent TGF- β 1 on the surface of GARP- or LRRC33- expressing cells, respectively. LRRC33:TGF- β 1 complexes are present on microglia and macrophages [7, 8], whereas GARP:TGF- β 1 complexes are present on regulatory T cells (Tregs) stimulated via the T cell receptor (TCR), B cells stimulated via the B cell receptor, endothelial cells, mesenchymal cells, and stimulated platelets and megakaryocytes [9-12]. Among these cells, Tregs are of particular interest in cancer, as they are known to suppress anti-tumor T cell responses via various mechanisms, which include production of active TGF- β 1 [13].

A tightly regulated process referred to as “TGF- β 1 activation” is required to release the mature cytokine from LAP, allowing binding to the T β R and subsequent autocrine or paracrine activity. Several cell type-specific mechanisms of latent TGF- β 1 activation have been described [1]. Activation from GARP:TGF- β 1 complexes on TCR-stimulated Tregs occurs via binding of integrin α V β 8 to an RGD motif in LAP [6, 14-17]. Mechanical pulling forces from the cytoskeleton transmitted by integrins to latent TGF- β 1 are thought to induce the deformation of LAP and release of mature TGF- β 1, which can now bind the T β R to trigger a signaling cascade characterized by phosphorylation of SMAD2 (pSMAD2) and SMAD3 in target cells [5].

Several strategies to inhibit the TGF- β signaling pathway have been attempted to treat cancer [18]. Most inhibit the serine-threonine kinase activity of the ubiquitously expressed T β R (small molecule inhibitors), or target mature TGF- β 1, 2 and 3 after their release from LAP (soluble T β Rs or anti-TGF- β mAbs) [18, 19]. Early clinical trials investigating these approaches in cancer have shown some clinical benefit but have sometimes led to severe cardiovascular toxicity, epithelial bleeding or appearance of pre-malignant tumors. These adverse events may be caused by the fact that most TGF- β -targeting therapies indistinctively inhibit signaling by all three TGF- β isoforms, irrespective of their cellular sources and targets.

RESULTS

Selective inhibition of TGF- β 1 activation by immunosuppressive cells might preserve or improve anti-tumoral efficacy while reducing toxicity. To this end, we previously developed mAbs that bind the human or mouse GARP:TGF- β 1 complex and block TGF- β 1 activation by TCR-stimulated Tregs [9, 16, 20]. Anti- GARP:TGF- β 1 mAbs showed therapeutic efficacy in murine models of solid tumors and blood cancer [9, 10]. The mAbs acted by blocking TGF- β 1 activation on GARP-expressing Tregs, as the anti-tumor effect was lost in mice carrying a selective deletion of the *Garp* gene in Tregs [9]. A mAb blocking the human GARP:TGF- β 1 complex is currently tested in phase 1 and 2 clinical trials in patients with advanced or metastatic solid tumors (NCT-03821935, NCT-05822752, NCT-061009272). There is currently no biomarker to predict which patients could benefit most from GARP:TGF- β 1 blockade.

We and others described the presence of GARP-expressing Tregs (GARP⁺FOXP3⁺ cells) in human tumors [9, 21, 22]. However, it is not known whether GARP-expressing Tregs release active TGF- β 1 in the tumor microenvironment in patients, nor whether this suppresses tumor-infiltrating effector T cells (TILs). In addition, cells other than Tregs express GARP in the tumor environment [20, 23]. They comprise mostly blood endothelial cells (BECs), which could also release active TGF- β 1 and contribute to suppression of anti-tumor TILs, although this has never been demonstrated either.

Here, we investigated the identity, abundance and distribution of GARP-expressing cells, as well as their spatial proximity with cells undergoing TGF- β 1 signaling (pSMAD2⁺ cells), in tumors of patients with colorectal, urothelial, lung or breast primary carcinomas or cutaneous melanoma metastases. Our results suggest that GARP-expressing Tregs represent a prominent source of immunosuppressive TGF- β 1 acting on T cells in human tumors, and that their abundance in tumor samples could serve to identify patients who may benefit most from GARP:TGF- β 1 blockade therapy.

Results

Multiplexed immunofluorescence analysis of human tissue samples from five cancer types and non-cancerous controls

We performed multiplexed immunofluorescence (mIF) staining of tissue sections and computerized image analyses to study the abundance, distribution and spatial interactions of GARP-expressing cells, T cells, blood vessels and cells undergoing TGF- β 1 signaling in a large series of tumors of different histological types. We analyzed 186 tumors including 24 primary colorectal carcinomas (CRC), 52 cutaneous melanoma metastases (CMM), 41 primary breast carcinomas (BC) and 12 breast ductal carcinomas *in situ* (DCIS), 35 primary non-small cell lung carcinomas (NSCLC), and 22 primary urothelial carcinomas (UC). Adjacent non-cancerous tissue was processed in parallel for 11 CRC, 5 DCIS, 25 NSCLC and 7 UC patients. Adjacent non-cancerous tissues were not available for CMM and BC. Images of a few representative samples are shown in **Fig. 1**. We also analyzed 10 tonsils as reference lymphoid tissues and useful technical controls. We used frozen tissues because none of the multiple anti-GARP antibodies that we tested yielded specific signals on formalin-fixed paraffin-embedded tissues. Tissue sections were stained with anti-GARP and anti-pSMAD2 antibodies, as well as antibodies to identify endothelial cells and blood vessels (CD34⁺), T cells (CD3e⁺) and FOXP3⁺ cells (**Fig. S1A-C**). Digital high-resolution images were obtained by slide scanning and analyzed with the HALO v3.3 software. Values of general interest such as Region Of Interest or ROI surface, nucleated cell count or cellular density are shown in **Fig. S2A**.

RESULTS

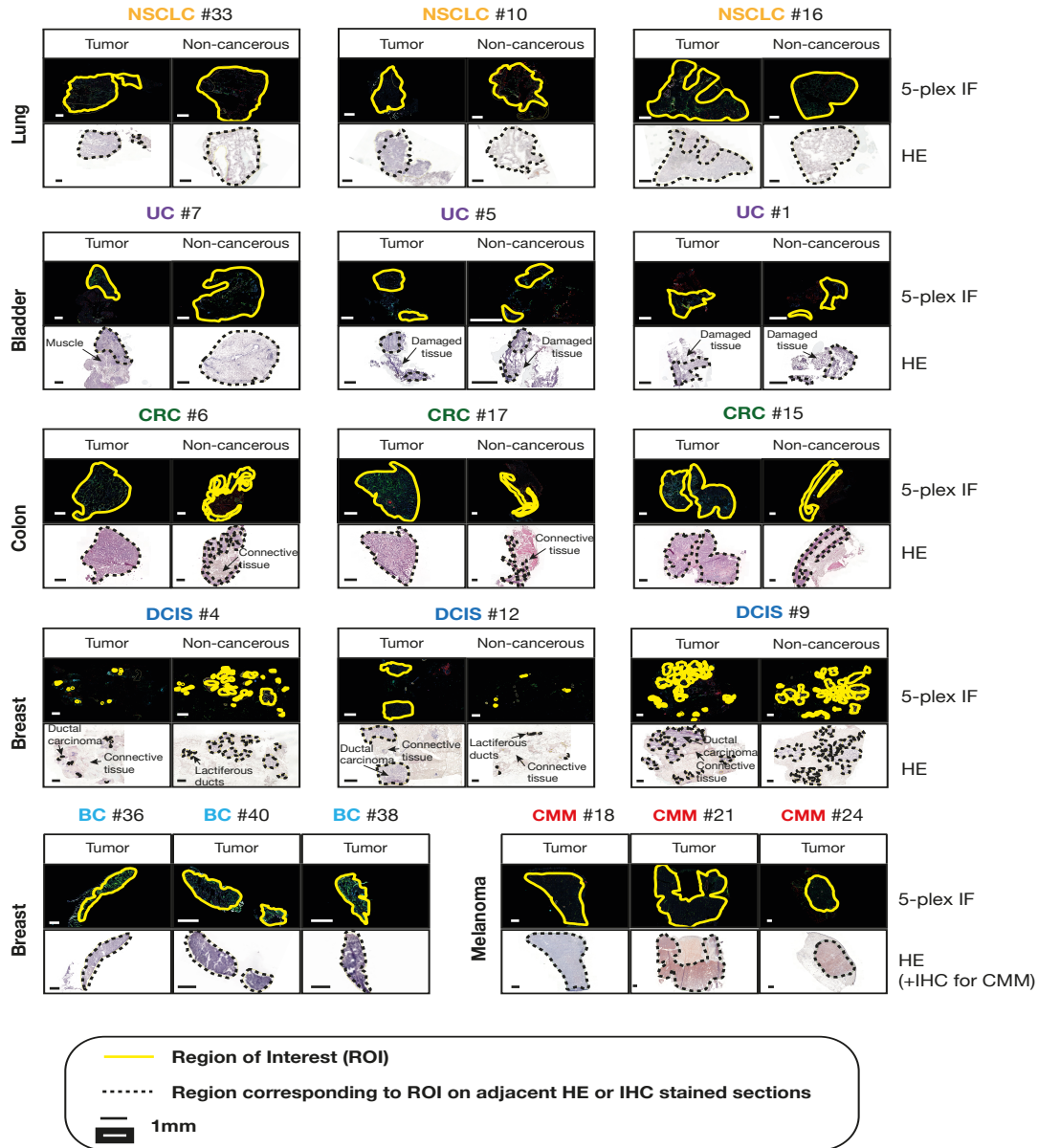


Figure 1: Representative images of ROI delineated on tumor and patient-matched non-cancerous tissues.

Two adjacent cryosections were cut from a series of human tumor samples, patient-matched non-cancerous tissues and tonsils. The first section was stained with hematoxylin and eosin or, in the case of melanoma, melanoma-specific antibodies. The second section underwent sequential mIF staining against CD3e, FOXP3, GARP, CD34 and pSMAD2, followed by nuclear counterstaining with Hoechst33342. High resolution digital microscopy images of both sections were acquired by slide scanning and analyzed with the HALO software. Regions of interest (ROI) comprising the visually identified tumor areas were defined on the first section and reported on the second. ROI of non-cancerous patient-matched samples delineated the same tissue histology as the cancer samples. Illustration of ROI selection in 3 representative tumor and patient-matched non-cancerous tissue are shown for each cancer type.

