

Multiple modes of action for antibodies targeting GARP- expressing cells in tumors

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ABBREVIATIONS

ACC	Adrenocortical carcinoma
ALK	Activin receptor-like kinase
Ang	Angiopoietin
APC	Antigen-presenting cell
ARE	AU-rich element
AREG	Amphiregulin
ATP	Adenosine triphosphate
B7-H1	B7 homolog 1
BATF	Basic leucine zipper transcriptional factor ATF-like
BCR	B cell receptor
BEC	Blood endothelial cell
BMP	Bone morphogenic protein
bp	Base pair
CAF	Cancer-associated fibroblast
CAM	Cytokine-induced adhesion molecule
CAR	Chimeric antigen receptor
ccRCC	Clear-cell renal cell carcinoma
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
cDNA	Complementary DNA
cKO	Conditional knock-out
Co-SMAD	Common SMAD
CSCC	Cutaneous squamous cell carcinoma
cSMAC	Central supramolecular activation cluster
CTB	Subunits B of cholera toxin
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein
DC	Dendritic cell
dLN	Draining lymph node
dnTGF-βRII	Dominant negative TGF- β RII
DSS	Dextran sodium sulfate
EBV	Epstein-Barr virus
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
EndoMT	Endothelial-mesenchymal transition
ETBR	Endothelin B receptor
F-TBRS	Fibroblastic TGF- β 1 response signature
Fab	Fragment, antigen-binding
FACS	Fluorescence-associated cell sorting
FasL	Fas ligand
Fc	Fragment crystallizable

FDA	Food and drug administration
FFPE	Formalin-fixed paraffin-embedded
GSK	Glycogen synthase kinase
GvHD	Graft-versus-host disease
GZMB	Granzyme B
HCC	Hepatocellular carcinoma
HHT	Hereditary hemorrhagic telangiectasia
HIF	Hypoxia-inducible factor
HL	Hodgkin lymphoma
HNSCC	Head and neck squamous cell carcinoma
hTERT	Human telomerase reverse transcriptase
HUVEC	Human umbilical cord endothelial cells
I-SMAD	Inhibitory SMAD
i.p.	Intraperitoneal
ICB	Immune checkpoint blockade
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IPRES	Innate anti-PD-1 resistance
IRSE	Interferon-sensitive response element
ITIM	Immunoreceptor tyrosine inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
KD	Knockdown
KO	Knock-out
LAP	Latency associated peptide
LCC	Large cell carcinoma
Lck	Lymphocyte-specific protein kinase
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharides
LRR	Leucine-rich repeat
LTBP	Latent TGF- β 1 binding protein
mAbs	Monoclonal antibodies
MDSC	Monocyte-derived suppressive cell
MHC	Major histocompatibility complex
mIF	Multiplex immunofluorescence
miRNA	MicroRNA
MK	Megakaryocyte
MMP	Metalloproteinase
mRNA	Messenger RNA
mTGF-β1	Mature TGF- β 1
NFAT	Nuclear factor of activated T cells
NK	Natural killer
NLR	Neutrophil-to-lymphocyte ratio
NSCLC	Non-small cell lung cancer
NSG	Nod scid gamma

nsSNV	Non-synonymous nucleotide variant
OE	Overexpression
ORR	Objective response rate
OS	Overall survival
OVA	Ovalbumin
PAI-1	Plasminogen inhibitor 1
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
PD-L	PD-1 ligand
PDCD1L1	PDCD1 ligand 1
PFS	Progression-free survival
PSGL-1	P-selectin glycoprotein ligand 1
pSMAD	Phospho-SMAD
pTreg	Peripheral Treg
R-SMAD	Receptor SMAD
RBP	RNA-binding proteins
RCC	Renal cell carcinoma
RNA-seq	RNA sequencing
rTGF-β1	Recombinant TGF- β 1
s.c.	Subcutaneous
SBE	SMAD-binding elements
SCID	Severe combined immunodeficiency
SHP	Src homology region 2 domain-containing phosphatase
SKP2	S-phase kinase-associated protein 2
SMC	Smooth muscle cell
Smurf	SMAD ubiquitination response factor
SNP	Single-nucleotide polymorphism
sTβRII	Soluble TGF- β RII
TAM	Tumor-associated macrophage
TBR5	TGF- β 1 response signature
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TEC	Tumor endothelial cell
TGF-β1	Transforming growth factor β 1
TGF-βRI	Type I TGF- β receptor
TGF-βRII	Type II TGF- β receptor
TIL	Tumor-infiltrating lymphocyte
TMB	Tumor mutational burden
TME	Tumor microenvironment
TRAMP	Transgenic adenocarcinoma of mouse prostate
Treg	Regulatory T cell
TSP-1	Thrombospondin-1
tTreg	Thymic Treg
UC	Urothelial carcinoma
UTR	Untranslated region

VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
WT	Wild-type

ABSTRACT

Cancer immunotherapies aim at inducing or reinvigorating immune responses directed against tumor cells. To date, approaches blocking the PD-1/PD-L1 axis with monoclonal antibodies (mAbs) have led to the most impressive successes in cancer patients and have become a standard of care for several cancer types. However, despite the fact that they induce durable and sometimes complete tumor regressions in a significant proportion of patients, blocking anti-PD-1 or PD-L1 mAbs still lack efficacy in most cases. Resistance or response to PD-1/PD-L1 blockade appears to mainly depend on the tumor type, although clinical efficacy also varies among patients with tumors of similar histological origin. Studies of the PD-1/PD-L1 axis in the tumor microenvironment aim at a better understanding of resistance mechanisms to design improved immunotherapeutic strategies.

TGF- β 1, a pleiotropic cytokine initially produced in a latent form, contributes to resistance to PD-1/PD-L1 blockade. Previous work in our laboratory showed that regulatory T cells, or Tregs, activate the cytokine *via* a mechanism that implies presentation of latent TGF- β 1 on the Treg surface by transmembrane protein GARP on the one hand, and pulling by integrin α V β 8 on the other. TGF- β 1 activation substantially contributes to the immunosuppressive function of Tregs, including dampening of anti-tumor effector T cell responses.

We developed antibodies against GARP:(latent)TGF- β 1 complexes that block TGF- β 1 activation by Tregs and evaluated their efficacy in murine models of cancer. We observed that anti-GARP:TGF- β 1 mAbs induce regression of MC38 and CT26 tumors in mice otherwise resistant to anti-PD-1 mAbs. Interestingly, the anti-tumor effect of combined PD-1/GARP:TGF- β 1 blockade was associated with different changes in the microenvironment of the two tumor types. In MC38 tumors, combined PD-1/GARP:TGF- β 1 blockade augmented T cell infiltration *via* blood vessels, most probably as a result of normalization and densification of the tumor vasculature and enhanced diapedesis. In CT26 tumors, the combination did not modify the density

of the immune cell infiltrate nor of the blood vasculature, but rather increased the activity of tumor-specific CD8 T cells already present in the tumor bed.

Altogether, our results in mouse models indicate that GARP:TGF- β 1 blockade can overcome resistance to PD-1 blockade by: *i*) augmenting the density of T cells in the tumor, and/or *ii*) reinvigorating the effector functions of T cells already present in the tumor. Anti-GARP:TGF- β 1 mAbs could thus be used in combination with PD-1/PD-L1 blockade in cancer patients expected to respond poorly to PD-1/PD-L1 blockade alone, whether this resistance is due to absent or low tumoral infiltration of T cells (*i.e.* so-called “cold” tumors), or whether it is due to the presence of abundant intratumoral T cells that are exhausted or have become inactive (*i.e.* so-called “inflamed” tumors).

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1 Biology of the PD-1/PD-L1 axis in anti-tumor immunity

1.1 Immune checkpoints

Immune checkpoints are also called “immunostats”, *i.e.* “rheostats” of the immune system, because they are key regulators of immunity [1]. These receptors are present at the surface of T lymphocytes that have been activated after engagement of their T cell receptor (TCR) by an MHC (Major Histocompatibility Complex) molecule loaded with an antigenic peptide. After binding to their ligand, generally expressed on antigen-presenting cells, immune checkpoints generate stimulatory or inhibitory signals that will guide T cell responses (Figure 1). For instance, inhibitory immune receptors, such as CTLA-4 and PD-1, are crucial in the maintenance of peripheral tolerance [2].

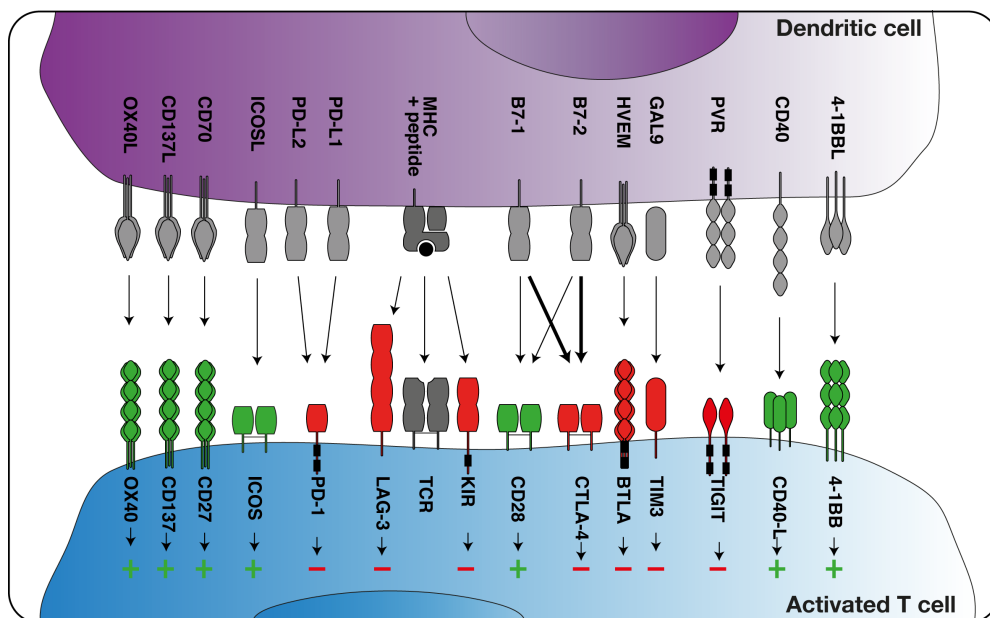


FIGURE 1. Stimulatory and inhibitory co-receptors on T cells (Courtesy of Pr. Pierre Coulie).

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Non-exhaustive view of stimulatory (green) and inhibitory (red) co-receptors on activated T lymphocytes. Stimulatory or inhibitory signaling, resulting from co-receptor engagement by their cognate ligand on a dendritic cell, is depicted by “+” or “-” symbols, respectively.

1.2 Discovery of PD-1 and of its role in immune regulation

In 2018, Tasuku Honjo and James Allison were awarded the Nobel Prize in Physiology and Medicine for their breakthrough discoveries in the field of cancer immunology and for opening new doors for cancer immunotherapy. They indeed discovered two inhibitory immune checkpoints expressed on the surface of activated T cells, namely CTLA-4 (Cytotoxic T Lymphocyte-Associated protein 4) and PD-1 (Programmed cell Death protein 1). The *PDCD1* gene, coding for PD-1, was discovered by the group of T. Honjo at the Kyoto University in 1992. The group showed that 2B4.11 (a murine T cell hybridoma) and LyD9 (a murine hematopoietic progenitor cell line) underwent apoptosis when stimulated or deprived of interleukin (IL)-3, respectively, and that the two cell lines expressed PD-1 while undergoing this classical type of programmed cell death [3]. The notion that PD-1 could be involved in immune regulation first came from the observation that it contains a consensus sequence found in the cytoplasmic tail of immune recognition receptors [3], then from the description of the phenotype of *Pdcd1*^{-/-} mice. *Pdcd1* deletion in C57BL/6 mice leads to spontaneous development of lupus-like glomerulonephritis and destructive arthritis at 6 to 9 months [4]. *Pdcd1* deletion in BALB/c mice induces a more severe phenotype, with the majority of mice dying within 30 weeks after birth from dilated cardiomyopathy. It was suggested that the latter phenotype was caused by T or B cell activity, because *Pdcd1* deletion in immunodeficient *Rag2*^{-/-} BALB/c mice does not lead to the same cardiomyopathy [5]. These observations, as well as the fact that PD-1 is upregulated on activated T cells, argued for a role of PD-1 in the maintenance of peripheral tolerance.

Since then, numerous studies have shown that PD-1 exerts deleterious functions in the context of cancer, notably because it decreases anti-tumor T cell responses.

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Strategies that aim at blocking PD-1 or its main ligand PD-L1 have been and are still being developed to this day, and generally consist in the use of monoclonal antibodies (mAbs). In this section, I will discuss the regulation of PD-1 expression and that of its ligands, as well as signaling by and functions of the PD-1/PD-L1 axis in cells of the tumor microenvironment (TME), focusing on T lymphocytes, antigen-presenting cells (APCs), endothelial cells and tumor cells. Finally, I will briefly describe efficacy and safety profiles of PD-1/PD-L1 blockade strategies that have been approved for the treatment of cancer patients.

