



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

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Abstract

Most cells produce latent transforming growth factor-beta 1 (TGF- β 1), but only very few activate the cytokine via cell type-specific mechanisms. TGF- β 1 favors cancer progression by suppressing anti-tumor T cell responses. Which cells produce this immunosuppressive TGF- β 1 in human tumors is unknown. Putative sources include cells expressing the glycoprotein A repetitions predominant (GARP) protein, comprising mostly activated regulatory T cells (Tregs) (GARP⁺FOXP3⁺ cells) and blood endothelial cells (BECs). We performed multiplexed immunohistochemistry and computerized image analyses on 186 tumor samples from 5 cancer types (colorectal, urothelial, lung and breast primary carcinomas and melanoma metastases), compared to patient-matched adjacent non-cancerous tissues. GARP⁺ Tregs were present in 29–75% of the various types of tumor samples. Their proportion was higher in tumors than non-cancerous tissues but unexpectedly it did not correlate with that of tumor-infiltrating T lymphocytes (TILs). The density of blood vessels was similar across samples, with more than half expressing GARP. The proportion of cells undergoing TGF- β 1 signaling, which express the phosphorylated form of mothers against decapentaplegic homolog 2 (pSMAD2), was approximately twice as high in tumors compared to non-cancerous tissues. In most tumor types, pSMAD2⁺ TILs were twice closer to the nearest FOXP3⁺ cell than after random repositioning, at a distance (~70 μ 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 suppressing nearby TILs. This local immunosuppression could be blocked with anti-GARP:TGF- β 1 antibodies, particularly to treat patients with tumors heavily infiltrated by GARP-expressing Tregs.

Keywords TGF- β 1 · GARP · Regulatory T cell · Tumor infiltrating lymphocyte · Quantitative multiplexed immunohistochemistry · Immunosuppression

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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 epithelial-to-mesenchymal transition, 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 TGF- β receptor (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 LTBP: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 the 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 the 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 arginine–glycine–aspartic acid (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.

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.

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

7 μm -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 (Supplementary Table S1). The following primary antibodies were used: anti-GARP [16], anti-pSMAD2 (Cell Signaling Technology Cat#3108, RRID:AB_490941), anti-FoxP3 (Thermo Fischer Scientific, Cat#MA5-14662, RRID:AB_10989334), anti-CD34 (Abcam, Cat#ab8536, RRID:AB_306607) and anti-CD3e (LSBIO, Cat#LS-B8765). More details about reagents used for staining are available in Supplementary Table S1. 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 [24]. 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. [25]. 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/200 μl) 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/0.8 $\times$ 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).

HE and IHC slides were scanned with a Panoramic P250 Flash III slide scanner (3DHitech), equipped with a 20x/0.8 $\times$ 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) (Supplementary 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 Supplementary Fig. 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