RESULTS

The proportion of activated Tregs in tumors is higher than in matched non-cancerous tissues, and does not correlate with the proportion of tumor-infiltrating CD3⁺ lymphocytes (TILs)

Human non-regulatory T cells can transiently upregulate FOXP3 upon TCR stimulation. Thus, FOXP3 staining is not sufficient to distinguish Tregs from other T cells in human tissues. In contrast, GARP is induced by TCR stimulation in Tregs but not in other T cells [14]. This allows to unambiguously distinguish activated Tregs (GARP⁺FOXP3⁺) from other T cells.

Proportions of activated Tregs among total cells varied greatly between tumor samples, with median values per tumor type ranging from 0 to 7×10^{-5} (**Fig. 2A**). Almost no or no activated Tregs were observed in matched non-cancerous colon, breast, lung and bladder tissues (median = 0), whereas tonsils had the highest median proportion (2×10^{-4}) across all tissues. GARP⁺ Tregs were detected in most tonsils (80%) with a minimal detected value of 1×10^{-5} . Proportions $\geq 1 \times 10^{-5}$ were observed in 75% of CRC, 63% of CMM, and 29-41% of BC and NSCLC and UC samples. Several samples in each tumor type contained no activated Tregs, although most contained FOXP3⁺ cells (**Fig. 2A and S2B**). The percentage of T cells (CD3⁺) among total cells was much lower in tumors (median 1% to 10%, depending on the tumor type) than in tonsils (median 39%), as expected, and was similar to that found in matched non-cancerous adjacent tissues (median 1% to 8%) (**Fig 2B**). Consequently, the ratio of activated Tregs to T cells was generally higher in tumors that contained activated Tregs than in tonsils (**Fig 2C**). This ratio was particularly high ($>1\%$) in 10 of 52 CMM and a few CRC, BC and UC samples. None of the non-cancerous tissue samples, including the 10 tonsils, had a high activated Treg/T cell ratio.

Unexpectedly however, the proportion of activated Tregs did not correlate with that of tumor infiltrating T lymphocytes (TILs; **Fig 2A and B**).

RESULTS

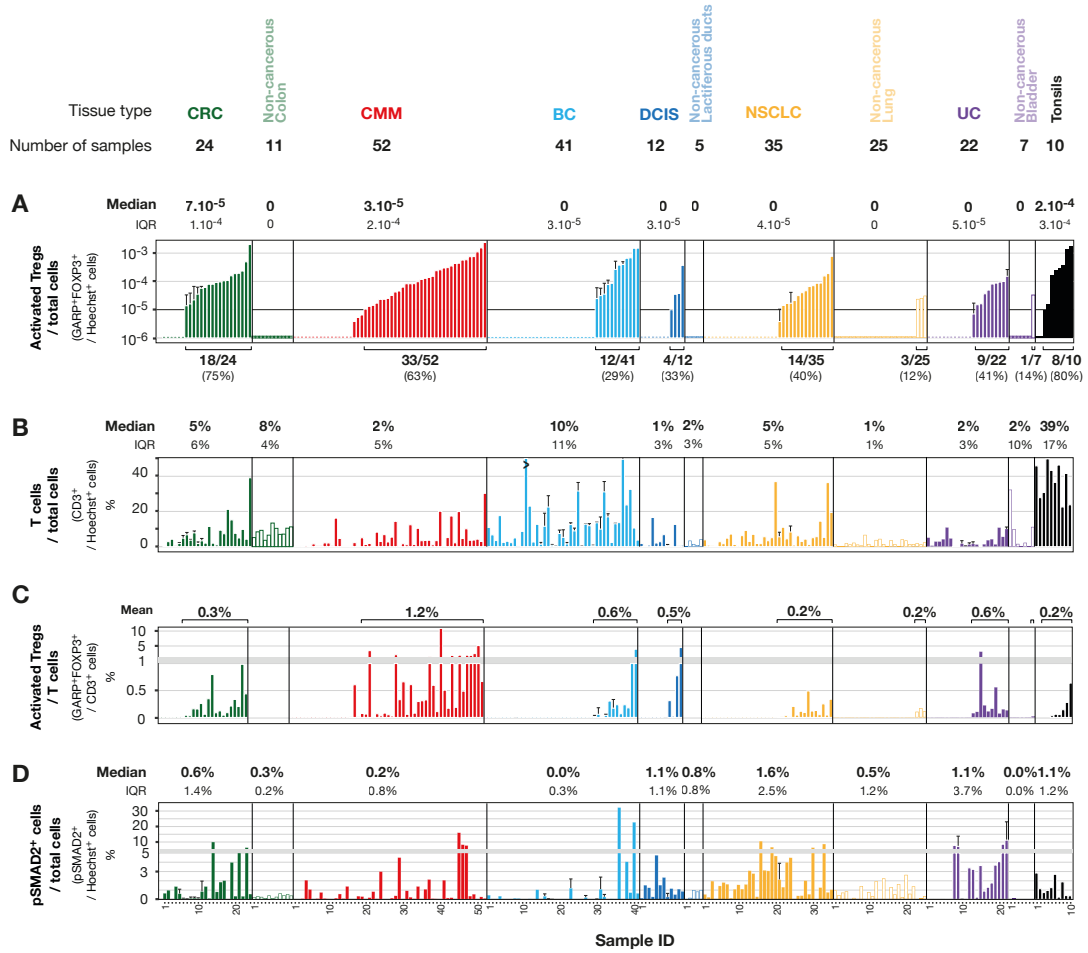


Figure 2: Quantitative analysis of activated Tregs, T cells and cells undergoing TGF- β 1 signaling in human frozen tissue sections.

CRC: colorectal carcinomas; CMM: cutaneous melanoma metastases; BC: breast carcinomas; DCIS: ductal carcinoma in situ; NSCLC: non-small cell lung carcinomas; UC: urothelial carcinomas. Each bar represents an individual sample (sample IDs indicated on the bottom), except when multiple samples from the same tumor were analyzed, in which case the bar represents the mean value of replicates, with the standard deviation displayed as a whisker. Samples are grouped and colored by tissue type and ordered by increasing frequency of activated Tregs (GARP+FOXP3+ cells). The median and interquartile range (IQR) per group is displayed on top of each panel. A. Proportion of activated Tregs in areas devoid of large GARP+ objects. The fraction of samples in which the proportion of activated Tregs is higher than the median in tonsils is indicated under the bar graph. B. Proportion of T cells (CD3+) in the ROI. C. Ratio of activated Tregs to T cells in areas devoid of large GARP+ objects. D. Proportion of pSMAD2+ cells in the ROI.

The density and proportion of GARP+ blood vessels in tumors are similar to that in matched non-cancerous tissues

The median proportions of blood endothelial cells (CD34+) and the median densities of blood vessels (CD34+ objects) were of the same order of magnitude in all tissue types (**Fig. S2C**). The median proportion of GARP+ blood vessels (GARP+CD34+ objects) among total blood vessels was also similar across all tissue types (47% to 82%), except for non-cancerous bladder tissue (16 $\pm$ 36%) (**Fig. S2C**). Large blood vessels (>100 μ m²) appeared more frequently positive for GARP than smaller vessels in all types of tissues ($p < 1.10^{-3}$) (**Fig. S3**).

RESULTS

The proportion of cells undergoing TGF- β 1 signaling is higher in tumors than in non-cancerous tissues, and correlates with that of activated Tregs in UC and BC

The proportion of pSMAD2⁺ cells varied greatly between samples. They were more abundant in NSCLC, DCIS, UC and CRC (medians: 1.6%, 1.2%, 1.1%, and 0.6% respectively) than in matched non-cancerous tissues (medians: 0.5%, 0.8%, 0.0%, and 0.3% respectively). They represented 20-30% of total cells in two BC samples and were almost absent in a few tumor samples (**Fig. 2D**). The proportion of pSMAD2⁺ cells correlated with that of activated Tregs in UC ($R = 0.63$), but not in CRC, DCIS, CMM and NSCLC (**Fig. S4**). A correlation was observed in BC as well ($R = 0.48$), but it was mainly driven by two samples which contained unusually high proportions of activated Tregs (0.5-1.0%) and pSMAD2⁺ cells (20-30%). No correlation was seen between the proportion of pSMAD2⁺ cells and the density of GARP⁺ blood vessels ($R < 0.40$).

TGF- β 1 activity is detected in all cell types analyzed

pSMAD2 was detected in T cells, FOXP3⁺ cells, BECs and other cells (CD3-FOXP3-CD34⁻) in most tissues. The proportion of pSMAD2⁺ cells tended to be higher among FOXP3⁺ cells, T cells or BECs than among other cells in CMM, NSCLC and UC samples (**Fig. S5A**). Proportions of FOXP3⁺ cells, T cells and BECs in pSMAD2⁺ cells were higher than in total cells in CMM and NSCLC samples indicated by arrows in **Fig. S5B**. Together, these observations suggest enriched TGF- β 1 activity in a compartment comprising FOXP3⁺ cells, BECs and T cells within tumors. However, whether Tregs or BECs could represent a source of TGF- β 1 exerting paracrine activity on nearby T cells cannot be determined based on proportions of pSMAD2⁺ cells.

In most tumors, pSMAD2⁺ TILs, but not other pSMAD2⁺ cells, are closer to FOXP3⁺ cells than expected by chance

Because the T β R is expressed ubiquitously and has a very high affinity for its ligands, we expect a close spatial proximity between a putative cellular source of TGF- β 1 (e.g. Tregs or BECs) and the cells on which it could exert immunosuppression (e.g. TILs). To assess this, we measured the distance between each pSMAD2⁺ TIL and the nearest FOXP3⁺ cell or CD34⁺ BEC. We used FOXP3⁺ and CD34⁺ cells, rather than GARP⁺FOXP3⁺ and GARP⁺CD34⁺, because we cannot discriminate if a FOXP3⁺ cell expresses GARP when it is located close to a BEC bordering a blood vessel. As expected, the distance between pSMAD2⁺ cells and neighbors of a given cell type was influenced by the frequency of the neighbor cell type. The median distance from pSMAD2⁺ TILs to the nearest FOXP3⁺ cells, which account for 0.03 to 0.9% of the cells, was 67 μ m, whereas their median distance to BECs, which account for 3 to 7% of the cells, was 46 μ m ("real" distances in **Fig. 3**). Therefore, to assess the significance of the spatial proximity between two cell types, we tested whether real distances were shorter than if the cells were randomly distributed. We digitally repositioned pSMAD2⁺ cells in the ROI and measured distances of the randomly repositioned cells to the nearest FOXP3⁺ cell or BEC (**Fig. S6**). We applied the same procedure to pSMAD2⁻ TILs and pSMAD2⁺ CD3⁻ cells.