1.3 Expression and regulation of PD-1 and its ligands

1.3.1 Expression and regulation of PD-1

PD-1 is constitutively expressed on immature CD4⁺ CD8⁻ double negative thymocytes, but also on B cells, monocytes, macrophages, natural killers (NKs), dendritic cells (DCs) and even on some tumor cells.

On T cells, PD-1 is considered as a marker of activation. Its expression is very low or absent on naïve and resting T cells and is upregulated upon activation induced by antigen recognition and co-stimulation [6, 7]. Following T cell activation, PD-1 expression is induced as a result of transient loss of DNA methylation of CpG regions in the *PDCD1* promoter. After antigen clearance, PD-1 levels decrease on effector T cells as a consequence of re-methylation of the *PDCD1* promoter. However, in situations where the antigen is not cleared, such as cancer and chronic infections, PD-1 expression is sustained, possibly *via* irreversible demethylation of the *PDCD1* promoter [8]. Continuous TCR (T Cell Receptor) signaling and inflammatory cytokine production likely contribute to sustained *PDCD1* demethylation because this region also contains binding sites for NFAT (Nuclear Factor of Activated T cells), that are activated by TCR-dependent pathways, and ISRE (Interferon-Sensitive Response Element), that are binding sites for transcription factors of the interferon pathways [8]. PD-1 mRNA (messenger RNA) expression and protein levels can also be regulated by several transcription factors and post-translational modifications, respectively. For instance, E3 ligase FBXO38 can poly-ubiquitinate PD-1 which

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leads to proteasome degradation [7]. Fucosylation by Fut8 (Fucosyl Transferase 8) was also highlighted as an important mechanism to stabilize PD-1 at the surface of cytotoxic T cells. Zhang and colleagues have shown that Fut8 knock-out (KO) mice displayed reduced tumor burden. This effect was abrogated by antibody-mediated depletion of CD8 T cells. The proportion of PD-1-expressing CD8 T cells and the expression level of PD-1 on T cells were both reduced in absence of this enzyme, as a result of enhanced PD-1 polyubiquitination. Cytotoxic T cells isolated from Fut8 KO mice exhibited increased activation and effector functions as compared with control littermates [9].

Anti-inflammatory cytokines such as TGF- β 1 (Transforming Growth Factor β 1) have also been described as important for the regulation of PD-1 expression on T cells. Park and colleagues reported that recombinant TGF- β 1 added to healthy donor peripheral blood mononuclear cells (PBMCs) stimulated with anti-CD3/CD28 considerably increased PD-1 expression, whereas IL-2, 6, 12 and TNF α only modestly increased PD-1 expression in the same setting [10]. Cyclosporin A (a calcineurin inhibitor that keeps transcription factor NFATC1 inactive) abolished the ability of TGF- β 1 to enhance PD-1 expression, indicating that TCR signals are required in addition to TGF- β 1. PD-1 induction by TGF- β 1 is mediated by SMAD3, as suggested by increased SMAD3 binding to the human *PDCD1* locus in response to TGF- β 1, and by the fact that SIS3, a specific inhibitor of phospho-SMAD3 (pSMAD3), inhibited TGF- β 1-mediated enhancement of *PDCD1* promoter activity. In mouse, the effect of TGF- β 1 on PD-1 expression was decreased in *SMAD3*, but not *SMAD2* conditional knock-out (cKO) T cells (*Cd4^{Cre} x SMAD3^{fl/fl}* or *SMAD2^{fl/fl}* cells, respectively). The authors also observed that the TGF- β 1/SMAD3-dependent enhancement of PD-1 expression was associated with decreased T cell function: *SMAD3* cKO mice challenged with MC38 tumor cells displayed an increased proportion of TNF α ⁺ CD8⁺ tumor-infiltrating lymphocytes (TILs) and of interferon- γ (IFN γ)⁺ or IL-2⁺ CD8⁺ T cells in the draining lymph node (dLN) [10].

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1.3.2 Expression and regulation of PD-L1 and PD-L2

Two ligands were identified for PD-1: PD-L1 and PD-L2. PD-L1, also called B7-H1 (B7 Homolog 1), PDCD1L1 (*PDCD1* Ligand 1) or CD274, was first identified by Dong and colleagues in a human cDNA (complementary DNA) library, in a screen for genes encoding homologs of B7-1 and B7-2 proteins [11]. PD-L1 is constitutively expressed at low levels on DCs, monocytes, mesenchymal stem cells, mast cells and nonhematopoietic cells such as vascular endothelial cells and fibroblasts [7]. On T cells, PD-L1 is induced by TCR stimulation and by γ -chain cytokines such as IL-2. LPS (lipopolysaccharides) and BCR (B Cell Receptor) activation, as well as IL-21 induce PD-L1 expression on B lymphocytes. IFN γ can further increase PD-L1 expression in monocytes and endothelial cells. TGF- β 1 has cell type-specific effects on PD-L1 expression. While it decreases PD-L1 expression on tubular epithelial cells and monocytes, TGF- β 1 increases PD-L1 expression on DCs and tumor-infiltrating CD8 T cells [12]. The PD-L1 promoter can also be the site of epigenetic control, as it contains a 220 bp (base pair)-long region rich in CpG motifs. Moreover, the *CD274* mRNA has a long 3' UTR (untranslated region) which contains an AU-rich element (ARE) and binding sites for microRNAs (miRNA) and RNA binding proteins (RBPs) that could be involved in post-transcriptional control [6]. At the post-translational level, degradation of the PD-L1 protein can be promoted by poly-ubiquitination by the E3 ligase cullin-3. Glycogen synthase kinase 3 β (GSK3 β)-dependent phosphorylation of PD-L1 leads to its ubiquitination by β -TrCP E3 ligase and further proteasome degradation. PD-L1 glycosylation prevents GSK3 β binding. CSN5 and CMTM6 can also stabilize PD-L1 by deubiquitination. The function and ability of PD-L1 to bind to PD-1 can be regulated by alternative splicing of the *CD274* mRNA. A shorter transcript lacking the third exon can be produced that will lead to the generation of a form of PD-L1 lacking an extracellular domain [7].

PD-L2 was later discovered by Tseng and colleagues who originally named it B7-DC because it was identified in a library of dendritic cell cDNAs [13]. PD-L2 is expressed on immature DCs and increased on DCs activated by GM-CSF and IL-4. PD-L2 is also constitutively expressed by macrophages, bone marrow-derived mast

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cells and peritoneal B1 cells. Its expression can be induced on B cells by LPS treatment and BCR stimulation [6].

PD-L1 and PD-L2 can be found on tumor cells, with PD-L1 more commonly present compared to PD-L2. There is no evidence that blocking binding of PD-1 to both ligands with anti-PD-1 mAbs leads to higher clinical benefit for cancer patients compared to blockade of PD-L1 alone, suggesting that PD-L1 is the dominant inhibitory ligand of PD-1 at least in human tumors [6]. PD-L1 has been shown to be upregulated on tumor cells as a result of mechanisms shared with non-tumor cells, such as IFN γ -mediated increase of *CD274* gene expression, but also as a result of tumor cell-specific mechanisms, such as the presence of genomic aberrations. An analysis performed on 22 cancer types, taking into account data from The Cancer Genome Atlas (TCGA), showed that increased copy number in chromosome 9p24.1, where the *CD274* locus is located, are linked with increased expression of this gene [14]. Increase of *CD274* mRNA in a fraction of human tumors have also been shown to correlate with loss of its 3' UTR region [7]. In addition, oncogenic transcription factors are involved in the transcriptional regulation of *CD274* in a variety of tumor cell types. For instance, there is a positive correlation between *MYC* and *CD274* expression in non-small cell lung cancer (NSCLC). Genetic or pharmacological inhibition of *MYC* leads to decreased PD-L1 levels in tumors [15]. HIF-1 α , a key transcription factor in the context of hypoxia, can also lead to upregulation of *CD274* mRNA levels in tumor cells of some cancer types such as melanoma, breast cancer, prostate cancer and NSCLC [16]. Other studies focusing on PI3K-Akt and MEK-ERK signaling have shown similar effects of these pathways on *CD274* expression [7, 17, 18]. It was recently shown that PD-L1 can be stabilized at the post-translational level in a tumor cell-specific manner [19]. In murine colon carcinoma MC38 cells, PD-L1 is palmitoylated by the palmitoyltransferase DHHC3, and this blocks PD-L1 ubiquitination thus suppressing lysosomal degradation. Moreover, glycosylation is thought to be a major contributor of PD-L1 stabilization in tumor cells because it prevents GSK3 β -dependent phosphorylation and subsequent ubiquitination [6].

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The potential use of PD-L1 expression as a prognosis marker in cancer patients has been extensively studied but remains controversial, as inconsistent results have been reported in retrospective clinical studies. In a subset of 117 patients with unresectable stage III NSCLC, Vrankar and colleagues have reported that PD-L1 expression has no prognostic value, because progression-free survival (PFS) and overall survival (OS) were similar in patients with high PD-L1 expression ($\geq 50\%$ of the cells are stained, as assessed by immunohistochemistry) compared to patients with low (1%-49% of the cells are stained) or no PD-L1 expression [20]. However, Tashima *et al* stated that the prognostic impact of PD-L1 could be influenced by the status of cancer immunity and that not taking this parameter into account could explain the lack of prognostic value of PD-L1 expression in tumors. In NSCLC patients, neutrophil-to-lymphocyte ratio (NLR) is associated with poor prognosis, and is correlated with increased survival in patients treated with PD-1/PD-L1 blockade therapies. As such, it is frequently used in NSCLC as a surrogate of the immune status. The authors confirmed that PD-L1 positivity does not provide a significant prognostic impact when looking at 83 patients with unresected stage I NSCLC in their globality. Interestingly, PD-L1 expression was correlated with lower OS in NLR-low (<2.2) patients, and this was not the case in NLR-high (≥ 2.2) patients [21]. In glioblastoma, a meta-analysis performed on 9 studies regrouping 806 patients showed that tumoral PD-L1 expression was significantly correlated with poor prognosis [22].

In addition to its potential prognostic value, there has been much speculations as to whether PD-L1 expression could be used as a predictive biomarker of response to anti-PD-1 and anti-PD-L1 mAbs. Immune checkpoint blockade (ICB) therapy has been shown to increase IFN γ production by T cells, which in turn enhances expression of PD-L1 in tumors. Thus, increased PD-L1 expression on tumor cells can actually be a read-out for IFN γ production by tumor-infiltrating T cells, and correlates with enhanced anti-tumor immune responses. This explains its use as an indicator of response to ICB in some cancer types, such as urothelial carcinoma and NSCLC [23]. In addition to its indicator value, the presence of PD-L1 on tumor cells

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is frequently used as a predictor of response to ICB, as patients with low or no tumoral PD-L1 generally experience less benefit from ICB therapies. However, it should not be used as a standalone predictive biomarker to select patients that are more likely to respond to PD-1/PD-L1 blockade, as some patients with PD-L1 expression do not experience clinical benefit after PD-1/PD-L1 blockade while others with PD-L1-low or negative tumors respond to these therapies [6, 23]. It has been proposed to combine PD-L1 expression with the presence of tumor-infiltrating lymphocytes (TILs) to increase the accuracy of predictive response to treatment. The presence of TILs seems required to observe clinical benefit after ICB, as most patients that have tumors with low density or absence of lymphocytes do not respond to ICB. Tumors harboring both PD-L1 expression and high TIL density have the best prognosis [23, 24].

1.4 Structure and interaction of PD-1 and its ligands

PD-1 and its ligands (PD-L1 and PD-L2) are type I transmembrane glycoproteins that belong to the superfamily of immunoglobulins (Ig) because they contain extracellular domains (Ig-V-like) first identified in immunoglobulins. They also contain a transmembrane domain, and a cytoplasmic tail that serves as a docking site for signaling or scaffolding proteins. More particularly, the cytoplasmic domain of PD-1 is composed of two tyrosine signaling domains termed ITIM (Immunoreceptor Tyrosine Inhibitory Motif) and ITSM (Immunoreceptor Tyrosine-based Switch Motif) that are required for recruitment of phosphatases SHP (Src Homology region 2 domain-containing Phosphatase)-1 and 2 [7, 12]. Some studies have shown that PD-L1 is also able to induce intracellular signaling, but this observation remains debated in the field. Effects of and signaling by PD-1 and PD-L1 will be discussed in a following section.