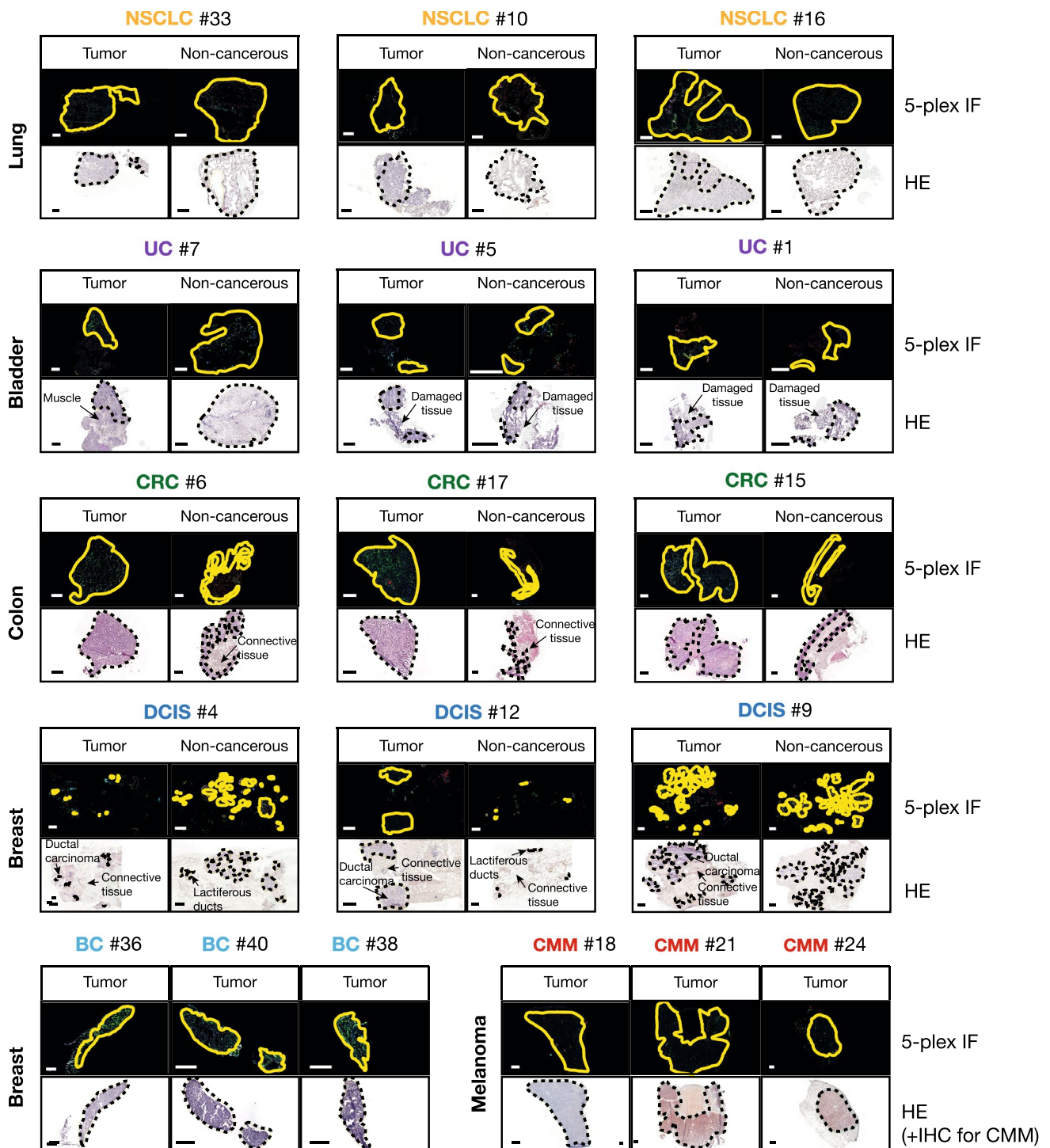


Fig. 1 Representative images of ROI delineated on tumor and patient-matched non-cancerous tissues. Two adjacent cryosections were cut from a series of human tumor samples, patient-matched non-cancerous tissues and tonsils. The first section was stained with hematoxylin and eosin or, in the case of melanoma, 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

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]. In the 186 tumor samples, the surface of the ROI was comprised between 0.1 and 340 mm² and contained 7.10^2 to 2.10^6 nucleated cells. The cellular density ranged from 5.10^2 to 1.10^4 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 (Supplementary 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 were analyzed 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

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), 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 (Supplementary 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 Supplementary Fig. S2A.