In about half of the tumors analyzed (32/67), the mean real distance of pSMAD2⁺ TILs to the nearest FOXP3⁺ cell was more than twice shorter than the random distance (**Fig. 3**). The mean Random/Real ratio of all 67 tumors was 2.3, indicating that FOXP3⁺ cells were on average twice as close to pSMAD2⁺ TILs than expected by chance (**Fig. 3**). In contrast, real distances of pSMAD2⁺ CD3⁻ cells or pSMAD2⁻ TILs to the nearest FOXP3⁺ cells were more than twice shorter in less than 20% of the tumors, with mean Random/Real ratios of 1.5 and 1.6, respectively. We calculated the distances from the three cell types (*i.e.* pSMAD2⁺ TILs, pSMAD2⁺ CD3⁻ cells or pSMAD2⁻ TILs) to the nearest BEC. Here again, less than 15% of the tumors had a mean distance more than twice

RESULTS

shorter than after repositioning, with mean Random/Real ratios ≤ 1.6 (**Fig. 3**). These observations held true when considering CRC, CMM and NSCLC cancer types independently, whereas no conclusion could be drawn for BC and UC due to the small number of samples analyzed (**Fig. S7**). Altogether, our results indicate that pSMAD2⁺ TILs, but not pSMAD2⁺ non-T cells, are closer to FOXP3⁺ cells, but not to BECs, than expected by chance. Distances between pSMAD2⁺ TILs and FOXP3⁺ cells (median: 67 μm) are compatible with short-range paracrine TGF- β 1 signaling. This supports the hypothesis that FOXP3⁺ cells, which include GARP-expressing Tregs, are a prominent source of active TGF- β 1 suppressing T cells in human tumors

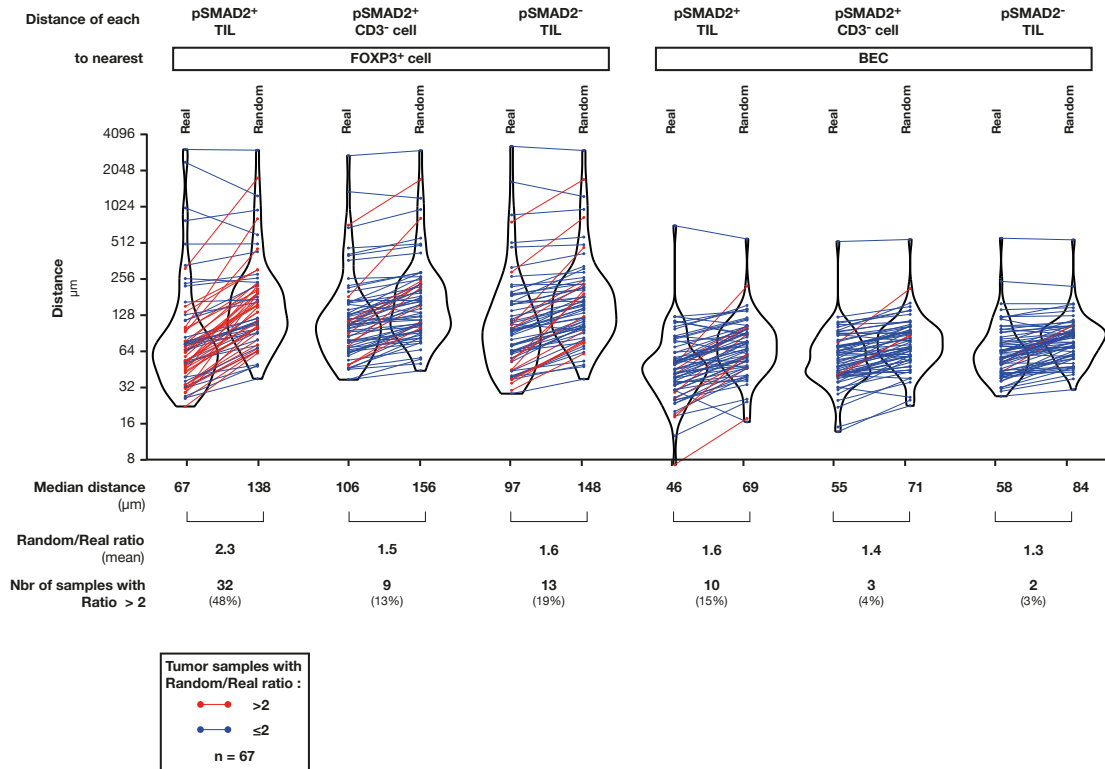


Figure 3: In tumors, TILs undergoing TGF- β 1 signaling (pSMAD2⁺) are closer to the nearest FOXP3⁺ cell than expected by chance.

Pairs of violin plots represent distances measured between each cell of the indicated cell type (pSMAD2⁺ or - TILs or non-T cells) and its nearest FOXP3⁺ or BEC neighbor. Each dot represents the mean distance in one tumor sample. Dots in each left and right violin plot represent either real or mean distances measured after 5 random repositioning of pSMAD2⁺ or - cells, respectively. Real and random distances from the same sample are connected by a line (blue line : random/real ratio ≤ 2 ; red line : random/real ratio > 2). Only tumor samples containing ≥ 50 pSMAD2⁺ TILs were analyzed (n=67).

Discussion

Studies evaluating the frequency and spatial distribution of activated Tregs infiltrating patient-derived tumor samples are scarce, because specific immunostaining of Tregs has remained challenging. One reason for this is that markers most frequently used to identify Tregs in human tissues lack Treg specificity. Both FOXP3 and CD25, the most frequently used markers, are also expressed by non-regulatory T cells after TCR stimulation. GARP is one of very few markers that appear on Tregs but not on other T cells upon TCR stimulation [24]. Unfortunately, most if not all available anti-GARP antibodies do not work on FFPE tissues. It is also worth noting that *GARP* mRNA expression correlates poorly with GARP protein levels, precluding the use of *in situ* hybridization as an alternative to immunostaining of FFPE tissues [24]. We circumvented these limitations by using an anti-GARP monoclonal antibody that specifically stains GARP-expressing cells in OCT-embedded frozen human tissues [16]. This allowed us to identify and analyze GARP⁺FOXP3⁺ cells (*i.e.* activated Tregs) by mIF and computerized image analysis in >200 samples of human cancerous and non-cancerous tissues.

In line with previous reports analyzing tumor-infiltrating FOXP3⁺ cells in various cancer types, we observed that frequencies of GARP-expressing Tregs were higher in tumors than in patient matched non-cancerous tissues [25-27]. Interestingly, we found activated Tregs in 29 to 75% of samples in the five different cancer types, but not in all. We could not evaluate whether the presence of GARP-expressing Tregs correlated with clinical prognosis, but we suggest that it could be tested as a biomarker to identify patients who could benefit most from GARP:TGF- β 1 targeting immunotherapy.

More unexpectedly, the frequencies of GARP-expressing Tregs did not correlate with that of CD3⁺ TILs in the large number (n=186) of patient-derived samples that we analyzed. This observation contrasts with previous reports by others, who used FOXP3 and/or CD25 as markers for Tregs [28-30]. It also contrasts with our own previous report, which analyzed GARP⁺FOXP3⁺ cells by mIF and estimated TIL abundance and activity by bulk RNAseq on a small number (n=19) of resected cutaneous melanoma metastases [9]. The lack of correlation between GARP-expressing Tregs and TILs observed here suggests that assessing the abundance and distribution of activated Tregs in human tumors will provide information that cannot be inferred from the analysis of tumor-infiltrating immune (T) cells alone.

Another aspect of our study consisted in trying to identify the cellular sources and targets of TGF- β 1 activity in human tumors. This has remained an outstanding question, due to the complex multi-step regulation of TGF- β 1 production and activation, the ubiquitous expression of TGF- β receptors, and the pleiotropy of TGF- β signaling and response. We show data that strongly support the fact that Tregs, but not BECs, are a prominent source of TGF- β 1 suppressing CD3⁺ TILs in approximately half of CMM, NSCLC and CRC cases. This may also hold true in UC and BC, the two other types of solid tumors that were studied here, although more samples will need to be analyzed to confirm this. The experimental argument we present is based on the observed proximity of FOXP3⁺ cells to pSMAD2⁺ T cells. Previous reports investigated the spatial proximity of FOXP3⁺ cells to T cells (effector T cells, whether or not these are under the influence of TGF- β 1 signals), and their impact on survival in cancer patients [28, 31-35]. Only one of these, focusing on early stage NSCLC, included a marker of TGF- β 1 activity, but it did not attempt to identify the cellular source of the cytokine [32]. Another one (in CRC) included random cell repositioning to assess the significance of distance measurements between FOXP3⁺CD25⁺ cells and CD8⁺ cells, but it did not include a marker of TGF- β 1 activity [33].

RESULTS

In addition to GARP-expressing Tregs, other reservoirs and putative cellular sources of TGF- β 1 are present in human tumors. We examined BECs, a majority of which also express GARP in tumors, but they do not appear to be a source of TGF- β 1 acting on TILs, at least not a source as prominent as Tregs. Other putative reservoirs were not examined here: they include lymphatic endothelial cells (LECs) and fibroblasts, which also present latent TGF- β 1 bound to GARP on their surface [23], and myeloid cells, which present latent TGF- β 1 bound to LRRC33 on their surface [36]. Large amounts of latent TGF- β 1 bound to LTBP are also deposited in the extra-cellular matrix.

Altogether, our results suggest that GARP-expressing activated Tregs infiltrate tumors of a subset of patients with CMM, BC, UC, CRC and NSCLC. Activated Tregs appear to represent a source of TGF- β 1 that acts on neighboring T cells in these tumors, thereby contributing to suppression of anti-tumor immune responses. Our results may open the path to the identification of biomarkers to identify patients with metastatic or locally advanced solid tumors who may best benefit from treatment with anti-GARP:TGF- β 1 antibodies, which are currently tested in early phase clinical trials.

Materials and Methods

Samples

Tumoral and non-cancerous tissue samples were obtained by surgery or biopsy, as surgical discard, or research-aimed material, after informed consent and under approval of the Commission d’Ethique Biomédicale Hospitalo-Facultaire, Brussels, Belgium (references 2021/03JUI/264, 2019/17JUI/261 and 2020/29JUI/345), or of the Comité d’Ethique Hospitalier of the Grand Hopital de Charleroi (reference ONCOGHdC2015-01 et ONCOGHdC2017-01). Tissue fragments were snap-frozen, embedded in Optimal Cutting Temperature Compound (Sakura) and stored at -80°C.