PD-1 displays a 3-fold higher affinity to PD-L2 compared to PD-L1, a difference attributed to the interface that PD-1 forms with each of its ligand. Indeed, the number of contacts formed between PD-1 and PD-L2 is higher due to the presence of a tryptophan at position 110 (W110) whereas the residue in the homologous position within the PD-L1 binding site is replaced by alanine (A121) [25]. Unlike other

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members of the family, PD-1 and its ligands mainly interact as monomers [26]. Binding between PD-1 and PD-L1 occurs mainly in *trans*, that is on different cells (*e.g.* between PD-1 on a T cell and PD-L1 on a tumor cell), but it can also occur in *cis* on certain tumor cells and tumor-infiltrating APCs that co-express both molecules. The latter type of interaction prevents the transmission of inhibitory signals inside T cells and might explain why, in some cases, anti-PD-1 mAbs fail to reinvigorate T cell activity. By binding to PD-1 expressed on APCs or tumor cells, anti-PD-1 mAbs might render PD-L1 available for binding to PD-1 expressed by T cells, thereby inhibiting T cell signaling. For that reason, combining PD-1 blockade with PD-L1 blockade might be an interesting strategy in refractory cancer types [26].

1.5 Signaling and functions of PD-1/PD-L1 in cells of the tumor microenvironment

Given the expression of PD-1 and its ligand PD-L1 by cells of the tumor microenvironment and the inhibitory signaling that results from PD-1 engagement, blocking the PD-1/PD-L1 axis with monoclonal antibodies has become a standard of care for cancer treatment. Even though such therapies brought major improvements in the field of cancer treatment, there is a high heterogeneity in the intensity as well as the durability of the response to PD-1 or PD-L1 blockade. This observation led to a great interest for the in-depth investigation of mechanisms by which the PD-1/PD-L1 axis transmits inhibitory signals, especially in tumors, with the goal to identify new therapeutic targets that could increase the overall efficacy of PD-1/PD-L1 blockade therapies in cancer patients [7, 26]. Figure 2 summarizes the main effects of PD-1/PD-L1 signaling in 4 cell types of the tumor microenvironment (effector T cells, tumor cells, dendritic cells, endothelial cells).

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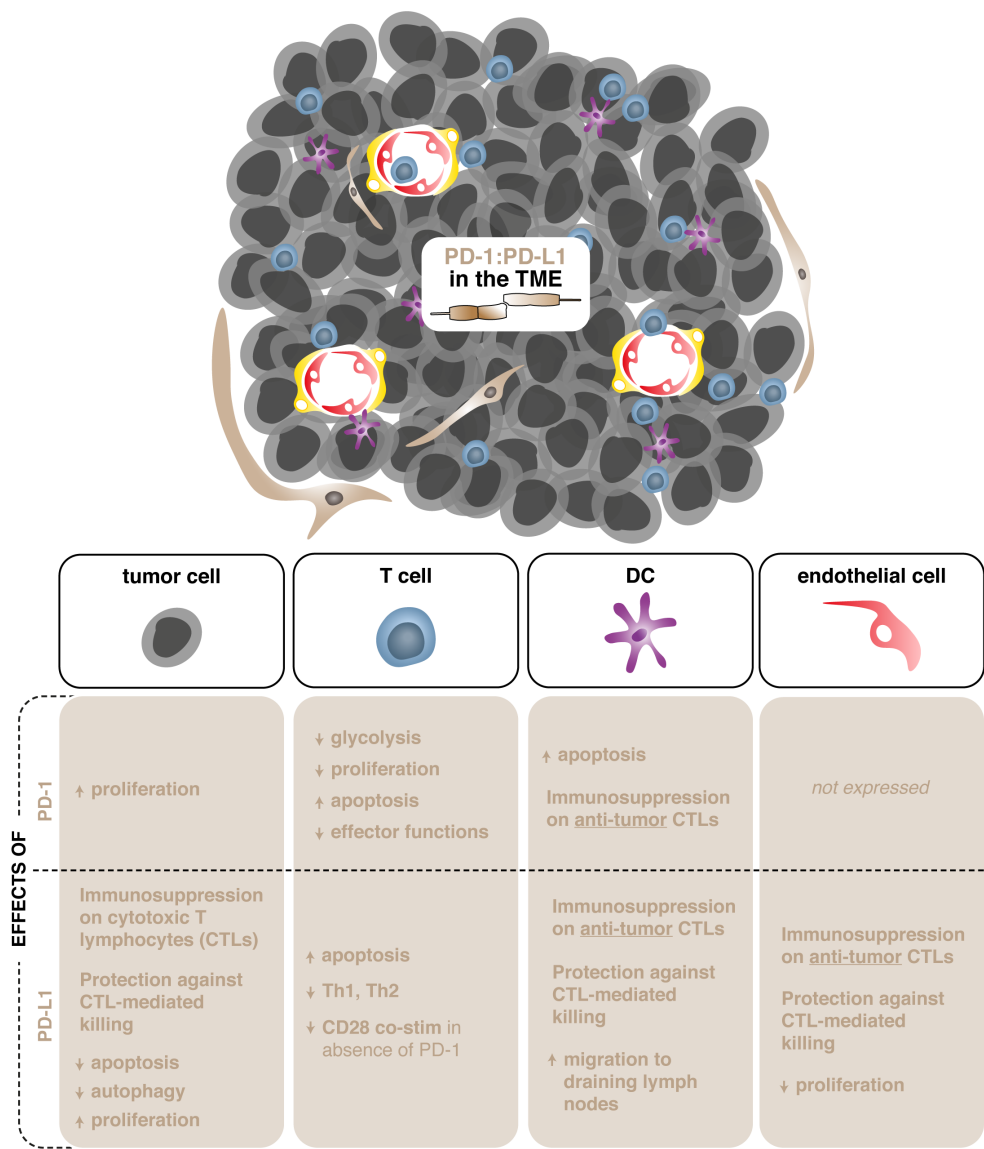


FIGURE 2. Overview of the effects of PD-1 signaling and PD-L1 expression and signaling on cells of the tumor microenvironment.

Effects of PD-1 signaling (upper panel) or PD-L1 expression and signaling (lower panel) on 4 cell types found in tumors, that are, from left to right, tumor cells, effector T cells, dendritic cells (DCs) and endothelial cells.

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1.5.1 Signaling and functions of PD-1/PD-L1 in T cells

Immune checkpoints such as PD-1 and CTLA-4 act as molecular brakes to prevent uncontrolled T cell activity. As such, they are crucial to maintain peripheral tolerance but can be deleterious in the context of cancer, because they dampen T cell-mediated anti-tumor immune responses. PD-1 exerts inhibitory signaling inside T cells in a different manner compared to CTLA-4. Whilst CTLA-4 decreases co-stimulatory signals by binding to B7 and thus competing with CD28 and preventing co-stimulatory signals, PD-1 rather tampers with signaling pathways that are crucial notably for T cell survival, proliferation, activation and effector functions. Because the precise role of PD-L2 in regulating T cell function remains to be fully elucidated, I will focus on the signal pathways that are tampered with following PD-1 binding to PD-L1. Effects of PD-1/PD-L1 in T lymphocytes are reviewed in [7], [26] and [27].

Which signaling pathway is the primary target of PD-1 still remains debated in the field. On the one hand, it is generally considered that PD-1 primarily inhibits cascades downstream of the TCR [26, 28]. On the other hand, a study published in *Science* suggests that it inhibits the cascade downstream of the co-stimulatory receptor CD28 [29]. Yet another publication has reported that both TCR and CD28 are equally targeted by PD-1 [30].

During T cell activation, and after engagement with PD-L1, PD-1 changes its conformation and accumulates at the Central SupraMolecular Activation Cluster (cSMAC), a dynamic intracellular signaling structure that is formed after TCR clustering at the site of antigenic recognition and that constitutes the immunological synapse [7, 27]. It is only after cross-linkage with TCR in such structures that PD-1 can transduce signals [6]. Tyrosine residues of the cytoplasmic tail of PD-1 are phosphorylated by Src family kinases thus serving as docking sites for tyrosine phosphatase SHP-2. PD-1 blockade in tumor-bearing mice that harbor a T cell-specific deletion of SHP-2 (*Cd4^{Cre} x Ptpn-11^{fl/fl}*) still induced tumor regression, indicating that another partner, later identified as SHP-1, could mediate PD-1 inhibitory signaling in the absence of SHP-2 [30, 31]. The recruitment of SHP-1/2 dampens the phosphorylation of ZAP70 by Lck (Lymphocyte-specific protein

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Kinase), an event that is important for distal signaling *via* ERK but also PI3K-dependent mTOR activation [7, 27] (Figure 3).

Upon activation, T cells switch their metabolic program towards glycolysis to have fast energy, a phenomenon known as the Warburg effect. Simultaneously, they increase glutamine uptake and catabolism to guarantee T cell fitness. PD-1 is thought to mediate rapid shift of T cell metabolism towards fatty acid β -oxidation while dampening glycolysis, glutaminolysis and metabolism of branched-chain amino acids and switching to a more oxidative environment. Such metabolic reprogramming following PD-1 engagement is thought to be mediated by inhibition of PI3K and ERK. Oxidative stress alters T cell function. In Tregs, which are particularly sensitive to oxidative environments, it leads to apoptosis and release of large quantities of ATP (Adenosine TriPhosphate) converted to adenosine, which drives a more immunosuppressive environment [26].

Inhibition of PI3K and ERK signaling leads to decreased transcription of SKP2 (S-phase Kinase-associated Protein 2), a part of SCF^{skp2} ubiquitin ligase that normally leads to the proteosomal degradation of CDK2 (Cyclin-Dependent Kinase 2) inhibitor CDKN1B (also called p27^{Kip1}). CDK2, as well as CDK4 and CDK6 are also inactivated by repressed transcription of CDC25A, a tyrosine phosphatase that normally removes inhibitory phosphorylations of these cyclin-dependent kinases. As a consequence of CDK inhibition, T lymphocytes are blocked at the G0-G1 phases of the cell cycle [27]. In addition, SHP-1/2 recruitment to the immunological synapse can lead to increased expression of basic leucine zipper transcriptional factors ATF-like (BATF), with a broad range of functional effects on T cells, among which alteration of effector functions [7] (Figure 3).

PD-1 engaged by PD-L1 can also signal independently of SHP phosphatases and upregulate the expression of E3-ubiquitin ligases, notably CBL-b, c-CBL and ITCH, that ubiquitinate chains of the TCR, leading to its removal from the surface possibly *via* endocytosis. Down-modulation of the TCR can constitute a way for tumor cells to escape the immune system, as the ability of T cells to recognize tumor-associated

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antigens is reduced by this process. Polyubiquitination of CD3 ζ by CBL-b and ITCH can also impair its phosphorylation and subsequent binding of ZAP70 [27] (Figure 3).

In brief, engagement of PD-1 on T cells leads to recruitment of SHP-1/2 or E3 ubiquitin ligases at the immunological synapse, resulting in inactivation of a plethora of signaling cascades downstream of the TCR and CD28. The functional outcomes of these modulation are diverse and comprise inhibition of T cell proliferation and survival (with eventual death of activated T lymphocytes), decreased activation and effector functions such as cytokine and granzyme/perforin production and altered metabolism.

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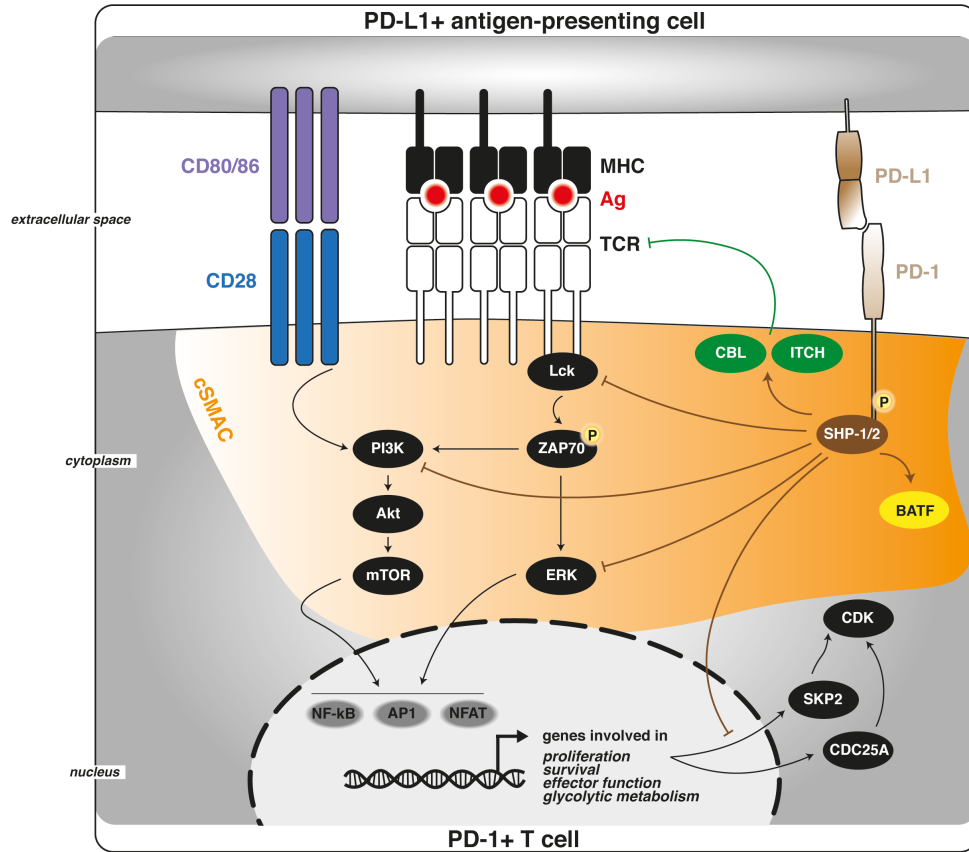


FIGURE 3. Overview of the inhibitory effects of PD-1 engagement on T cell signaling.