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

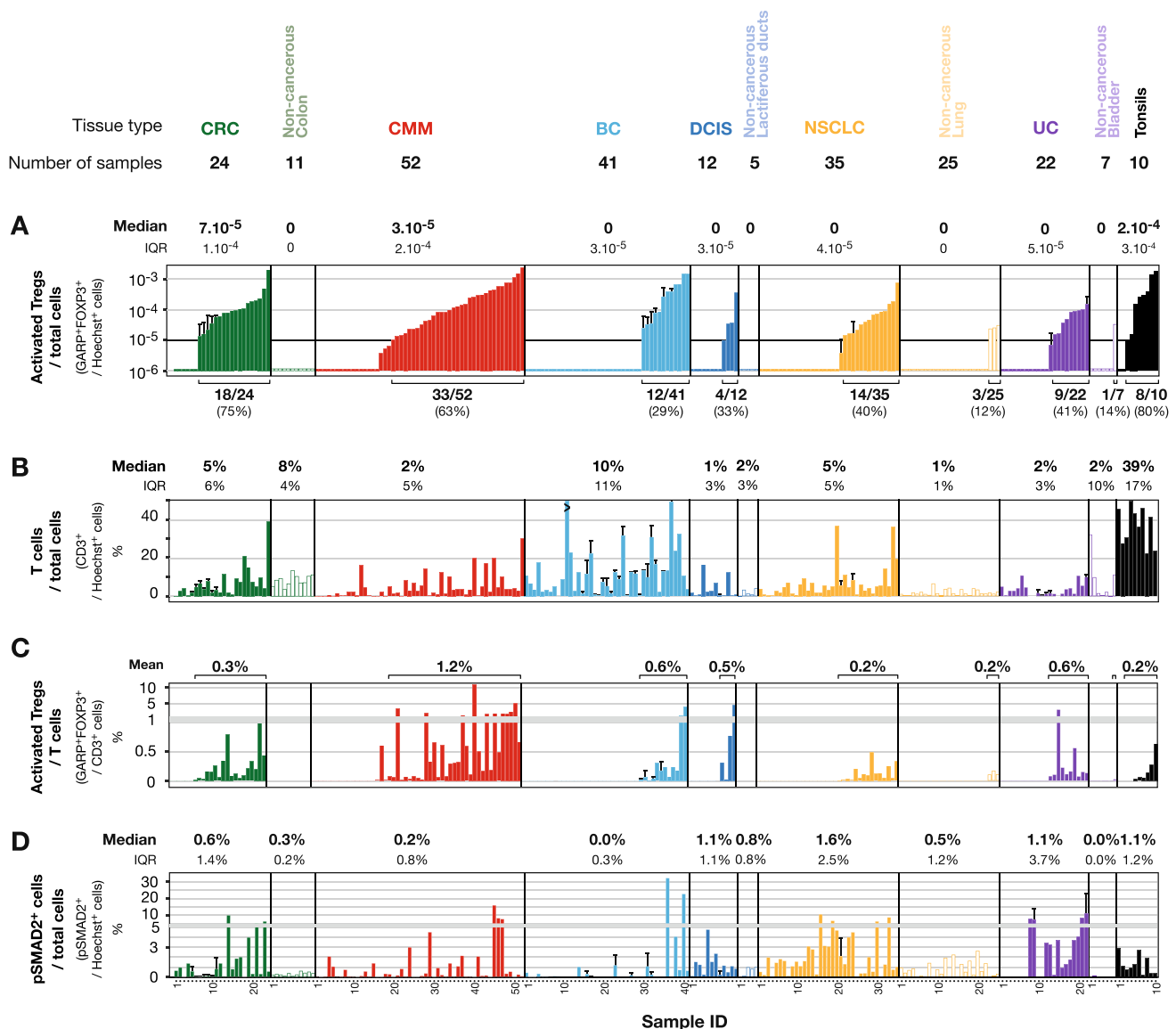


Fig. 2 Quantitative analysis of activated Tregs, T cells and cells undergoing TGF- β 1 signaling in human frozen tissue sections. CRC: colorectal carcinomas; CMM: cutaneous melanoma metastases; BC: breast carcinomas; DCIS: ductal carcinoma in situ; NSCLC: non-small cell lung carcinomas; UC: urothelial 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 or mean and interquartile range (IQR) per group is displayed on top of each panel. A. Proportion of activated Tregs in the ROI. 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 the ROI. D. Proportion of pSMAD2+ cells in the ROI

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 Supplementary Fig. S2B). The percentage of T cells (CD3+) among total cells was much lower in tumors (median 1–10%, 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 1–8%) (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 2B).

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 (Supplementary Fig. S2C). The median proportion of GARP⁺ blood vessels (GARP⁺CD34⁺ objects) among total blood vessels was also similar across all tissue types (47–82%), except for non-cancerous bladder tissue ($16 \pm 36\%$) (Supplementary Fig. S2C). Large blood vessels ($>100 \mu\text{m}^2$) appeared more frequently positive for GARP than smaller vessels in all types of tissues ($p < 1.10^{-3}$) (Supplementary Fig. S3).