Staining and immunostaining of tissue sections.

7µm-thick tissue cryosections were mounted on a microscope slide, fixed in formaldehyde 4% during 5 min, and washed in Tris-buffered saline (TBS) supplemented with 0.005% Tween20 (TBS-T). One tissue section per sample underwent multiplexed immunofluorescence (mIF) consisting of 5 sequential cycles of staining, one per target antigen (**Suppl. Table 1**). In each cycle, the sections were incubated with a primary antibody of distinct species or isotype, then with a matched secondary antibody coupled to horseradish peroxidase (HRP), and finally with a distinct tyramide-coupled fluorophore. Between each cycle, the enzyme was inactivated with a peroxidase blocking reagent. Finally, the slides were counterstained with Hoechst33342 (ThermoFisher), mounted with Fluorescent Mounting Medium (Dako) and covered.

In parallel with mIF, an adjacent or neighboring tissue section was also processed. For non-melanoma tissues, it was stained with hematoxylin and eosin (HE) (Roth). For melanoma samples, it underwent immunohistochemistry (IHC) with an antibody cocktail specific for melanocytes and melanoma cells [37]. It was fixed for 5 min in 4% formaldehyde, treated with Bloxall (Vector Laboratories) for 15 min, rinsed with TBS-T, permeabilized and blocked with Normal Goat Serum Blocking Solution 2.5% (Vector Laboratories) for 30 min, incubated for 1h with a cocktail of 4 antibodies (Biocare, #165), washed in TBS-T, incubated with EnVision+ System-HRP Anti-mouse (Agilent), washed twice in TBS-T, treated with 3-amino-9-ethylcarbazole substrate (ThermoFisher), washed in TBS-T, counterstained with hematoxylin (Roth), mounted with IHC Mounting Medium (Enzo) and covered (IHC slides). All the steps were performed at room temperature.

Chemical synthesis of ATTO425-tyramide

ATTO425-tyramide was synthesized according to a modified procedure of Hopman et al [38]. Briefly, a solution of tyramine hydrochloride (Sigma-Aldrich) (1.6 mg/ml) in dimethyl sulfoxide (DMSO) and a small excess of N-methylmorpholine (Sigma-Aldrich) (2 µl) was added in equimolar amounts to a solution of ATTO425 NHS ester (ATTO-TEC) (5 mg/200µl) in DMSO under stirring. The reaction was allowed to proceed in the dark at room temperature for two hours. Finally, ATTO425-tyramide was purified by reverse phase high pressure liquid chromatography, lyophilized and dissolved in DMSO to prepare a 3 mg/ml stock solution.

Digital Scanning

mIF slides were scanned with an Axioscan.z1 slide scanner, equipped with a Colibri 7 light source, a 20x/0.8x Plan-Apochromat objective (all from Zeiss), an Orca Flash 4.0 V3 camera (Hamamatsu) and Dylight405-C, SpAqua-C, Cy5-A filter sets (all from Semrock), Green3, GoldFish and Red2 filter sets (all from Chroma).

RESULTS

HE and IHC slides were scanned with a Panoramic P250 Flash III slide scanner (3DHistech), equipped with a 20x/0.8x Plan-Apochromat objective (Zeiss) and a Quartz Q-12A180 camera (Adimec).

Data Analysis

The digital images were analyzed with the HALO v3.3 software and its HighPlex v4.1.3 and Object Colocalization v1.0 modules (Indica Labs) (**Fig. S1A**). Tumoral and normal epithelial regions of interest (ROI) were selected based on the IHC or HE images and reported on the mIF images (**Fig. 1 and S1B**). We determined ROI surface, number of nucleated cells (Hoechst⁺), proportion of marker-positive cells among all cells and counts of blood vessels by computerized image detection of individual cells and of phenotypically similar cells grouped as objects. The spatial localization of each cell and blood vessel (CD34⁺ object) was also registered as X and Y coordinates. Quantitative analyses were carried out with in-house computer scripts written in the R programming language (v.4.3.1).

GARP immunostaining is not restricted to FOXP3⁺ and CD34⁺ cells. It is also frequently detected in the extracellular matrix, including perivascular areas. In these structures, it can be difficult to assess whether a FOXP3⁺ cell is also GARP positive. Therefore, we validated by visual inspection all or a random fraction of at least 10% of the FOXP3⁺GARP⁺ cells identified by the software. For each sample, the number of validated FOXP3⁺GARP⁺ cells was calculated as the total number of FOXP3⁺GARP⁺ cells obtained by automated counting times the proportion of validated cells. The proportion of FOXP3⁺GARP⁺ cells was obtained by dividing the respective validated counts by the total number of cells [9]. All the other parameters were measured in the entire ROI. In the 186 tumor samples, the surface of the ROI was comprised between 0.1 and 340 mm² and contained 7.10² to 2.10⁶ nucleated cells. The cellular density ranged from 5.10² to 1.10⁴ cells/mm². BC, DCIS and non-cancerous breast samples were smaller compared to other tissues, with a median ROI surface of 3 and 0.2 mm², respectively (**Fig. S2A**).

We also determined the Euclidean distances between each pSMAD2⁺ CD3⁺ cell (pSMAD2⁺ TIL) and the nearest FOXP3⁺ cell or CD34⁺ BEC. We then randomly relocated the same number of pSMAD2⁺ TILs in the ROI and measured the distances again. The random relocation process was weighted with the local density of nuclei in the ROI. Cells had a greater probability of being randomly relocated in regions of the ROI with a higher density of nuclei. The random relocation was repeated 5 times in total. We carried out the same procedure with either pSMAD2⁻ TILs or pSMAD2⁺ CD3⁻ cells. Only tumors in which more than 50 pSMAD2⁺ CD3⁺ cells were counted were analyzed.

Statistical analyses were done using R (v.4.3.1). Differences between GARP positivity among large and small blood vessels was done using a pairwise Wilcoxon two-tailed test (stats package). Pearson's correlation coefficients and p values of linear regressions were calculated using the *stat_cor* function (ggpubr package).

RESULTS

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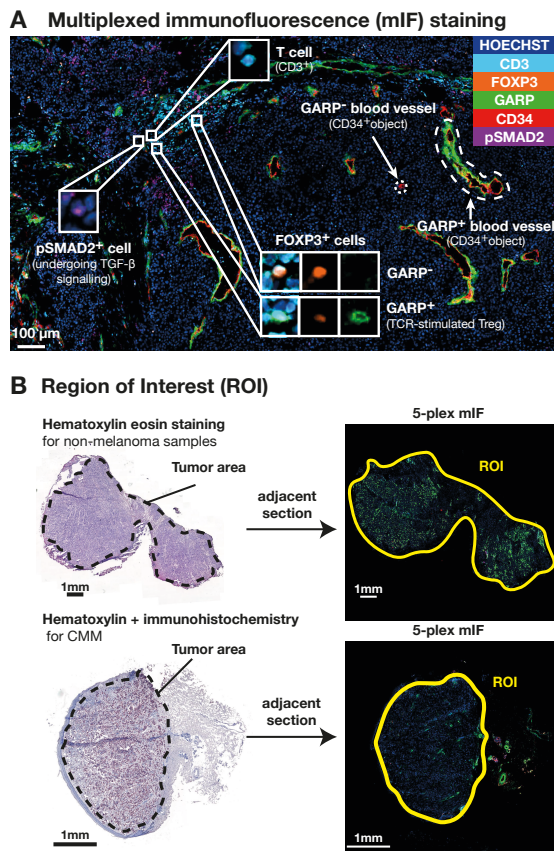
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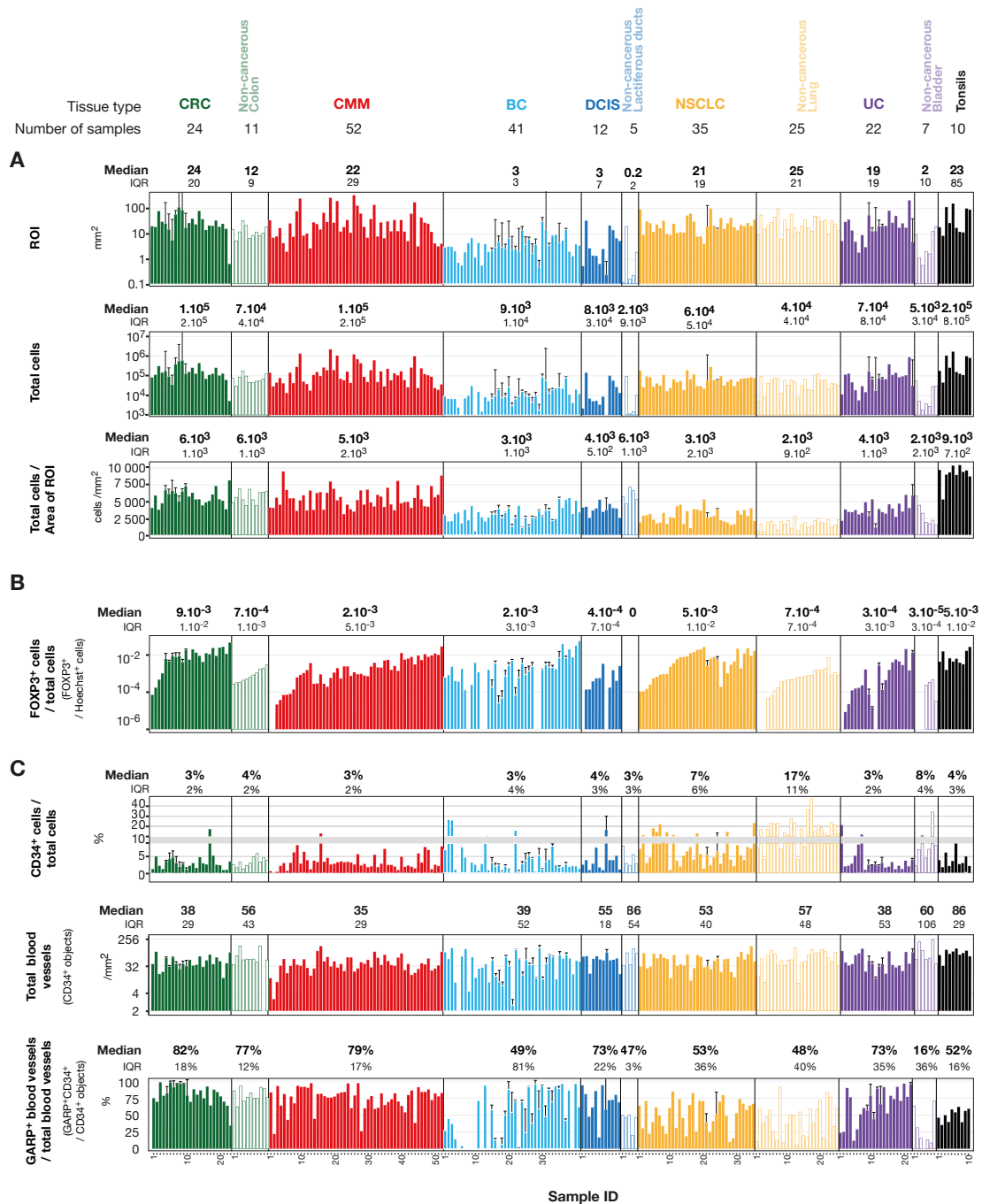
Supplementary Material



Supplemental figure S1: Experimental approach.