T cell receptor (TCR) and CD28 cluster following recognition of antigen (Ag) presented by major histocompatibility (MHC) molecules and CD80/86 binding, respectively. This forms an area of reinforced signaling (called cSMAC) leading to T cell proliferation, survival and activation as well as favoring glycolytic metabolism, all driven by ZAP70, PI3K and ERK signaling pathways. After binding to PD-L1-expressing antigen-presenting cell, SHP-1 or 2 is recruited to the cytosolic phosphorylated tail of PD-1, and this leads to: *i*) inhibition of Lck-dependent phosphorylation of ZAP70, *ii*) inhibition of PI3K and ERK, *iii*) decreased activity of cyclin-dependent kinases (CDK), *iv*) expression of BATF transcription factors that alter T cell function, and *v*) upregulation of E3 ubiquitin ligases (CBL-B, c-CBL and ITCH) that lead to TCR internalization and decrease TCR-dependent signaling.

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The ability of PD-L1, also upregulated on T cells in response to antigen presentation and inflammatory signals, to induce signaling inside T cells is still debated by controversial studies. One study showed that PD-L1 can induce phosphorylation of p38, leading to apoptosis in CD8 T cells [32]. Another study showed that PD-L1 engagement at the surface of CD4 T cells transmits STAT3-dependent signaling and that this partially favors differentiation towards Th17 and reduces Th1 and Th2 polarization. The authors also evidenced that PD-L1 signaling mitigates activation of cytotoxic T lymphocytes (CTLs), as *Pd1* KO CD8 T cells exhibited enhanced effector functions [33]. In some instances, PD-L1 can also bind to CD80 on T cells and prevent co-stimulation by competing with CD28 ligation [34], a mechanism similar to that of CTLA-4. This is possible because the affinity of binding of CD80 to PD-L1 is approximately 3 times stronger than that of binding to CD28. Thus, CD80 will preferentially bind to PD-L1 when T cells are in resting conditions. However, PD-L1 will preferentially bind to PD-1 when present at the surface of activated T cells because the affinity of the PD-1/PD-L1 interaction is about 4 times higher than that of CD80/PD-L1 [35-37].

1.5.2 Signaling and functions of PD-1/PD-L1 in tumor cells

Although PD-L1 is thought to transmit inhibitory signals that lead to apoptosis or decreased function and proliferation in T cells, it rather prevents apoptosis and enhances proliferation of cancer cells. PD-L1 serves as a defense mechanism for tumor cells, by activating several pathways in a PD-1 dependent, but also in a PD-1 independent manner. Indeed, Azuma and colleagues showed that PD-L1⁺ tumor cells display increased resistance to CTL-mediated killing *in vitro* compared to PD-L1⁻ parental cells and that blocking anti-PD-L1 antibodies can restore lysis of tumor cells by CTLs [34]. Although they cannot exclude that this was due to decreased T cell activity following lymphocytic PD-1 engagement with PD-L1⁺ tumor cells, they postulated that it could also be due to signaling inside tumor cells following engagement of PD-L1. They used a truncated form of PD-L1 in which the intracellular domain was replaced by GFP, that displays normal binding to PD-1 but impaired capacity to transmit a putative signal to P815 tumor cells. They showed

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that T cells efficiently lysed P815 expressing the truncated form of PD-L1 whereas P815 cells expressing full-length PD-L1 were protected from CTL lysis. They also showed that PD-L1-expressing P815 cells are resistant to apoptosis in a T cell-free system [34]. This suggests that PD-L1 transmits anti-apoptotic signals preventing tumor cell death, and shows that tumoral PD-L1 can function in an immune-independent fashion, that is without the presence of PD-1-expressing T cells. Clark *et al* showed that PD-L1 decreases tumor cell autophagy, as shRNA-mediated knockdown (KD) of PD-L1 in ID8agg and B16 murine tumor cell lines led to significantly higher levels of LC3-II *versus* LC3-I compared to control cell lines [38]. They showed equivalent results for the human ovarian cancer cell line ES2. Using RNA-seq, they observed that several genes involved in the mTOR pathway were downregulated in PD-L1 KD ID8agg cells. mTOR is a serine/threonine kinase important for cell proliferation and metabolism and is involved in many cellular processes including repression of autophagy. In B16, ID8agg and ES2 cells, PD-L1 augmented mTORC1 signaling, as PD-L1 KD in these cell lines led to lower levels of phospho-P70S6K^{T389}. Chloroquine, used to inhibit autophagy, had opposing effects on mTORC1 in ID8agg and B16 cells. Thus, effects of PD-L1 on autophagy could be independent of the mTOR pathway [38]. PD-L1 signaling inside tumor cells could however contribute to their survival and proliferation by increasing the mTOR pathway. Another study focusing on Hodgkin lymphoma (HL) observed that the growth and survival of PD-L1-expressing HL cell lines was increased following *in vitro* treatment with agonistic anti-PD-L1 mAb. This was associated with activation of the MAPK pathway and increased mitochondrial oxygen consumption. All these events were reversed by treatment with nivolumab, an anti-PD-1 mAb, suggesting that this type of signaling downstream of PD-L1 occurs after binding to PD-1 [39].

Kleffel and colleagues have shown that human and murine melanomas contain a subpopulation of tumor cells expressing PD-1. Using *Pdcd1*-knockdown (KD) and *Pdcd1*-overexpression (OE) in B16 murine melanoma cell lines, they could show that PD-1 expression in tumor cells increases their tumorigenicity in a lymphocyte-independent manner, as *Pdcd1*-KD decreased and *Pdcd1*-OE increased tumor

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growth in both immune-competent C57BL/6 and immunodeficient NSG (Nod Scid Gamma) mice. This requires interactions between melanoma-expressed PD-1 and its ligand PD-L1, because the growth of *Pdcd1*-OE melanomas was attenuated in PD-L1^{-/-} Rag^{-/-} mice. In B16 cells, PD-1 engagement leads to increased mTOR signaling, suggesting that PD-1 signaling in tumor cells, as PD-L1, can increase their proliferation and survival [40].

1.5.3 Signaling and functions of PD-1/PD-L1 in dendritic cells

Experiments performed in murine tumor models underlined the importance of PD-L1 on myeloid cells, and more particularly on dendritic cells, in anti-tumor immune responses and to generate responses to immune checkpoint blockade. Indeed, MC38 and E.G7 tumors grew slower when they were implanted to DC-conditional PD-L1 knockout mice (*Cd11c^{Cre} x Pdl1^{fl/fl}*) and did not respond to anti-PD-L1 mAbs, as shown by Peng *et al.* After transplantation of MC38 cells stably expressing EGFP (MC38-EGFP), EGFP⁺ DCs displayed much higher levels of PD-L1 in the draining lymph node and in the tumor as compared to EGFP⁻ DCs, which indicates that PD-L1 expression is increased on DCs upon antigen uptake. This could be the result of IFN γ production by CD8 T cells because both IFN γ blockade and CD8 T cells depletion reduced PD-L1 levels [41]. Transmission of PD-1-mediated inhibitory signals in T cells during antigen presentation could lead to suboptimal T cell priming and activation. The authors also showed that PD-L1⁺ DCs were less susceptible to T cell-mediated killing compared to PD-L1-deficient DCs, which suggests that PD-L1 could protect DCs from cytotoxicity exerted by tumor-specific CTLs. Such protective effects rely on close contact of DCs with T cells, because it was abrogated in a permeable transwell assay where DCs and T cells are physical separated [41].

Lucas and colleagues have shown that PD-L1 reverse signaling is required for DC migration to the lymph nodes during an active immune response (induced by Toll-like receptor agonist poly(I:C) or by bacterial infection). More precisely, they identified a TSS motif in the cytoplasmic tail of PD-L1 that is crucial for CCR7 and CXCR4 signaling and that leads to homing of DCs to the draining lymph nodes following inflammation [42]. Although the author did not show it in this context,

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involvement of PD-L1 in DC migration in inflammatory conditions could be particularly relevant to cancer, where DCs migrate from the tumor site to the draining lymph nodes to prime and activate T cells with tumor-associated antigens.

Zhao *et al* have shown that PD-1 and PD-L1 are co-expressed on tumor-infiltrating dendritic cells and that they can bind to each other in *cis*. This inhibits the ability of dendritic PD-L1 to bind to PD-1 in *trans* at the surface of T cells and to induce inhibitory signaling in this cell type [43]. Dendritic PD-1 or CD80 binding to PD-L1 in *cis* could also contribute to PD-L1 reverse signaling in DCs in the absence of PD-1-expressing T cells [42, 43]. PD-1 negatively impacts survival of DCs. Park and colleagues have shown that PD-1 KO DCs are less apoptotic and exhibit increased MAPK1 and CD40-CD40L signaling. Proliferation and IFN γ production by OT-I and OT-II cells (ovalbumin-specific CD8 and CD4 T cells, respectively) was increased by ovalbumin-pulsed PD-1-deficient DCs, suggesting that DC-expressed PD-1 decreases T cell priming and activation [44].

1.5.4 Signaling and functions of PD-1/PD-L1 in endothelial cells

PD-L1 expression by endothelial cells greatly varies depending on the organ where they are localized, cell activation status and the species. PD-L1 can be induced or upregulated on endothelial cells under the influence of pro-inflammatory cytokines such as IFN γ or TNF α . Other stimuli, such as VEGF (Vascular Endothelial Growth Factor) can also lead to PD-L1 upregulation on endothelial cells. Shear stress can decrease PD-L1 expression, *via* activation of tetrahydrobiopterin (BH $_4$), a nitric oxide synthase cofactor that will prohibit transcription of the *CD274* gene. Some authors speculate that this could explain the difference in PD-L1 levels between arterial and venous vessels or during vasoconstriction and vasodilation but convincing evidence is still lacking to date [45].

Because they are in close contact with circulating immune cells, endothelial cells are classically described for the regulation of T cell recruitment but can also induce peripheral tolerance thanks to PD-L1 expression. Endothelial PD-L1 binds to PD-1 at the surface of T cells, generating inhibitory signaling that will lead to

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downregulation of T cell proliferation and activation, as described above [45-48]. In mice transplanted with B16-OVA (ovalbumin) tumors, tumor endothelial cells (TECs) can act as antigen-presenting cells, because they harbor MHC (Major Histocompatibility Complex) class II molecules at their surface and they are positive for OVA (SIINFEKL)-H2K^b complexes, which indicates that they are able to cross-present tumor-derived OVA on MHC class I molecules [48]. The ability of endothelial cells to act as antigen-presenting cells and to cross-present antigens to CD8 T cells was also highlighted by others [49, 50]. In the B16-OVA model, TECs exerted contact-dependent immunosuppressive activity specifically on OVA-specific CD8 T cells, as they decreased their proliferation, cytotoxicity and production of pro-inflammatory cytokines such as IFN γ and TNF α [48]. Such effects were reverted by PD-L1 blockade. Moreover, in co-cultures of TECs and OT-II cells, the proportion of Tregs and the supernatant levels of IL-10 and TGF- β 1 were increased [48]. Overall, it appears that endothelial cells can contribute to tumor immune evasion by decreasing T cell responses in an antigen-dependent fashion. Concomitantly, PD-L1 expression on endothelial cells was associated with poor prognosis in advanced NSCLC [47].

Similarly, PD-L1 serves as a molecular shield for endothelial cells to resist CD8 T cell-mediated cytotoxicity and thereby reduce vascular injury that could lead to pro-atherogenic inflammatory vascular diseases. This occurs *via* downregulation of T cell effector functions after PD-1 engagement, but interestingly it has also been suggested to occur *via* intracellular signaling after PD-L1 engagement in endothelial cells themselves. This is reviewed in [45]. Intracellular signaling by PD-L1 possibly involves Notch, because Notch is both an inducer of PD-L1 expression and a homeostatic regulator of endothelial cells; it enables them to overcome apoptosis and to build-up functional blood vessels from a damaged vessel. However, although endothelial PD-L1 can dampen T cell-mediated damage of the vessels, opposing effects have also been described. Indeed, PD-L1 is thought to decrease vascular integrity because PD-L1^{high} endothelial cells lose the expression of VE cadherin and ZO-1, two major molecular components involved in tight junction formation.