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 GARP⁺FOXP3⁺ activated Tregs and FOXP3⁺ cells in UC ($R=0.62$ and 0.45 , respectively) and BC ($R=0.48$ and 0.64 , respectively), but not in CRC, DCIS and CMM ($R \leq 0.40$; $p \geq 0.05$) (Supplementary Fig. S4). The correlations observed in BC were mainly driven by two samples which contained unusually high proportions of activated Tregs (0.05–0.15%), FOXP3⁺ cells (3–4%) and pSMAD2⁺ cells (20–30%). In NSCLC, the proportion of pSMAD2⁺ cells correlated with that of FOXP3⁺ cells ($R=0.68$) but not activated Tregs ($R=-0.047$). This discrepancy can be explained by the difficulty to visually validate GARP expression by FOXP3⁺ cells in lung samples, due to high vascularization and thus particularly abundant perivascular areas with intense GARP immunostaining. No correlation was seen in any tumor type between the proportion of pSMAD2⁺ cells and the density

of blood vessels or GARP⁺ blood vessels (Supplementary Fig. S4).

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 (Supplementary 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 Supplementary 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 0.03–0.9% of the cells, was 67 μm , whereas their median distance to BECs, which account for 3–7% of the cells, was 46 μm (“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 (Supplementary 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

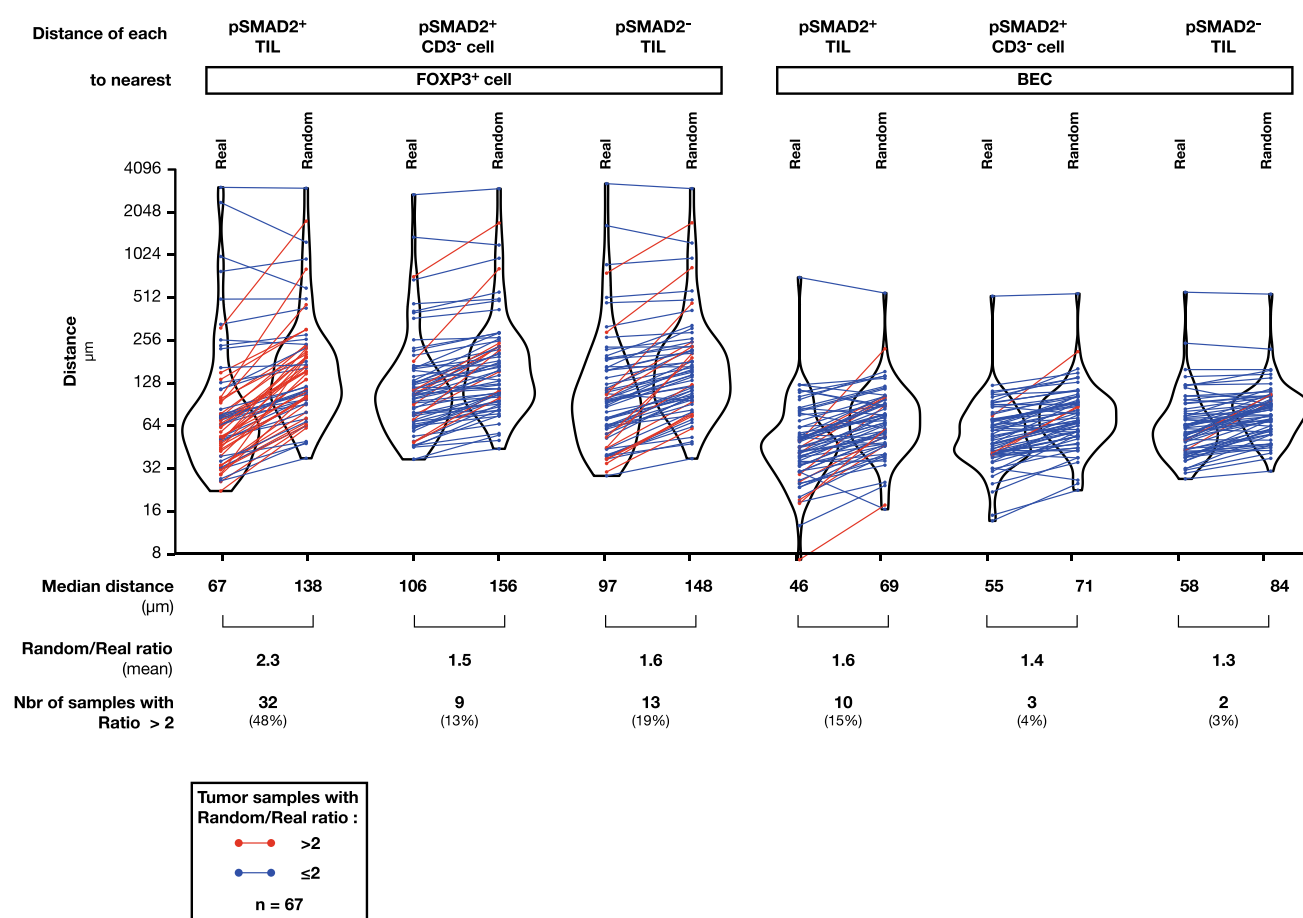


Fig. 3 In tumors, TILs undergoing TGF- β 1 signaling (pSMAD2⁺) are closer to the nearest FOXP3⁺ cell than expected by chance. Pairs of violin plots represent distances measured between each cell of the indicated cell type (pSMAD2⁺ or - TILs or non-T cells) and its nearest FOXP3⁺ or BEC neighbor. Each 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 ($n = 67$)

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 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 (Supplementary 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 μ m) 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.

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 [15]. Unfortunately, most if not all available anti-GARP

antibodies do not work on formalin-fixed paraffin-embedded (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 [15]. 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 [26–28]. Interestingly, we found activated Tregs in 29–75% 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 [29–31]. 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 [29, 32–36]. 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 [33]. 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 [34].