Two adjacent cryosections were cut from a series of human tumors, patient-matched non-cancerous tissues and tonsils. The first section was stained with hematoxylin and eosin or, in the case of melanoma, was stained by immunohistochemistry with melanoma-specific antibodies. The second section underwent sequential multiplexed immunofluorescence (mIF) staining against CD3e, FOXP3, GARP, CD34 and pSMAD2, followed by nuclear counterstaining with Hoechst33342. High resolution digital microscopy images of both sections were acquired by slide scanning. Regions of interest (ROI) comprising the visually identified tumor areas were defined on the first section and reported on the second. A. Illustrative image of a melanoma section after mIF staining, showing T cells (CD3⁺), blood endothelial cells (CD34⁺), GARP-expressing blood endothelial cells (GARP⁺CD34⁺), FOXP3⁺ cells, activated Tregs (GARP⁺FOXP3⁺) and cells undergoing TGF- β 1 signaling (pSMAD2⁺). Individual cells defined by their nucleus were identified and recorded with their phenotype by the HALO software. The software also allowed to group blood endothelial cells from individual blood vessels and identify them as objects. The spatial coordinates of cells and objects was recorded. B. Illustrative image of ROI selection. Top panels: non-melanoma sample. Bottom panels: melanoma sample. Left panels: ROI selection (black dotted line). Right panels: reported ROI on mIF images (yellow line).

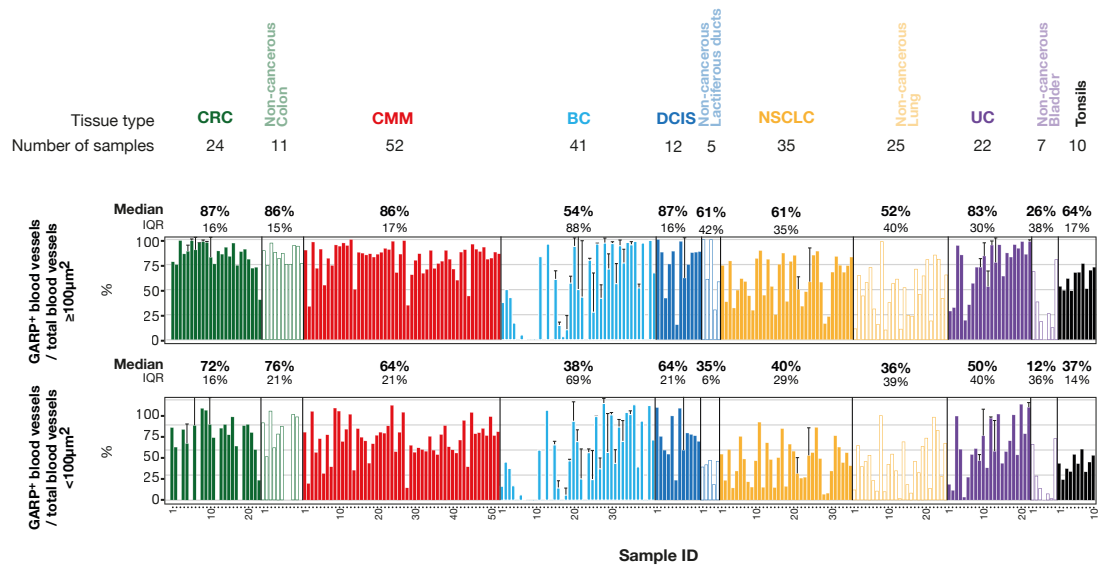
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Supplemental figure S2: Additional parameters measured by computerized analyses of mIF images

Each bar represents an individual sample (sample IDs indicated on the bottom), except when multiple samples from the same tumor were analyzed, in which case the bar represents the mean value of replicates, with the standard deviation displayed as a whisker. Samples are grouped and colored by tissue type and ordered by increasing frequency of activated Tregs (GARP+FOXP3+ cells). The median and interquartile range (IQR) per group is displayed on top of each panel. A. Surface of ROI, total number of cells counted as Hoechst+ nuclei and cell density. B. Proportion of FOXP3+ cells. C. Proportion of BECs (CD34+ cells) and density of blood vessels (CD34+ objects) in the ROI. Proportion of GARP+ blood vessels (GARP+CD34+ objects) among blood vessels.

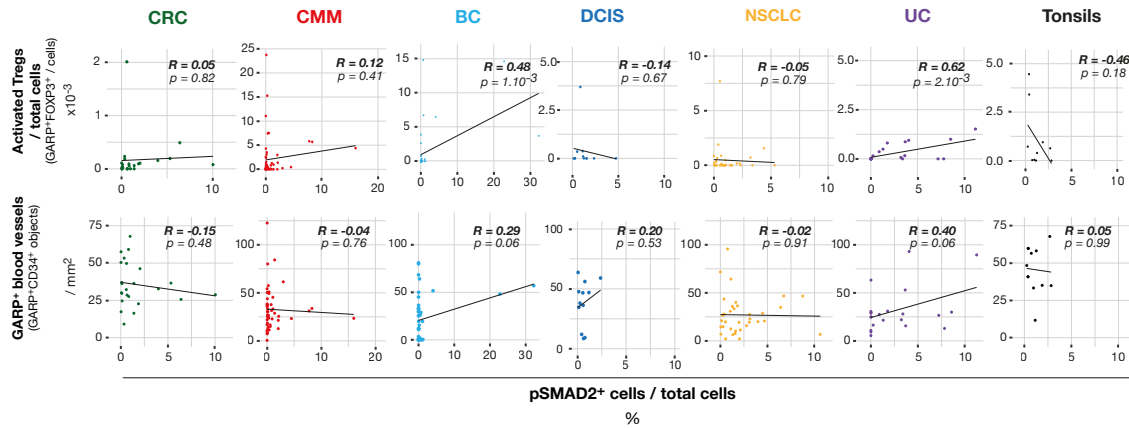
RESULTS



Supplemental figure S3: Large blood vessels appeared more frequently GARP+ than smaller vessels

Percentage of GARP+ blood vessels among vessels larger (top) or smaller (bottom) than 100µm².

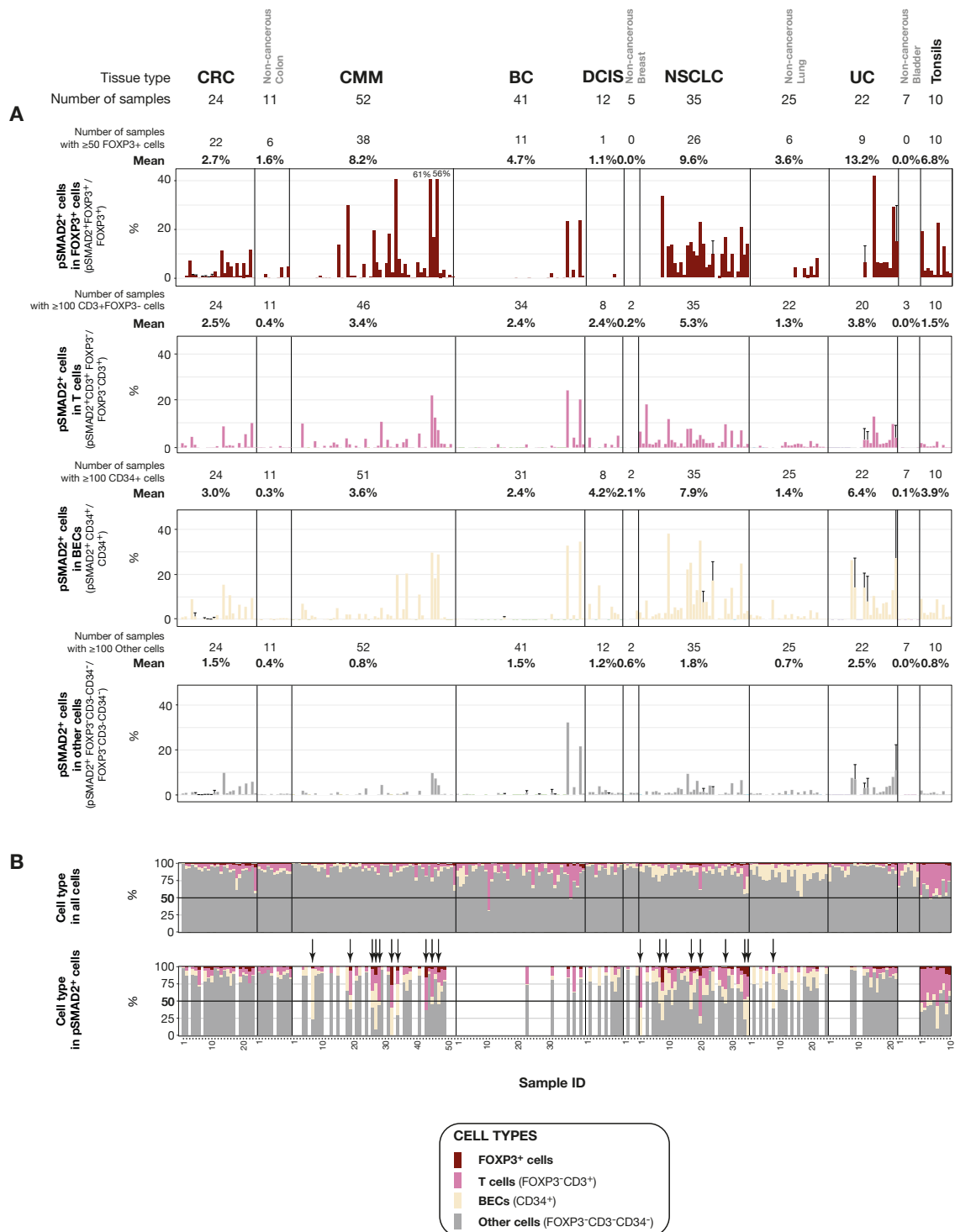
RESULTS



Supplementary figure S4: Absence of correlation between the proportion of TGF- β 1 stimulated cells and either the proportion of activated Tregs or the density of GARP+ blood vessels.