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Moreover, PD-L1 upregulates angiopoietin-2 (Ang-2) that can lead to vessel destabilization by competing with Ang-1 for binding to receptor Tie2, thereby disrupting protective signaling in vascular stability induction [45]. Using MS1, a vascular endothelial cell line obtained from pancreatic sarcoma, Jin and colleagues have shown that PD-L1 decreases angiogenesis and dampens endothelial proliferation. *In vitro*, PD-L1 inhibition by siRNA (Small Interfering RNA) in MS1 cells increases the mRNA levels of VEGFR2 (Vascular Growth Factor Receptor 2) and endothelial proliferation. *In vivo*, they observed that the expression level of VEGFR2, as well as the density of CD31^{high} LYVE1⁻ vessels was significantly higher in PD-L1 KO mice after suture-induced angiogenesis in the cornea compared to wild-type littermates [51]. These observations highlight the fact that effects of PD-L1 in endothelial cells are multi-faceted. They appear to depend on the tissue localization of endothelial cells, pathological conditions or the inflammatory context. This illustrates that a lot remains to be understood in this area.

1.6 Overview of existing PD-1/PD-L1 blockade therapies

PD-1/PD-L1 blockade is being achieved thanks to the use of monoclonal antibodies directed either against PD-1 or PD-L1. Anti-PD-1 mAbs used in humans are pembrolizumab, nivolumab and cemiplimab.

Pembrolizumab has been tested in several phase I, II and III clinical trials in NSCLC cancer patients, where it has shown relevant efficacy. A phase III clinical trial tested the efficacy of pembrolizumab combined to chemotherapy (carboplatin or cisplatin and pemetrexed) in 616 previously untreated non-squamous NSCLC. Objective response rate (ORR) reached 48% in the group of patients treated with combination of pembrolizumab and chemotherapy compared to 19% in patients receiving chemotherapy only. Progression-free survival (PFS) was 8,8 months in the combination group compared to 4,9 months in patients treated with chemotherapy. Similar results were shown in another phase III trial combining pembrolizumab and chemotherapy (carboplatin plus docetaxel or nab-paclitaxel) in 559 metastatic untreated squamous NSCLC patients (ORR: 58% in combination vs 38% in chemotherapy alone; PFS: 6,4 months in combination vs 4,8 months in

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chemotherapy alone). Pembrolizumab showed a favorable safety profile, with less than 10% of serious immune-related adverse events in monotherapy and with no further toxicity when combined to chemotherapy [52]. Another clinical trial testing pembrolizumab as a monotherapy on 210 patients with relapsed/refractory Hodgkin lymphoma showed impressive efficacy as tumor burden was decreased in more than 90% of patients. Complete response rate reached 22% [53]. For these reasons, pembrolizumab has become a standard of care for NSCLC and HL patients. It has also been approved for the treatment of melanoma, cutaneous squamous cell carcinoma (CSCC), head and neck squamous cell carcinoma (HNSCC), urothelial carcinoma (UC) and renal cell carcinoma (RCC), that are advanced and/or refractory to conventional therapies such as chemotherapy. It is still being investigated in other tumor types such as adrenocortical carcinoma (ACC) [54], and cancer of the digestive and female reproductive tract [55-57].

Efficacy and safety of nivolumab as a monotherapy was demonstrated in a clinical trial where 296 patients with various cancer types were enrolled. Durable (*i.e.* lasting 1 year or more) responses were observed in patients with NSCLC (18%), melanoma (28%) and RCC (27%). Fourteen percent of patients experienced manageable high-grade adverse events such as colitis, hepatitis or hypophysitis [58]. In a phase III trial combining nivolumab and chemotherapy (dacarbazine) in previously untreated metastatic melanoma patients, addition of nivolumab increased the objective response rate as compared to chemotherapy alone (40% vs 14%, respectively), as well as the overall survival (38,5 months vs 11,2 months, respectively). The rate of high-grade adverse events was comparable in both groups (approximately 15%) [59]. Nivolumab was approved for the treatment of inoperable or metastatic melanoma in combination with ipilimumab (anti-CTLA-4 mAb) in UC and gastric or esophageal cancers in combination with chemotherapy.

Cemiplimab is currently recommended for the treatment of advanced or metastatic CSCC and investigations are being performed for the treatment of myeloma, lung and cervical cancer [60].

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Anti-PD-L1 antibodies currently used in the clinics are atezolizumab, avelumab and durvalumab. In a meta-analysis performed on 14 clinical trials and 3 266 patients with (N)SCLC, RCC or UC, Tie *et al* showed that atezolizumab treatment leads to ORR, 6-month PFS and 1-year OS of 21%, 36% and 55%, respectively, with similar results obtained when grouped by tumor type. Moreover, it displays an acceptable safety profile, because high-grade adverse events occurred in only 13% of participants. Atezolizumab has been approved by US Food and Drug Administration (FDA) for the treatment of UC and NSCLC [61].

Mechanisms of PD-1/PD-L1 blockade have mainly been elucidated in murine tumor models using other anti-PD-1 or anti-PD-L1 antibodies, and greatly vary depending on the model used. It can lead to T cell reinvigoration, increased T cell proliferation or trafficking. Few mechanistical studies have been performed on humans to date. Effects of anti-PD-1 and anti-PD-L1 mAbs on the tumor microenvironment will be discussed in a following section. As evidenced by results gathered thanks to various clinical studies, response to PD-1/PD-L1 blockade is highly heterogenous, as some cancer types are refractory to anti-PD-1 and/or anti-PD-L1 mAbs and not all patients within one tumor type respond. This supports the idea that resistance mechanisms, which can be primary (*i.e.* present before treatment) or acquired (*i.e.* appearing during treatment), can occur. Several studies have highlighted the immunosuppressive cytokine TGF- β 1 as a potential pathway leading to resistance to immune checkpoint blockade, and thus an interesting target to improve the efficacy of these treatments in refractory tumors. In the following section, the biology of TGF- β 1 and its functions in anti-tumor immunity will be discussed.

2 Biology of TGF- β 1 signaling in anti-tumor immunity

The TGF- β superfamily is composed of 32 proteins regrouped in 2 subfamilies of TGF- β s and Bone Morphogenic Proteins (BMPs). Three different genes encode 3

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isoforms of TGF- β (β 1, β 2 and β 3), which share 71 to 79% amino-acid identities in the sequence of the mature cytokine. Gene expression signatures indicative of TGF- β signaling are often correlated with poor prognosis or lack of response to ICB therapies in cancer patients [62]. TGF- β signatures have been linked to resistance to PD-1/PD-L1 blockade in melanoma and colorectal cancer in humans [62-64]. Comparative analysis performed on RNA sequencing (RNA-seq) data from TCGA revealed that TGF- β 1 is the most prevalent isoform expressed in the majority of human cancer types [65]. The authors observed a stronger correlation between TGF- β gene signatures and *TGFB1* mRNA levels than with *TGFB2* and *TGFB3* mRNA levels in human tumors, suggesting also that TGF- β 1 is the main isoform contributing to TGF- β signaling in human tumors. Lastly, they showed that expression of *TGFB1*, but not of *TGFB2* or *TGFB3*, correlated with a transcriptional signature of innate resistance to anti-PD-1 (IPRES), suggesting that TGF- β 1 is the isoform that contributes to primary resistance to ICB in cancer patients [65]. For these reasons, I will focus this part of the introduction on the effects of TGF- β 1 in the tumor microenvironment.

2.1 Production of, activation of and signaling by TGF- β 1

2.1.1 Production of latent TGF- β 1

TGF- β 1, as other isoforms, is produced as a pre-pro-protein that contains the sequence of the signal peptide, of the latency-associated peptide (LAP) and the mature cytokine. After signal peptide cleavage and homodimerization *via* the formation of interchain disulfide bonds, the resulting pro-TGF- β 1 precursor is cleaved by pro-protein convertase furin into mature TGF- β 1 (C-terminal portion) and LAP (N-terminal portion). The two dimeric fragments remain non-covalently associated and form a complex called “latent TGF- β 1”. LAP embraces mature TGF- β 1 (mTGF- β 1) and thereby maintains it inactive by preventing binding to the TGF- β receptor. To exert biological functions, mature TGF- β 1 must be released from LAP, a process referred to as “TGF- β 1 activation” [66] (Figure 4).

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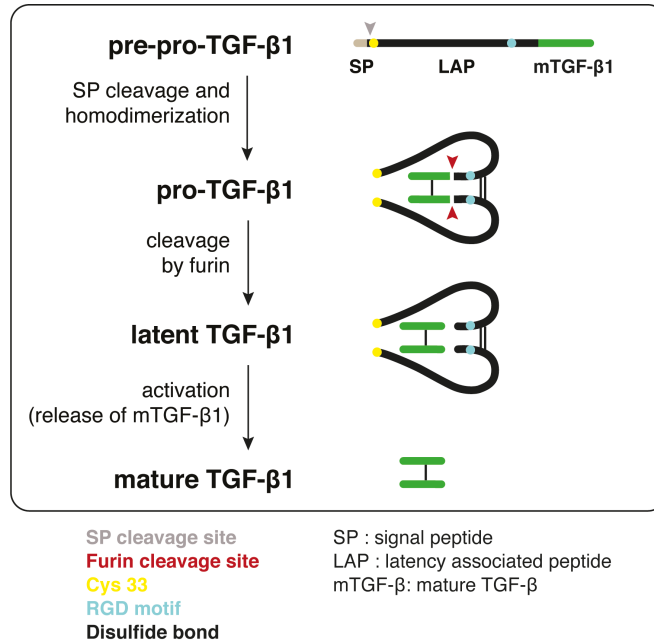


FIGURE 4. Schematic representation of TGF-β1 production (from [66]).

TGF-β1 is produced in a pre-pro-protein that homodimerizes after signal peptide (SP) cleavage to form pro-TGF-β1. Cleavage by furin leads to formation of latent TGF-β1, composed of mature TGF-β1 (mTGF-β1) non covalently associated to and embraced by the latency-associated peptide (LAP). To bind to its receptor, TGF-β1 must be released from LAP, a process called “TGF-β1 activation”.

The group of Timothy Springer solved the structure of porcine latent TGF-β1 in 2011 [67]. Each LAP monomer is composed of a rigid “arm” domain and a straitjacket domain that maintains mature TGF-β1 enclosed in LAP. Each “arm” domain contains 2 anti-parallel, four-stranded β-sheets. Each straitjacket domain is composed of an α1 helix and a long flexible loop that connects the α1 helix to the arm domain, called the latency lasso. The latency lasso encircles the tips of mature TGF-β1 which interact with the TGF-β receptor after activation of the cytokine, thus maintaining latency (Figure 5).

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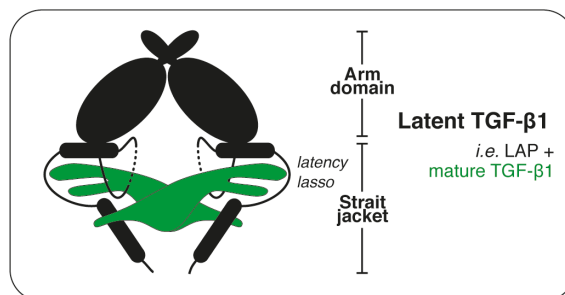


FIGURE 5. Schematic representation of TGF- β 1 structure.

Latent TGF- β 1 is composed of an “arm” domain and a “straitjacket” domain. The latency lasso connects both domains and maintains mature TGF- β 1 (green) enclosed in LAP (black).

2.1.2 Reservoirs of latent TGF- β 1

Almost all cell types can produce latent TGF- β 1, which is either secreted as a soluble form in the extracellular space or bound to other proteins thanks to formation of disulfide bonds with LAP. These latent TGF- β 1-tethering proteins include:

- **Latent TGF- β 1 binding proteins (LTBPs).** LTBP-1, -3 and -4 covalently bind to TGF- β 1 *via* formation of disulfide bonds with LAP. LTBPs do not contain a transmembrane domain. After secretion, LTBP:latent TGF- β 1 complexes tether latent TGF- β 1 in the extracellular matrix (ECM), where they bind to ECM components such as fibrillin and fibronectin [68].
- **LRRC32, more commonly called GARP.** GARP belongs to the superfamily of leucine-rich repeat (LRR) proteins. Due to the presence of such leucine repeats, GARP has a typical horseshoe-like 3D structure [69]. Contrarily to LTBPs that anchor latent TGF- β 1 in the ECM, GARP allows for the presentation of latent TGF- β 1 at the cell surface. GARP also binds covalently to TGF- β 1 *via* formation of disulfide bonds with LAP. It is the same cysteines (Cys33) in the two LAP monomers that bind to either 2 cysteines in one GARP molecule, or 2 cysteines in one LTBP molecule. Binding of latent TGF- β 1 to GARP or to LTBPs is thus mutually exclusive. GARP:latent TGF- β 1 complexes are expressed on the

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surface of TCR-stimulated Tregs [70, 71], BCR-stimulated B cells [72], endothelial cells [73, 74], fibroblasts [75], megakaryocytes (MKs) and platelets [71, 74], mesenchymal stromal cells [76, 77] and hepatic stellate cells [78]. We and others have shown that Tregs, B cells and platelets can activate TGF- β 1 in a GARP-dependent manner [70, 72, 79].

- **LRRC33.** LRRC33 is a leucine-rich repeat (LRR) protein closely related to GARP. It allows for presentation of latent TGF- β 1 on the surface of myeloid cells, which include macrophages and microglia cells (macrophage-like cells of the nervous system) [80].