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 [37]. Large amounts of latent TGF- β 1 bound to LTBP are also deposited in the extracellular 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.

Statement of translational relevance

Whereas TGF- β 1 limits the efficacy of cancer immunotherapy by suppressing T cells in the tumor microenvironment, it also exerts cytostatic effects on cancer cells themselves. Therefore, pharmacological inhibition of TGF- β 1 irrespective of its cellular sources and targets bears the risk of adverse events. Several types of cells can produce active TGF- β 1, via cell type-specific mechanisms. Among these are activated Tregs, which present latent TGF- β 1 bound to GARP for activation by integrins on their surface. Anti-GARP:TGF- β 1 antibodies blocking TGF- β 1 activation by GARP-expressing cells, but not by other cells, are currently tested in the clinics. We show here that GARP⁺ Tregs are present in a fraction (29–75%) of tumors samples from 5 cancer types and appear to represent a source of TGF- β 1 suppressing nearby T cells. The presence of GARP⁺ Tregs in tumor samples could serve as a biomarker to identify patients most likely to respond to immunotherapy with anti-GARP:TGF- β 1 antibodies.

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Author contribution P.V.M., N.V.B. and S.L. conceived and designed the overall project. E.M., F.A.N., M.V.D.E., A.D., J.C. and N.V.B. provided biobanked patient-derived samples. P.V.M., D.M. and N.V. prepared and stained the samples for mIF. P.V.M. and N.V.B. developed the computerized image analyses approaches. P.V.M., G.D.S., A.N., N.V.B. and S.L. analyzed the data. P.V.M., N.V.B. and S.L. wrote the manuscript with input from all the authors.

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Declarations

Conflict of interest Patents pertaining to blocking antibodies against human GARP:TGF- β 1 have been filed under the Patent Cooperation Treaty (International application Number PCT/IB2019/053753), with Sophie Lucas as inventor and UCLouvain as applicant. Anti-human GARP:TGF- β 1 antibodies have been licensed to AbbVie.

Data availability Any information required to reanalyze the data reported in this paper is available from the lead contact upon request. This includes the in-house computer scripts written in the R programming language (v.4.3.1) that were developed to carry out the quantitative image analyses.

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