Correlation plots for each tumor type showing the proportion of pSMAD2+ cells and the proportion of activated Tregs (top panels), and the proportion of pSMAD2+ cells and the GARP+ blood vessel density (lower panels). Each dot corresponds to one sample. Solid black lines represent the linear regression obtained by the least-square method. Pearson's correlation coefficients are shown in the upper right corners.

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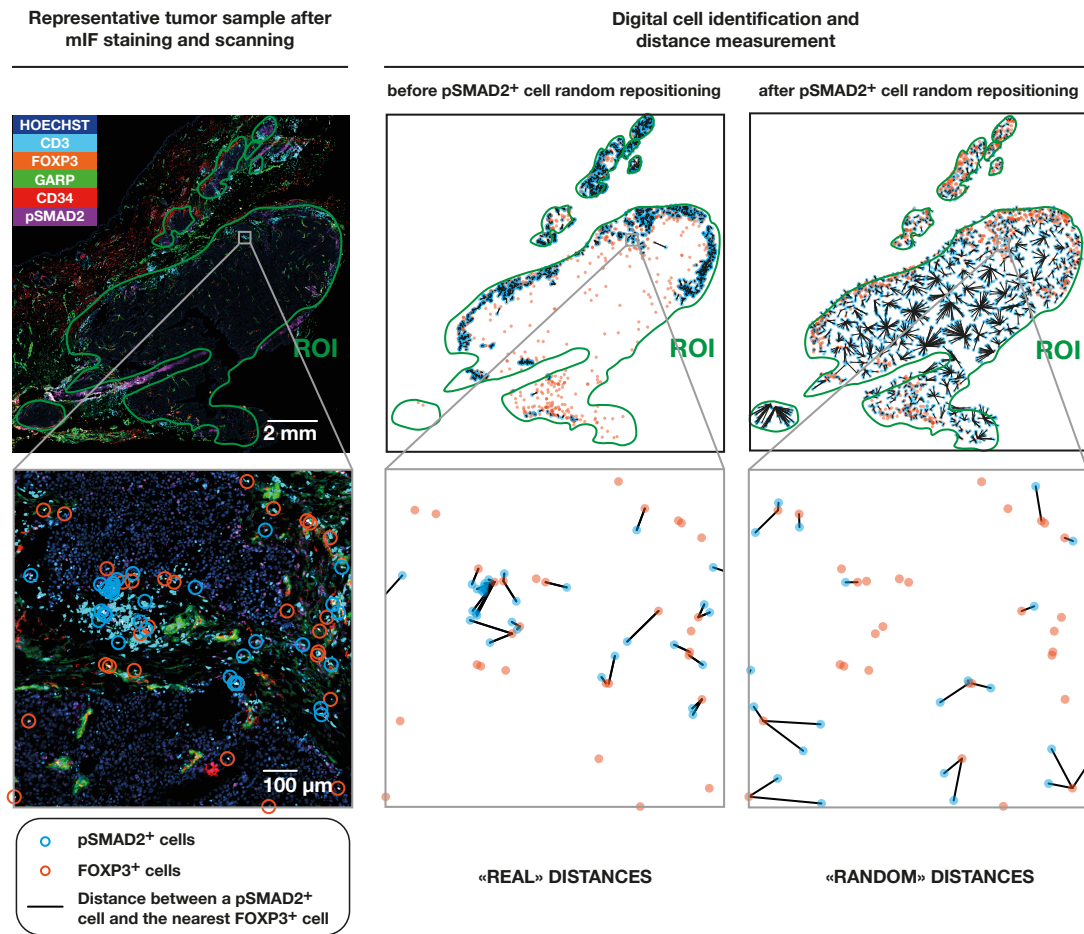
Supplementary figure S5: Distribution of cells undergoing TGF- β 1 signaling by cell type.

Each bar represents an individual sample (sample IDs indicated on the bottom), except when multiple samples from the same tumor were analyzed, in which case the bar represents the mean value. Samples are ordered by increasing frequency of activated Tregs (GARP⁺FOXP3⁺ cells). A. Percentages of pSMAD2⁺ cells among FOXP3⁺ cells, T cells (CD3⁺FOXP3⁺), BECs (CD34⁺) and other cells (from top to bottom). For each respective panel, samples with < 50 FOXP3⁺ cells, or < 100 T cells, or < 100 BECs or < 100 other cells were not analyzed. The mean proportions and the number of analyzed samples are displayed on top of each panel. B. Bar plot of the proportion of FOXP3⁺ cells, T cells (CD3⁺FOXP3⁺), BECs (CD34⁺) and other cells (FOXP3⁺CD3⁺CD34⁺) among all cells (top panel) and

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among pSMAD2⁺ cells (lower panel). Samples with <100 pSMAD2⁺ cells were not analyzed. In the lower panel, samples where the cumulative proportion of FOXP3⁺ cells, T cells and BECs in pSMAD2⁺ cells exceeded 50% were indicated with black arrows.

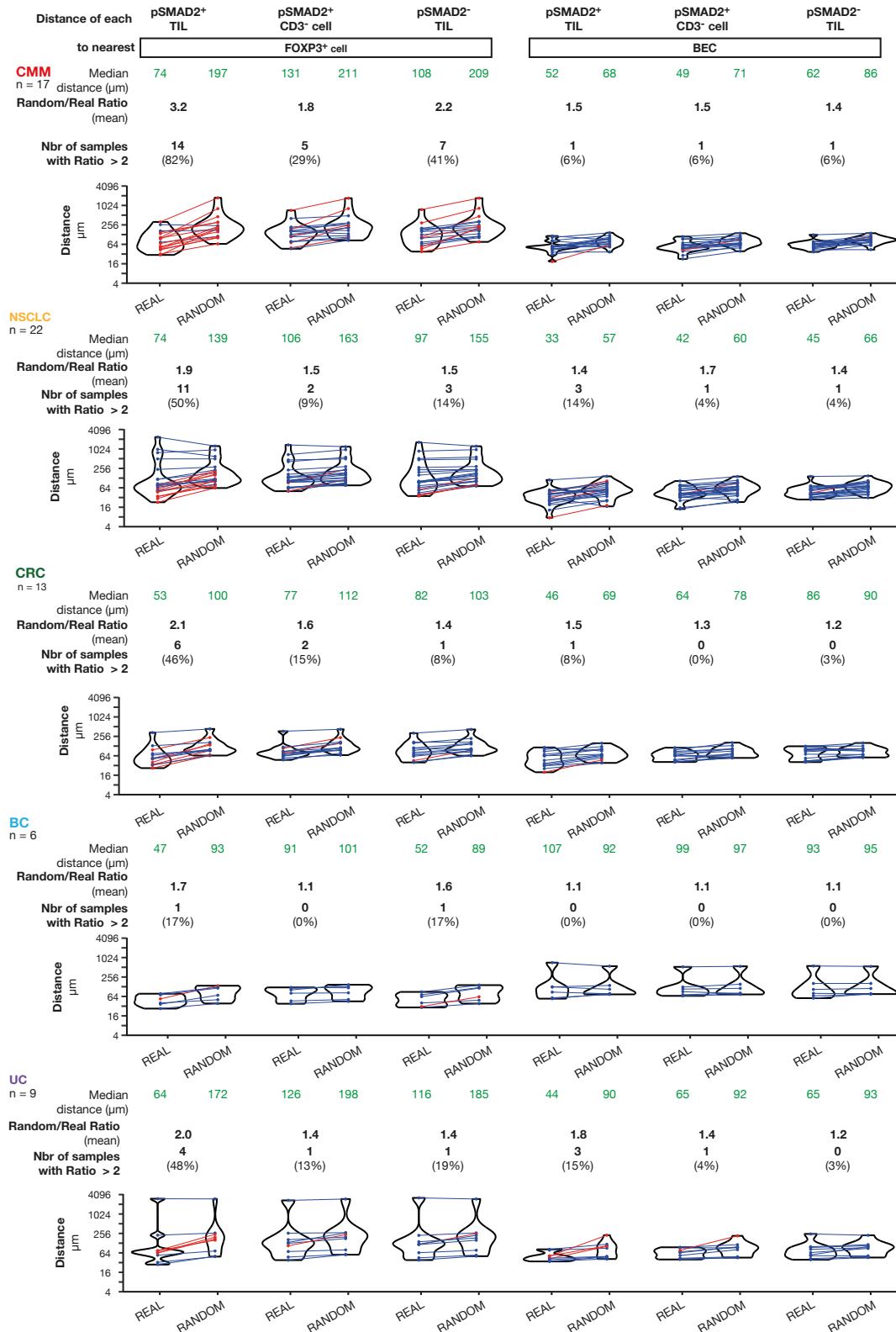
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Supplementary figure S6 : Assessment of proximity between TGF-β1 stimulated cells and either FOXP3+ cell or BEC

Illustrative example of a CMM (Sample ID #42) analyzed by mIF and image analysis (left panels). The location and phenotype of each cell were recorded. The distance between each pSMAD2⁺ T cell and the nearest FOXP3⁺ cell was calculated (central panels). The pSMAD2⁺ T cells were digitally removed and randomly repositioned within the same ROI. The distances were recalculated as above (right panels). This process was repeated a total of five times. The same approach was applied with either pSMAD2⁺CD3⁻ cells or CD3⁺pSMAD2⁻ cells instead of CD3⁺pSMAD2⁺ cells, and also with BECs instead of FOXP3⁺ cells.

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Supplemental figure S7: Same as figure 3, detailed by type of tumor.