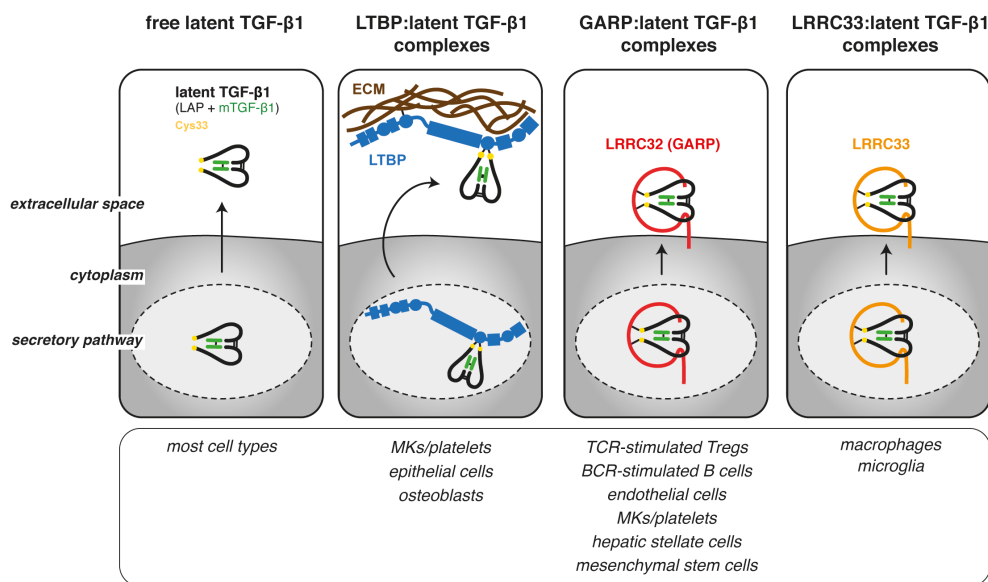


FIGURE 6. Reservoirs of latent TGF- β 1 in various cell types.

Most cell types secrete free latent TGF- β 1. In some cell types, latent TGF- β 1 is produced bound to latent TGF- β 1 binding proteins (LTBPs) that tether it to the extracellular matrix (ECM). Other cells are able to present latent TGF- β 1 at their surface *via* transmembrane proteins such as LRRC32 (GARP) or LRRC33.

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2.1.3 Activation of TGF- β 1

2.1.3.1 General considerations about known mechanisms of TGF- β 1 activation

Two major mechanisms were described for TGF- β 1 activation *in vivo*, and imply thrombospondin-1 (TSP-1) or RGD-binding integrins. These mechanisms appear to be cell-type specific, with only a few cell types able to activate latent TGF- β 1. I will discuss cell-type specific aspects of TGF- β 1 activation further below.

TSP-1 is a large homotrimeric protein that is secreted in the ECM by multiple cell types. TSP-1-dependent TGF- β 1 activation was suggested to be mediated by the non-covalent binding of a KRFK motif in TSP-1 to a LSKL motif in LAP. This interaction interferes with the association between mature TGF- β 1 and LAP and thus favors release of the cytokine and binding to its receptor [81]. *Tsp1*^{-/-} mice harbor a similar phenotype than that of *Tgfb1*^{-/-} mice, the latter dying at 3-4 weeks of age due to multifocal inflammation syndrome [82]. This confirms that TSP-1 plays a role in TGF- β 1 activation at least during development. However, symptoms of *Tsp1*^{-/-} mice are milder compared to *Tgfb1*^{-/-}, which suggests that additional, non-redundant mechanisms of *in vivo* TGF- β 1 activation exist [83].

Integrins are surface proteins that are formed as heterodimers of non-covalently associated α and β subunits. A subset of integrins are designated RGD-binding integrins because they bind to an RGD (arginine-glycine-aspartate) motif in their ligands [84]. This motif is present on the C-terminal portion of the “arm” domain of the LAP [67]. *Tgfb1*^{RGE/RGE} mice phenocopy *Tgfb1*^{-/-} mice. *Tgfb1*^{RGE/RGE} mice express a form of latent TGF- β 1 in which the aspartate of the RGD motif has been replaced by glutamate (thus becoming RGE), precluding integrin binding [85]. These observations strongly suggest that RGD-binding integrins are a major mechanism of TGF- β 1 activation. Integrins α V β 1, α V β 3, α V β 5, α V β 6 and α V β 8 were shown to bind and activate latent TGF- β 1 *in vitro*. In the context of cancer, many cell types express RGD-binding integrins. Integrin α V β 1 is expressed by fibroblasts, α V β 3 and α V β 5

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are expressed by tumor cells and endothelial cells, $\alpha V\beta 6$ by epithelial cells and $\alpha V\beta 8$ by dendritic cells, monocytes, fibroblasts, tumor cells and Tregs [86, 87].

Insights as to how RGD-binding integrins activate TGF- $\beta 1$ have been mainly deciphered for integrins $\alpha V\beta 6$ and $\alpha V\beta 8$. Integrin $\alpha V\beta 6$ -mediated TGF- $\beta 1$ activation involves anchorage of integrin $\alpha V\beta 6$ to actin/myosin filaments of the cytoskeleton by its cytoplasmic tail on the one hand, and immobilization of TGF- $\beta 1$ in the ECM by LTBP_s or at the cell surface by GARP or LRRC33 on the other. It is thought that contraction of the cytoskeleton generates pulling forces exerted by integrin $\alpha V\beta 6$ on immobilized latent TGF- $\beta 1$, which lead to unfolding of the latency lasso of LAP, with subsequent release of mature TGF- $\beta 1$, or perhaps simple unmasking of receptor binding sites without release of the cytokine in a soluble form [67, 88, 89]. The mechanism of TGF- $\beta 1$ activation *via* integrin $\alpha V\beta 8$ remains to be fully elucidated, but it is expected to differ from that of integrin $\alpha V\beta 6$. Indeed, anchorage of integrin $\alpha V\beta 8$ to the cytoskeleton does not seem to be required for this process, as deletion of the cytoplasmic tail of the $\beta 8$ subunit in human colon epithelial cells (SW480) does not impair its ability to activate TGF- $\beta 1$ [90]. Recruitment of metalloproteinases (MMP) by integrin $\alpha V\beta 8$ was reported to be required in some cell types, leading to degradation of LAP and thus TGF- $\beta 1$ activation. In other cell types such as Tregs, TGF- $\beta 1$ activation does not require MMPs [86, 91]. Recently, it was proposed that binding of integrin $\alpha V\beta 8$ to LAP induces a conformational change in LAP that provokes unmasking of binding sites of mature TGF- $\beta 1$ to its receptor, and leads to active TGF- $\beta 1$ signaling without actual release of the mature cytokine in the extracellular milieu (Figure 7a) [92].

Only very few cell types have been shown to activate latent TGF- $\beta 1$. I will describe below what is known about TGF- $\beta 1$ activation by Tregs and endothelial cells, two cell types that are at the core of the research articles presented in the “Results” section of my thesis.

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2.1.3.2 Activation of TGF- β 1 by Tregs

Regulatory T cells, or Tregs, are a subset of CD4 T cells that exert immunosuppressive functions. They are crucial for induction and maintenance of peripheral immune tolerance, but they play deleterious roles in the context of cancer by reducing anti-tumor immune responses [93]. In mice, Foxp3 expression is a specific marker of Tregs and is required for Treg differentiation and function. Foxp3 deficiency leads to the development of autoimmune-mediated multiorgan inflammation causing a phenotype called “scurfy” in mice, and the very severe IPEX (Immunodysregulation Polyendocrinopathy Enteropathy X-linked) syndrome in humans [94]. Our laboratory and other groups have shown that human and mouse Tregs are able to activate TGF- β 1 after TCR stimulation, *via* a mechanism that requires GARP and integrin α V β 8 and that does not involve MMPs. In human Tregs, GARP and α V β 8 integrin interact with each other as shown by immunoprecipitation assays. Antibodies directed against GARP or the β 8 subunit block TGF- β 1 activation by TCR-stimulated human Tregs *in vitro* and block human Treg-mediated immunosuppression in a murine model of xenogeneic graft versus host disease (GvHD) [75, 86]. More recently, we showed that targeting GARP-expressing Tregs with blocking anti-GARP:TGF- β 1 mAbs exerts anti-tumor efficacy in mice. This work [73] is described in the first research article of the “Results” section below. Resolution by X-ray crystallography of the tridimensional structure of GARP:TGF- β 1 complexes bound to the Fab (antigen-binding fragment) of a blocking anti-human GARP:TGF- β 1 mAb allowed us to better understand how such antibodies block TGF- β 1 activation by Tregs. Blocking anti-GARP:TGF- β 1 mAbs do not prevent interactions between LAP and integrin α V β 8. Rather, because they bind complex conformational epitopes comprising amino acids from GARP, LAP and mature TGF- β 1, we postulated that anti-GARP:TGF- β 1 mAbs secure the straightjacket exerted by LAP on mature TGF- β 1, preventing LAP deformation following integrin α V β 8 binding [69] (Figure 7b).

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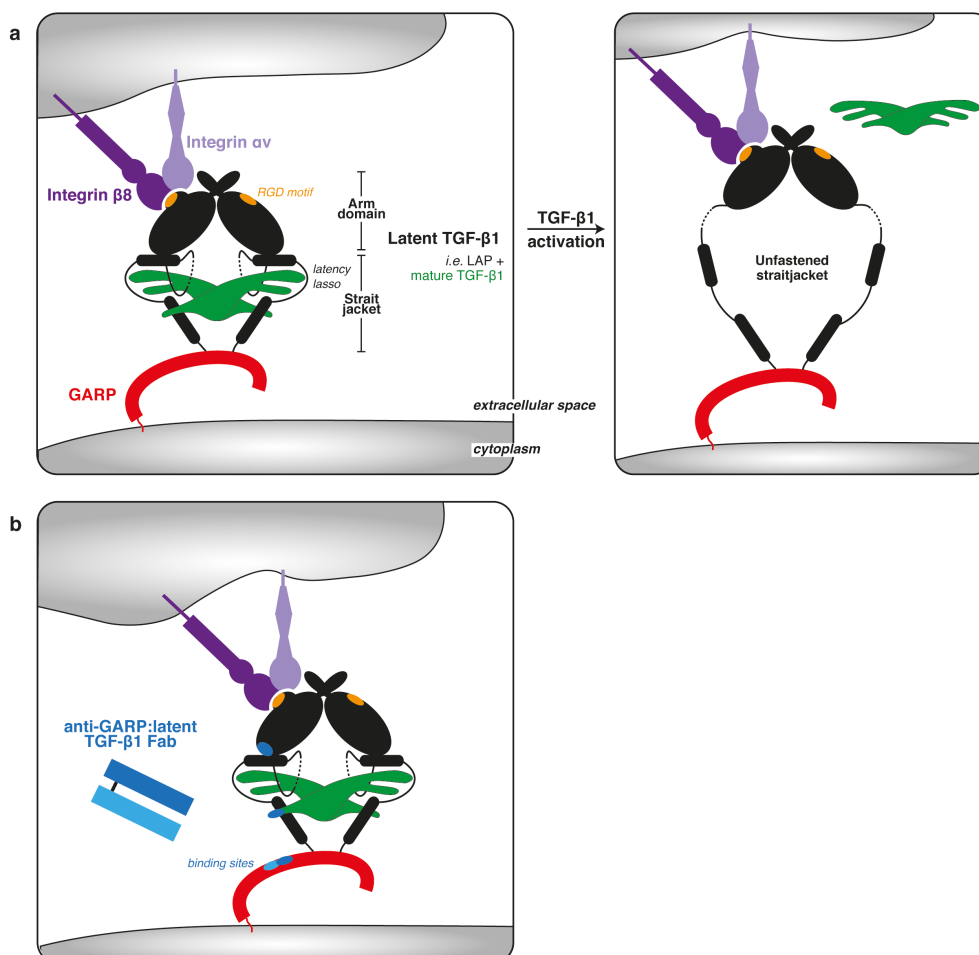


FIGURE 7. Schematic representation of TGF-β1 activation by Tregs and blockade by anti-GARP:TGF-β1 mAbs.

(a) Tregs present latent TGF-β1 at their surface *via* transmembrane protein GARP, and binding of integrin αVβ8 on the same Treg (*cis*) or expressed by another Treg (*trans*) presumably leads to a change of conformation of LAP that leads to unfastening of the straitjacket. It has been proposed that this renders mature TGF-β1 able to bind to its receptor without being released from LAP.

(b) Anti-GARP:latent TGF-β1 mAbs contact amino acids of GARP, LAP and mature TGF-β1 at the same time (blue spots), and we propose that this secures the interaction between mature TGF-β1 and LAP and prevents conformational change of the LAP.