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Cycle	Peroxidase Block	Protein Block	Primary Antibody	Secondary Antibody	HRP Substrate
1	Peroxidase-Blocking Solution ¹	Home-made blocking solution ³	Anti-GARP antibody ⁴ (MHG-6, 5µg/mL), 90 min	EnVision+ System-HRP Anti-mouse secondary antibody ¹ , 1 h	Tyramide AF-488 Congugate ⁶ in Borate Buffer, 10 min
2	Bloxall ²	Normal Goat Serum 2.5% ²	Anti-phospho SMAD2 antibody ⁵ (138D4, 4.5 µg/mL), 90 min	EnVision+ System-HRP Anti-rabbit secondary antibody ¹ , 1h	Tyramide AF-555 Congugate ⁶ in Borate Buffer, 10 min
3	Bloxall ²	Normal Goat Serum 2.5% ²	Anti-FOXP3 antibody ⁶ (450, 5µg/mL), 15 min	Peroxidase AffiniPure Goat Anti-Mouse subclass 3 specific secondary antibody ⁸ , 1 h	Tyramide AF-647 Congugate ⁶ in Borate Buffer, 20 min
4	Bloxall ²	Normal Goat Serum 2.5% ²	Anti-CD3ε antibody ⁷ (CD3-12, 5µg/mL), 90 min	ImmPRESS HRP Goat Anti-Rat ² , 1 h	Tyramide ATTO-425 Congugate (in-house), 10min
5	Bloxall ²	Normal Goat Serum 2.5% ²	Anti-CD34 ⁶ antibody (QBEnd-10, 0.25µg/mL), 90 min	Peroxidase AffiniPure Goat Anti-Mouse subclass 1 specific secondary antibody ⁸ , 1 h	Tyramide AF-594 Congugate ⁶ in Borate Buffer, 20 min

¹ Agilent, ² Vector Laboratories, ³ TBS-T 2% milk, 5% bovine serum albumin and 1% human Ig (IgIV, CSL Behring), ⁴ ref [16], ⁵ Cell Signaling Technology, ⁶ ThermoFisher, ⁷ LSBio, ⁸ Jackson ImmunoResearch, ⁹ Borate Buffer containing 0.1M boric acid, 3M NaCl, 0.1% Tween 20 , pH7.8, supplemented with 0.003% H₂O₂.

Supplemental table 1: The reagents, amounts and incubation times used in multiplexed immunofluorescent staining.

7µm-thick cryosections were dried for 15min, were fixed in 4% formaldehyde for 5min and underwent 5 consecutive cycles of fluorescent immunostaining. In each cycle, the sections were sequentially incubated in a peroxidase blocking reagent, a protein blocking reagent, a primary antibody directed against the target antigen, a secondary antibody directed against the primary antibody and coupled to horseradish peroxidase (HRP) and a fluorescent HRP substrate. The sections were rinsed with tris-buffered saline supplemented with 0.005% Tween20 (TBS-T) between each incubation except after the protein block. At the end, the slides were counterstained with Hoechst33342 (ThermoFisher) and sealed. All steps were performed at room temperature.

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Discussion & Perspectives

1 Why T lymphocytes seem to be more preferentially stimulated by TGF- β 1 produced by Tregs in tumors than other cells?

In many tumors in our series of samples, we measured shorter distances between FOXP3⁺ cells and TGF- β 1-stimulated TILs (pSMAD2⁺CD3⁺ cells) than between FOXP3⁺ cells and other cell types (pSMAD2⁺CD3⁻ cells). This suggests that some Tregs may produce TGF- β 1 that preferentially targets TILs, supporting the contribution of Tregs in tumor immunosuppression by a TGF- β 1-dependent mechanism. This is consistent with experimental observations. In a murine model of tridimensional tumor cell culture, CD8⁺ TILs were found to be targeted by TGF- β 1 produced by nearby Tregs [145]. The mechanism underlying this preferential stimulation is not known. Below we propose a few possible explanations.

TILs could be preferentially located close to Tregs in the tumor environment. Upon Treg activation and TGF- β 1 release, the nearby TILs would be preferential targets due to pre-existing proximity between non-activated Tregs and TILs. The frequent colocalization of FOXP3⁺CD3⁺ and FOXP3⁻CD3⁺ cells observed in multiplexed histological stainings supports this hypothesis. In addition, we measured shorter distances of pSMAD2⁻ T cells, which represent most T cells (>95%), and their nearest FOXP3⁺ cell than after random repositioning of the pSMAD2⁻ T cells, although this proximity was not as pronounced as that seen between pSMAD2⁺ TILs and Tregs. This TIL and Treg proximity, irrespective of a Treg activation and TGF- β 1 release, may result from a common attraction mechanism for these cells from the blood stream into the tumor bed, for example mediated by the same chemokines [146]. The CCR6, CCR10 and CXCR3 chemokine receptors are expressed by both TILs and Tregs in various human tumors. Chemokines recognized by these receptors include CCL20, CCL28, CXCL9, CXCL10 and CXCL11. They are produced by various cells of the tumor micro-environment and their expression is upregulated in several cancer types [146]. TIL and Treg proximity might thus result from similar motility features of these two types of T cells in the tissues.

It is also important to remember that the release of active TGF- β 1 from GARP:TGF- β 1 complexes requires at least a third molecular partner in addition to GARP and latent TGF- β 1 itself. The RGD-binding integrin- α v β ₈ is currently the only third molecule identified that controls release of active TGF- β 1 by Tregs. However, other RGD-binding integrins or other molecules might be implicated as well. Such other partners, if expressed by TILs and acting in *trans* on nearby activated Tregs, could direct the released TGF- β 1 preferentially towards their own TGFBR than those of other cells. It must be noted here that no expression of the *ITGB6* or *ITGB8* genes encoding the β chains of integrins known to activate TGF- β 1, was detected in conventional T helper cells isolated from the blood of non-cancerous donors [98].

It will be important to investigate this preferential TIL stimulation by Treg-derived TGF- β 1 in human tumors, as well as its molecular mechanism, as it supports the focused effect of anti-GARP:TGF- β 1 antibody therapy on Treg-mediated immunosuppression.

2 Why don't we observe a correlation between the proportion of pSMAD2⁺ cells and that of GARP-expressing Tregs?

FOXP3⁺GARP⁺ cells define activated Tregs, i.e. Tregs that have encountered their cognate antigen and, among other, release active TGF- β 1 in their immediate vicinity. In turn, this TGF- β 1 binds the TGF- β receptor of neighboring cells which activate their TGF- β signaling pathway resulting in SMAD2 phosphorylation. Considering this, one would expect to observe a correlation between FOXP3⁺GARP⁺ and pSMAD2⁺ cells if and only if a number of conditions are met. Let us consider these conditions one by one.

First, there should be contrasted proportions of FOXP3⁺GARP⁺ cells in the sample set in order to be able to determine whether the correlation exists. This is obviously the case in our data set.

Secondly, TGF- β 1 should be the only cytokine of the TGF- β superfamily able to trigger SMAD2 phosphorylation that is active in our tumors. TGF- β 2 and TGF- β 3, given their known biological function and the fact that they are rarely expressed in tumors other than glioblastoma, are unlikely to play an active role in the tumor environment. Among non-TGF- β members of the superfamily, myostatin, nodal and activins are the only ones that signal through SMAD2 and SMAD3 phosphorylation. These members are known to be mostly expressed and active only in neural and muscle tissue, as well as tumors that derive therefrom. For these reasons, we hypothesize that the SMAD2 signals observed in a small subset of the cells present in our tumors are probably induced by TGF- β 1, which is to our knowledge expressed at higher level than TGF- β 2, TGF- β 3 and non-TGF- β superfamily members in most of these cancer types. We will verify in publicly available RNAseq data whether non-TGF- β 1 members signaling via SMAD2 and SMAD3 are expressed in the five cancer types that we analyzed herein. At this stage, another source can not be formally excluded, though, which would be a possible explanation for this lack of correlation.

Thirdly, for a strict correlation to be observed, activated Tregs should be the only source of active TGF- β 1 in the tumors. As we have seen, the active TGF- β 1 cytokine can be produced by various cell types and several molecular mechanisms control its release. Among other possible sources are ECM-associated LTBP proteins, LRRC33-expressing myeloid cells, and other GARP-expressing cells. The latter comprise principally blood and lymphatic endothelial cells as well as fibroblasts. In addition, GARP immunoreactivity associated with acellular or paucicellular fibrotic areas was repeatedly observed in our tumor samples. The precise origin of this is unknown. The presence of GARP in the ECM could result from the deposition of soluble GARP, complexed or not with latent TGF- β 1, that was shed from GARP-expressing cells such as fibroblasts [70]. All these cells and structures are present in the tumor environment, and it is very possible that they contribute to at least part of the pSMAD2 signals. In addition, it was shown that TGF- β 1 can be released from soluble GARP *in vitro*, leading to FOXP3 expression in Tregs and to reduced proliferation and cytokine production by effector T cells [147].

Fourthly, part of the TGF- β 1 signaling might be undetected due to the reorientation of the TGF- β 1 signaling towards the ALK1 pSMAD1/5 pathway instead of the ALK5 pSMAD2/3, a phenomenon that seems to occur frequently in proliferating BECs. This switch in signaling is caused by the expression of co-receptors of the TGFBR, such as endoglin [148] or neuropilin-1 (NRP1) [149] among BECs. However, this hypothesis is less likely, as *in vitro* TGF- β 1 stimulated BECs still exhibit basal levels of pSMAD2 when ALK1 is activated.

Another explanation may also stem from the fact that the various cell types under the influence of TGF- β 1 signals in tumors do not all receive the signal from the same TGF- β 1 cellular source. This is highly probable: we observed TGF- β 1 signals (pSMAD2⁺) in all cell types that we could identify in the tumors (i.e. T cells, BECs and other cells), and the frequencies of pSMAD2⁺ cells were higher than that of GARP-expressing Tregs. Cells other than immune cells, which include cancerous cells and non-immune cells in the tumor microenvironment could very well receive TGF- β 1 signals from non-Tregs sources. In that case, a global correlation between the proportion of pSMAD2⁺ cells and that of GARP-expressing Tregs would be highly improbable.

Finally, it is important to address some technical limitations of our approach. We study tissue sections, which means two-dimensional representations of a three-dimensional tissue. We usually take one sample from one single resected tissue or one or a few biopsies from a single tumor left in place. We do this at a given time during the evolution of the malignancy, and thus miss the time dimension. We consider that this sample represents the tumor. We define activated Tregs by their FOXP3 and GARP phenotype, knowing that at least the latter marker is expressed transiently after activation. This transient presence also holds true for the TGF- β 1 stimulus itself, as well as the SMAD2 phosphorylation in the stimulated cell. Many cells, particularly immune cells are mobile in tissues. Even though these features are not supposed to directly influence the correlation between the proportion of cells producing a cytokine and those responding to it, they affect the precision of the measurements, and thus indirectly the correlation.

Among new techniques available, spatial transcriptomics may help us to harness the great complexity of TGF- β 1-mediated communications in tumors, including the cell-dependent diversity of molecular pathways and genes that respond to TGF- β 1 signaling, while preserving the spatial relationships between cells.

3 Do blood endothelial cells activate TGF- β 1 in human tumors?

This question was previously addressed in experimental models in our laboratory [150]. We studied GARP-dependent activation of the TGF- β 1 by murine and human BEC cell lines exposed to various stimuli. The production of TGF- β 1 was measured in co-culture with a reporter cell line stably transfected with a construct containing a luciferase gene under the control of a TGF- β responsive promoter. No evidence of TGF- β 1 activation by any of the GARP⁺ BECs (HUVEC and MS1) or GARP⁻ BECs (EA.hy926 and C166) with any of the tested stimuli (AERG, VEGF, IL-6, IL-1 β and TNF α) was observed. This negative result does not allow to exclude BEC-mediated TGF- β 1

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activation in human tissues, as we are far from having tested all possible BEC stimuli in a few experimental conditions.

We have shown here that a majority (~75%) of BECs express GARP, both in tumors and in non-cancerous matched tissues. Our data do not support the idea that CD34⁺ BECs are a major source of TGF- β 1 acting on TILs, and perhaps even on other cells, in tumors. Indeed, we did not observe a clear enrichment of pSMAD2⁺ cells in CD34⁺ cells compared with other cells, suggesting an absence of autocrine TGF- β 1 signaling of BECs. This absence of clear enrichment pSMAD2⁺ cells was the case as well in FOXP3⁺ Tregs, however. In contrast to Tregs though, we did not find clear evidence of a paracrine TGF- β 1 signaling mediated by BECs in TILs when we examined spatial proximity of BECs with pSMAD2⁺ TILs (or other non-T pSMAD2⁺ cells, for that matter): both pSMAD2⁺ TILs and pSMAD2⁺ non-TILs were nearly as close from CD34⁺ cells as in a random situation.

The absence of pSMAD2 signal associated with BECs may be a consequence of blood vessel quiescence. In tumors, one of the most frequent mechanisms of neoangiogenesis is angiogenic sprouting [139]. During this process, a proangiogenic stimulus is sent from the tumor and is recognized by a highly restricted portion of the blood vessels. This recognition causes the BECs to switch from a quiescent to an activated state. The activated portion of the blood vessel forms a sprout, which evolves from a single tip BEC to a mature blood vessel surrounded by mural cells. The large remaining portion of the original blood vessel is not concerned by the proangiogenic sprouting stimuli. In these portions, BECs stay in quiescence, which is notably characterized by impaired TGF- β 1 signaling, caused by an active ERK1/2 pathway and by the production of bone morphogenetic protein 9 [151]. To confirm that TGF- β 1 signaling is present only in proliferating BECs, a novel mIF panel could be set up and tested on tumor sections. Markers of the panel would include CD34 to visualize the blood vessels, pSMAD2 to highlight the cells undergoing TGF- β 1 signaling and endoglin to indicate the proliferating BECs [152]. We could also add a marker of cellular proliferation such as KI67. An enrichment of pSMAD2⁺ cells among endoglin⁺ CD34⁺ BECs compared to endoglin⁻ CD34⁺ BECs would indicate preferential TGF- β 1 signaling in BECs under angiogenic condition. As the implication of GARP on TGF- β 1 activation by BECs remains to be unraveled, GARP could also be included as a marker in the panel in order to decipher whether its expression on proliferating BECs has an impact on the presence of pSMAD2. Finally, as mural cells are essential in angiogenesis and favor TGF- β 1 activation when co-cultured with BECs, PDGFR or α -SMA could be added to investigate whether a close contact between mural cells and endoglin⁺ CD34⁺ BECs is associated with pSMAD2 signal in and around proliferating BECs.

4 Is it possible to automate the quantification of activated Tregs in human tissue sections?

The identification and counting of the GARP⁺FOXP3⁺ cells in tissue sections was a significant challenge in my thesis due to the complex distribution of GARP expression. Besides activated Tregs, GARP immunostaining is observed in endothelial cells, perivascular areas and in the extracellular matrix (ECM). Consequently, a large proportion of GARP⁺FOXP3⁺ cells, as identified by image analysis softwares correspond to FOXP3⁺ cells overlapping GARP⁺ structures, such as vascular or fibrotic areas. In these cases, it is not possible to confidently determine whether GARP staining comes from the FOXP3⁺ cell or from its immediate environment.

We used and compared two different approaches to quantify the activated Treg population as accurately as possible. In the Visual Validation approach, all the GARP⁺FOXP3⁺ cells in the tissue are counted automatically by the software, including the false positives resulting from overlap of FOXP3⁺GARP⁻ cells by GARP⁺ background. A representative proportion of these cells is selected randomly, and each selected cell is assessed visually and either validated as a true GARP⁺FOXP3⁺ cell or rejected as a false GARP positive cell. The total automated count is then multiplied by the proportion of validated cells among the selection. This approach is labor-intensive and prone to variability between operators. Alternatively, in the Area Exclusion approach, large GARP objects are identified and selected, and the smallest rectangles hosting these objects are excluded from the total tissue area. The GARP⁺FOXP3⁺ cells are then counted by the software in the remaining tissue.

We carried out and compared the two approaches in our series of tumor and tonsil samples (**Figure 18**). On average, the absolute number of activated Tregs obtained by Visual Validation was 5 ± 6 times (mean $\pm$ sd) lower than that obtained by Area Exclusion. The proportion of activated Tregs among Hoechst⁺ cells, compared between the two approaches, correlated strongly in CRC, CMM, BC, DCIS, UC and tonsils ($r = 0.8-1$, $p < 1.10^{-3}$). In NSCLC, the correlation was weaker ($r = 0.7$, $p < 1.10^{-3}$). In a number of NSCLC samples (11/35), no activated Tregs were detected with the Visual Validation method, whereas the Area Exclusion method identified some. This discrepancy could originate from the architecture of the pulmonary vasculature, which contains a high density of capillaries. These capillaries, due to their small size similar to single cells, are not identified as large GARP⁺ objects by the image analysis software. In summary, automated quantification of activated Tregs tends to overestimate their numbers compared to the manual approach and is suitable for most, but not all, cancer types.

In total, by applying the Visual Validation approach on the 300 tissue fragments, approximately 17,000 GARP⁺FOXP3⁺ cells identified by the software were also inspected visually, among which 912 were validated as activated Tregs. We could use these images and validation results to train machine learning algorithms, such as those available in the QuPath or HALO softwares, to automatically recognize activated Tregs and distinguish them from false positive cells.

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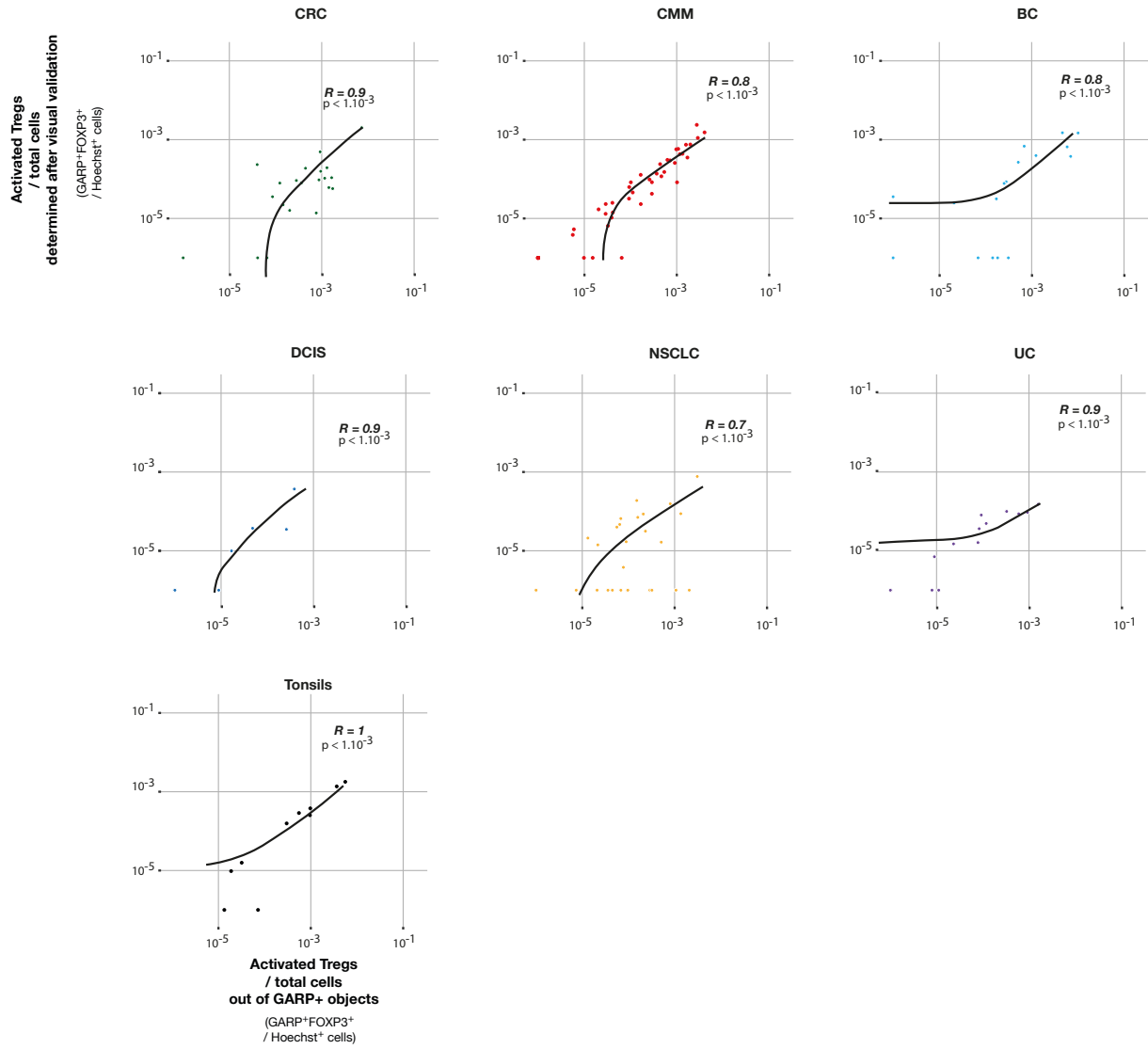


Figure 18: Comparison between manual (Y axis) versus automated (X axis) quantification of activated Tregs. Values equal to zero were set to 1.10^{-6} .

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