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2.1.3.3 Activation of TGF- β 1 by endothelial cells

In vitro studies have reported that endothelial cells can produce active TGF- β 1 when they are exposed to stimuli such as proinflammatory cytokines (IL-1 β and TNF α) or hypoxia [95, 96]. In mature blood vessels, the endothelium is surrounded by mural cells, that are pericytes in capillaries, and smooth muscle cells (SMCs) in larger vessels such as arteries and veins, and that play critical roles in the regulation of blood vessel stability and permeability [97]. Other studies provide evidence that TGF- β 1 activation involves cooperation between endothelial cells and adjacent mural cells. Ding and colleagues showed that co-culture of endothelial cells and 10T1/2 cells, *i.e.* precursors for pericytes and SMCs, leads to increased differentiation of 10T1/2 measured by expression of SM22 α . Culture of 10T1/2 cells with TGF- β 1 also induced SM22 α expression and, interestingly, addition of neutralizing anti-TGF- β 1 antibody in co-culture of endothelial cells and 10T1/2 cells abolished such effect [98]. Two other publications reported similar results, showing that endothelial cell proliferation or movement, respectively, was decreased in co-culture with pericytes and SMCs and that such effect was reverted by neutralization of TGF- β 1 [99, 100]. The underlying molecular mechanism behind endothelial-pericyte cooperation to activate TGF- β 1 is still unknown, but requires contact between both cell types.

Endothelial cells express GARP:TGF- β 1 complexes in healthy tissues as well as in tumors [73, 74]. To my knowledge, no publication has investigated GARP as a potential mode of TGF- β 1 activation by endothelial cells and mural cells to date. The group of Hans Deckmyn has shown that GARP deletion specifically in endothelial cells in mice (*Tie2^{Cre} x Garp^{fl/fl}*) did not affect tail bleeding time or thrombus formation [74]. Interestingly, *Itgb8*^{-/-} and *Tgfb1*^{-/-} mice both display vascular abnormalities that lead to mid-gestation death [101]. This suggests a possible implication of integrin α V β 8-dependent activation of TGF- β 1 in the vascular compartment, at least during development.

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2.1.4 Signaling pathways of mature TGF- β 1

TGF- β 1 signaling is reviewed by Batlle and Massagué in [102]. It occurs *via* type I (also known as activin receptor-like kinases or ALK) and type II serine/threonine kinase receptors that exist as homodimers in resting state. Upon ligand binding, homodimers of type I and type II receptors interact with each other to form heterotetramers, and this leads to their auto- and transphosphorylation. Once phosphorylated, these kinases become activated and subsequently phosphorylate intracellular signaling molecules SMADs. SMAD proteins are grouped in 3 categories: receptor SMADs (R-SMADs 1, 2, 3, 5 and 8), inhibitory SMADs (I-SMADs 6 and 7) and common SMAD (co-SMAD 4). TGF- β 1 signaling occurs in most cells *via* ALK5/TGF- β RII, that triggers recruitment and phosphorylation of R-SMADs 2 and 3. Endothelial cells also express ALK1, whose signaling leads to phosphorylation of R-SMADs 1, 5 and 8. Phospho-R-SMADs then bind to co-SMAD4 and translocate to the nucleus where they can act as transcription factors through direct binding to DNA containing SMAD-binding elements (SBEs). In addition, TGF- β 1 signaling leads to expression of inhibitory SMADs that downregulate its pathway and thus establish a negative feedback loop. The SMAD pathway represents the canonical signaling pathway of TGF- β 1 (Figure 8). TGF- β receptors can also signal using non canonical pathways which include PI3K-Akt, MAPK or Rho GTPase pathways [102].

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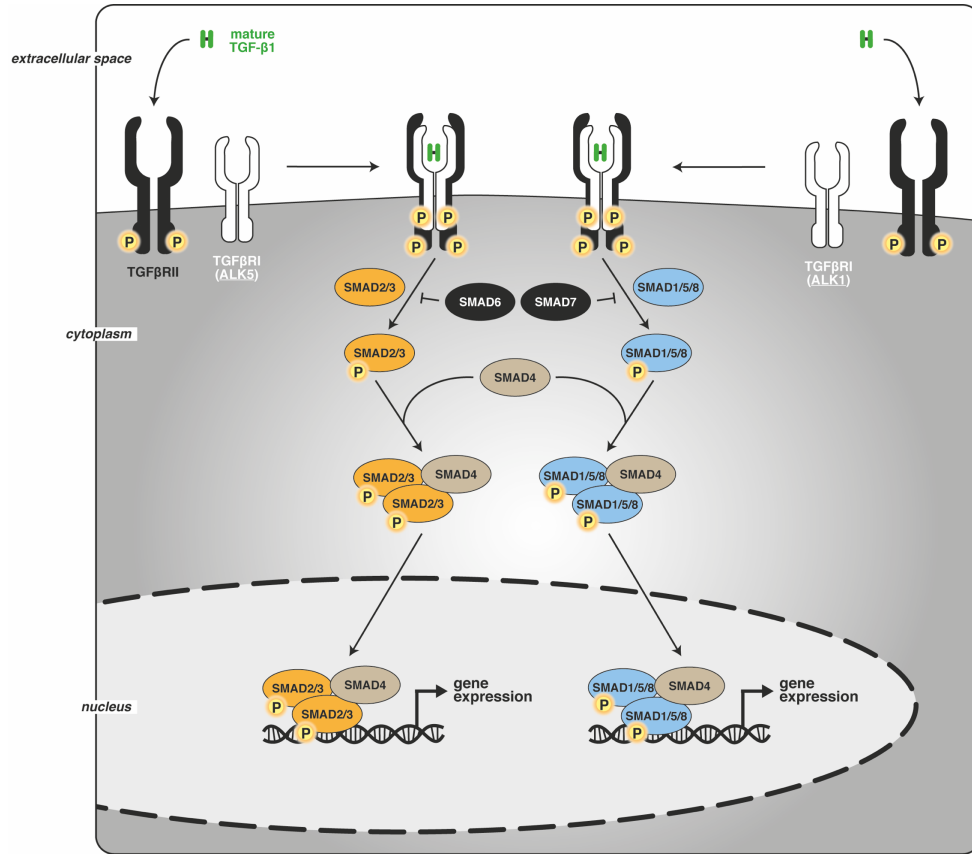


FIGURE 8. Canonical pathway of mature TGF-β1 signaling.

After binding of mature TGF-β1 to dimeric type II TGF-β receptor (TGF-βRII), heterotetramers are formed with dimeric type I TGF-β receptor (TGF-βRI) ALK5 (left) or ALK1 (right). Activated kinase of the TGF-β receptor complex phosphorylates receptor SMADs (R-SMADs) 2 and 3 or 1, 5 and 8, respectively. Phospho-R-SMADs then bind to co-SMAD4, and rapidly migrate to the nucleus to regulate gene expression.

Endoglin and betaglycan are co-receptor or type III TGF-β receptors, that modulate TGF-β signaling in various manners (reviewed in [103] and [104]). Endoglin expression is mainly restricted to endothelial cells, where it can be phosphorylated by kinases of type I and type II TGF-β receptors. Phosphorylated endoglin is involved in SMAD-independent signaling pathways regulating cell proliferation and adhesion.

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It negatively impacts ALK5 signaling, by causing the dissociation of ALK5 from complexes with TGF- β RII, and has been shown to decrease SMAD3-mediated cellular processes. Endoglin thus counteracts the TGF- β 1-mediated cytostasis. Endoglin was also described to increase ALK5/SMAD2 signaling by dampening the expression of Smurf2 (SMAD Ubiquitination Response Factor 2), an inhibitor of SMAD2, and potentiate ALK1 signaling. Transmembrane betaglycan is more ubiquitously expressed and enhances TGF- β signaling by increasing its presentation to TGF- β RII. Both endoglin and betaglycan exist in soluble forms, that decrease TGF- β 1 signaling by competing for binding to its receptor [103, 104].

2.2 Effects of TGF- β 1 on cells of the tumor microenvironment

TGF- β 1 exerts many deleterious effects in the context of cancer, by acting on multiple cells of the tumor microenvironment, and notably generates an immunosuppressive environment that supports tumor growth. This section will discuss the effects of mature TGF- β 1 on cancer cells, cells of the immune system, namely dendritic cells, effector T cells and Tregs, as well as on endothelial and mural cells. These effects are summarized in Figure 9.

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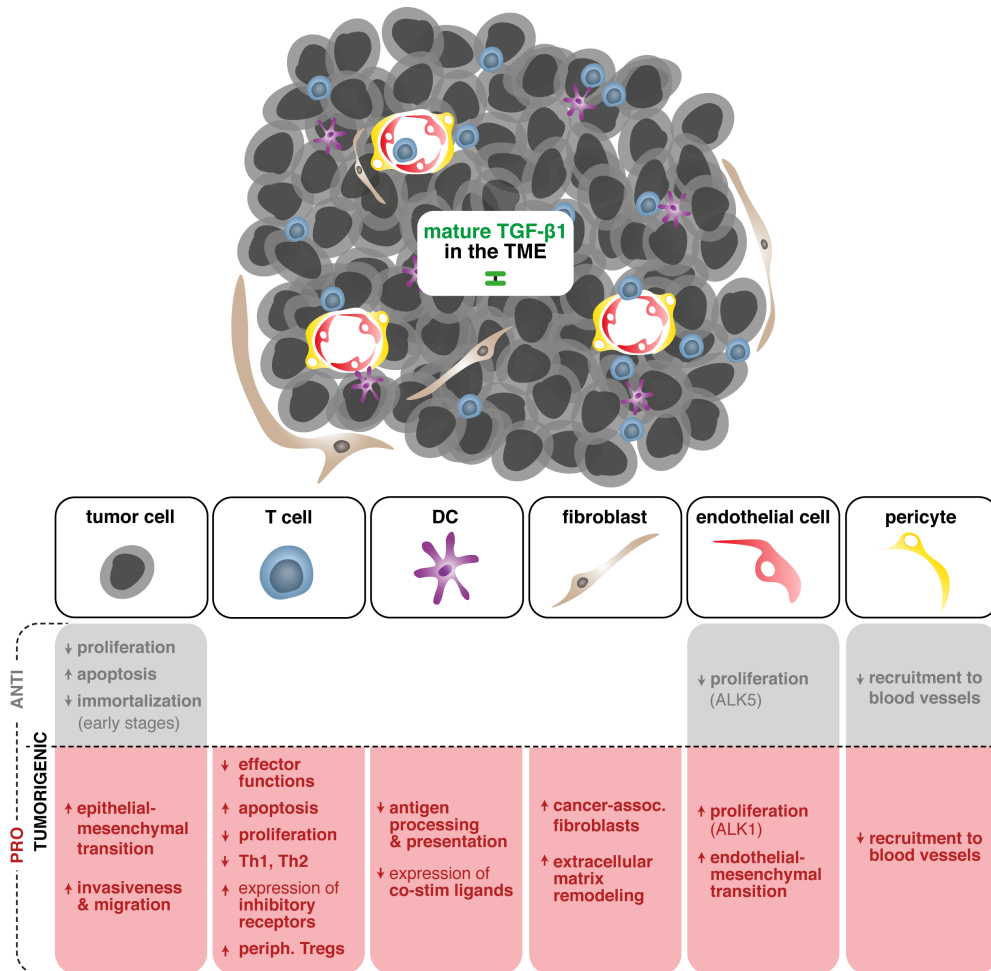


FIGURE 9. Overview of effects of mature TGF- β 1 on cells of the tumor microenvironment.

Summary of anti-tumorigenic (upper panel) and pro-tumorigenic (lower panel) of mature TGF- β 1 on cells of the tumor microenvironment (TME), namely, from left to right, tumor cells, T cells, dendritic cells (DCs), fibroblasts, endothelial cells and pericytes.

2.2.1 Effects of TGF- β 1 on cancer cells

TGF- β 1 exerts a biphasic role on tumor cells, both inhibiting or supporting tumor growth, depending on the stage of carcinogenesis.

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During early stages of cancer development, TGF- β 1 acts as a tumor suppressor. This is supported by the observation made by Böttinger and colleagues that expression of a dominant negative type II TGF- β receptor (dnT β RII) in mouse increases incidence of chemically-induced mammary and pulmonary tumors [105]. Similarly, inactivating mutation of TGF- β RII has been shown to increase the growth of human colon carcinoma HCT116 cells in nude mice [106]. TGF- β 1 signaling prevents tumor development by exerting cytostatic effects on pre-malignant cells. More precisely, it acts on three different arms. Firstly, TGF- β 1 induces cell cycle arrest by inducing expression of cyclin-dependent kinase inhibitors (CDKIs) [107]. Moreover, it exerts antiproliferative effects by dampening the expression of major growth promoting factors such as MYC. Repression of MYC further contributes to expression of CDKIs, reinforcing a negative feedback loop that tampers with cancer cell proliferation [108]. Secondly, TGF- β 1 promotes apoptosis by various molecular mechanisms that are cell-type dependent [102]. And thirdly, TGF- β 1 prevents cell immortalization by repressing the expression of human telomerase reverse transcriptase (hTERT), an enzyme whose role is to add DNA repeats at the telomeres to prevent their shortening and subsequent senescence induced by chromosome instability [109].

During cancer progression, there is a selective pressure towards tumor cells that harbor mutations in components of the TGF- β 1 pathway to abolish its cytostatic effect on tumor cells. Inactivating mutations in type II and type I TGF- β receptors have been reported in several types of human cancer, with higher frequencies for tumors with microsatellite instability due to mutations in mismatch repair genes. Mutations affecting SMAD elements, particularly of SMAD2 and SMAD4, are also common in human tumors [102]. TGF- β 1 signaling can also be reduced in human tumors as a result of increased expression of the SMAD transcriptional repressor SnoN [110] or by overexpression of inhibitory SMAD7 [111]. Activation of pro-oncogenic pathways such as MAPK, PI3K, Ras or MYC can also override TGF- β 1 signaling in cancer cells [112]. Thus, the tumor suppressor activities of TGF- β 1 are decreased, while other tumor promoting functions of TGF- β 1 prevail.

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TGF- β 1 promotes cancer invasion and metastasis by inducing epithelial-mesenchymal transition (EMT). The EMT is the biological process by which epithelial cells acquire a mesenchymal cell phenotype, leading to enhanced migratory capacities and invasiveness. TGF- β 1 upregulates the expression of EMT transcription factors Snail and Slug, ZEB1 and ZEB2, and Twist in a SMAD-dependent manner [113]. TGF- β 1-induced EMT leads to decreased cell adhesion and confers migratory and invasive properties to cancer cells. Moreover, TGF- β 1 increases the expression of MMPs, leading to remodeling of the extracellular matrix that allows cancer cells to cross the ECM barrier and reach distant metastatic sites [114].

2.2.2 Effects of TGF- β 1 on immune cells

2.2.2.1 Effects of TGF- β 1 on dendritic cells

Dendritic cells are professional antigen-presenting cells that play crucial roles in the initiation of anti-tumor immune responses because they mediate T cell priming and activation in response to tumor-associated antigens. Mice that harbor myeloid-specific deletion of TGF- β RII (*LysM^{Cre} x Tgfb^{2fl/fl}*) showed better tumor control that was linked to enhanced antigen-presenting properties and ability to stimulate proliferation of antigen-specific T cells [115]. TGF- β 1 dampens DC maturation [116], *i.e.* the process by which they migrate from peripheral tissues to adjacent lymph nodes or the spleen and during which expression of MHC molecules and co-stimulatory ligands is upregulated to yield robust antigen presentation and skew the balance towards reactivity rather than tolerance against the given antigen [1]. More precisely, TGF- β dampens DC-mediated antigen presentation in lymphoid organs and in the tumor by decreasing expression of MHC class II at their surface and by inhibiting DCs' endocytic and phagocytic functions [117-119]. Moreover, TGF- β 1 promotes the switch of DC towards a phenotype of immature myeloid cells that exert immunosuppressive effects [118].

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2.2.2.2 Effects of TGF- β 1 on effector T cells

TGF- β 1 appears to play crucial roles in T cell homeostasis, as suggested by the phenotype of *Tgfb1* KO mice that die at the age of 3-4 weeks due to early-onset multifocal inflammatory disorder. This phenotype was shown by Diebold and colleagues to be lymphocyte-mediated because deletion of *Tgfb1* in SCID (Severe Combined ImmunoDeficiency) mice, that lack mature B and T cells, did not lead to the development of such inflammatory disorder [82]. Moreover, complete ablation of TGF- β 1 signaling in T cells (*Cd4^{Cre} x Tgfb^{2fl/fl}*) phenocopied *Tgfb1^{-/-}* mice [120]. Use of a dominant negative (dnT β RII) or a soluble (sT β RII) form of TGF- β RII leads to milder inflammatory phenotype in mice and thus allowed for the study of the impact that TGF- β 1 can have on effector T cells.

In tumors, TGF- β 1 can dampen anti-tumor T cell responses in diverse ways. Notably, it has been shown to inhibit expression of the cytotoxic apparatus of CD8 T cells, like perforin and granzyme B, as well as the secretion of pro-inflammatory cytokines such as IFN γ and TNF α . Indeed, TGF- β RII-deficient CD8 T cells produce higher amounts of effector function molecules. The group of James Allison showed that dnT β RII mice had decreased levels of SMAD2 and SMAD3 phosphorylation in T lymphocytes located in lymph nodes draining TRAMP (Transgenic Adenocarcinoma of Mouse Prostate) tumors. These mice were significantly protected from tumor development, thanks to higher density of tumor-infiltrating T cells and higher expression of *Irfng* and *Gzmb* by T cells in the draining lymph nodes [121]. In addition, TGF- β 1 tampers with TCR signaling and with IL-2 expression and secretion, thereby decreasing T cell proliferation [122, 123]. It can also induce T cell apoptosis by enhancing expression of Fas ligand (FasL) [122]. As far as CD4 T cells are concerned, TGF- β 1 has been described to decrease their differentiation into Th1 and Th2 phenotypes by downregulating transcription factors T-bet and GATA-3, respectively, and to favor a Th17 transcription program. Lastly, TGF- β 1 induces expression of inhibitory receptors on T cells, such as PD-1 [10].

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Thus, in the context of cancer, TGF- β 1 has been shown to inhibit T cell growth, promote T cell death and favor T cell dysfunctionality by dampening effector functions and increasing expression of inhibitory receptors.

2.2.2.3 Effects of TGF- β 1 on Tregs

In peripheral tissues, TGF- β 1 promotes differentiation of naïve T cells into Tregs (referred to as peripheral Tregs or pTregs, as opposed to thymic Tregs or tTregs) by upregulating the transcription of Foxp3 [124]. During T cell priming and activation by DCs, the ratio of effector and regulatory T cells is crucial to determine the reactivity against the antigen [1]. This cytokine could thus contribute to the induction of partial tolerance against some tumor antigens, by skewing the balance towards a higher proportion of Tregs. Multiple mechanisms have been described for Treg-mediated immunosuppression, and our laboratory postulated that production of active TGF- β 1 constitutes a major mean for Tregs to suppress T cell responses, especially in the context of cancer. Chen *et al* confirmed this by showing that the cytolytic functions of tumor-specific CD8 T cells was decreased by Tregs in CT26 tumor-draining lymph nodes and that this was linked to active TGF- β 1 production because expression of dnT β RII by tumor-specific CD8 T cells abolished such immunosuppression [125].

2.2.3 Effects of TGF- β 1 on fibroblasts

Cancer-associated fibroblasts (CAFs) are highly proliferative and express higher levels of proteases and proteins of the extracellular matrix compared to normal fibroblasts. CAFs are thought to be an important contributor of cancer invasion and metastasis, by providing a scaffold of ECM proteins for tumor cells to track along and a structural support for vessels [126]. TGF- β 1 signaling, whose pro-fibrotic properties have been extensively described in other pathologies [127], leads to desmoplasia, *i.e.* growth of fibrous or connective tissue, in tumors by inducing a reactive phenotype in CAFs. More precisely, a study performed by Casey and colleagues showed that TGF- β 1 increased the percentage of myofibroblasts *in vitro* in a culture of fibroblasts isolated from patients bearing invasive breast cancer but not in a culture of fibroblasts isolated from healthy patients [126]. TGF- β 1 can act

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directly on fibroblasts, but also induce the transition of various cell types into myofibroblast-like cells by increasing expression of α -smooth muscle actin (SMA). Myofibroblasts are considered as key effector cells in fibrotic states. TGF- β 1 stimulates synthesis of type I, III and IV collagens by myofibroblasts. Additionally, TGF- β 1 exerts functions in matrix synthesis and preservation. For instance, it can augment collagen stability *via* post-translational modifications that enhance its cross-linking. TGF- β 1 also induces expression of protease inhibitors such as PAI-1 (Plasminogen Inhibitor-1), which will decrease extracellular matrix degradation [128]. Excessive deposition of components of the extracellular matrix and stabilization of the latter as a result of TGF- β 1 production creates abnormal tissue architecture, and leads to altered organ function by mechanisms that are still poorly understood.

Two papers suggest that TGF- β 1 could restrict penetration of immune cells in the tumor bed. Tumors are generally grouped in three categories based on their phenotype of immune infiltration: immune inflamed or “hot” (characterized by high density of immune cells that are located inside the tumor bed), immune desert or “cold” (characterized by absence or very low density of immune cells) and immune excluded (characterized by confinement of immune cells at the border of the tumor) [129]. Studies published by Mariathasan *et al* and Tauriello *et al* suggest that TGF- β 1 contribute to the establishment of the immune excluded phenotype, by supporting the formation of a fibroblastic barrier at the border of the tumor. They have shown that a pan-fibroblast TGF- β 1 response signature (F-TBRS) was associated with lack of response to atezolizumab and reduced overall survival in patients with metastatic urothelial cancer. In a mouse model of mammary carcinoma (EMT6), Mariathasan and colleagues have shown that TGF- β blockade potentiates the effect of anti-PD-L1 mAb thanks to optimal positioning of T cells inside the tumor bed. This was correlated with a reduction of the F-TBRS signature, but not of TGF- β response signature associated with immune cells, suggesting that TGF- β blockade leads to reprogramming of peritumoral stromal fibroblasts [62]. In a mouse model that closely resembles that of advanced human colorectal cancer, Tauriello and colleagues

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showed that immune exclusion was associated with higher levels of TGF- β 1 signaling in the stromal margin compared to other regions of the tumor [63].

2.2.4 Effects of TGF- β 1 on endothelial cells and pericytes

TGF- β 1 plays crucial roles in regulating endothelial cell plasticity, as mice that harbor a global deletion of TGF- β 1 or of TGF- β 1 signaling elements (ALK1 KO, ALK5 KO, TGF- β RII KO, endoglin KO) die at the embryonic stage due to abnormal formation of the primitive vascular network that exhibit hyper-dilatation and leakiness. Knock-outs of ALK5 or TGF- β RII specifically in endothelial cells (*Alk1^{Cre} x Alk5^{fl/fl}* or *Tgfb^{fl/fl}*, respectively) phenocopied mice lacking receptors in all cells, harboring defective yolk sac vasculogenesis and intracerebral hemorrhage. This shows that endothelial cells are the primary target of TGF- β 1 at least during embryogenesis [130-133]. In humans, several cardiovascular disorders have been linked to alterations of TGF- β 1 signaling. For instance, hereditary hemorrhagic telangiectasia (HHT), which is characterized by the development of mucocutaneous telangiectasias (*i.e.* dilatation of tiny blood vessels) and arteriovenous malformations in diverse organs, has been linked to mutations in endoglin, ALK1 and SMAD4 [130].

Effects of TGF- β 1 on blood vessels are dual, both promoting and inhibiting angiogenesis (reviewed in [130] and [134]). The nature of TGF- β 1 effect is thought to be conditioned by the balance between signaling *via* ALK1 and ALK5, regulating the activation state of endothelial cells. On the one hand, during the activation phase of angiogenesis, endothelial cell sprouting is favored by ALK1 signaling that stimulates endothelial cell migration, proliferation and tube formation *via* upregulation of *Id1*. TGF- β 1 has also been proposed to induce angiogenic sprouting by promoting endothelial-mesenchymal transition (EndoMT), a process that mainly takes place during embryonic development and by which endothelial cells acquire fibroblastic characteristics and experience loss of adhesion and tight junction molecules. Although it remains to be fully elucidated, reversible EndoMT could favor angiogenic sprouting because loss of cell-cell contact is necessary for endothelial cells to detach from existing vessels [130, 134]. EndoMT also increases blood vessel

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permeability. On the other hand, ALK5 signaling inhibits proliferation of endothelial cells during the resolution phase of angiogenesis. Expression of fibronectin and PAI-1 are increased by ALK5 signaling, and this decreases endothelial migration but increases vessel maturation by stabilizing interactions between endothelial cells and surrounding mural cells. Depending on the context, TGF- β 1 can thus exert both pro- and anti-angiogenic functions, that are also dependent on its concentration (high doses decrease proliferation of endothelial cells and low doses enhance their proliferation) [130, 134, 135]. Interestingly however, ALK1 and ALK5 signaling are not mutually exclusive in endothelial cells, as they can also physically interact with each other. Indeed, ALK5 is required for proper ALK1 signaling, because it stimulates the recruitment of ALK1 in the TGF- β receptor complex and the kinase activity of ALK5 is necessary for maximal ALK1 activation. Moreover, ALK1 can antagonize SMAD2/3, representative of ALK5 signaling. TGF- β -mediated endothelial plasticity could thus be fine-tuned by a crosstalk between ALK1 and ALK5 [130].

Proliferating endothelial cells predominantly express TGF- β co-receptor endoglin (as compared to betaglycan) and its expression is highly upregulated in tumor-associated endothelial cells. Endoglin expression is used as a marker of tumor angiogenesis and poor prognosis in cancer patients. Immunoblockade of endoglin has been successfully used as an anti-angiogenic therapy in human tumors transplanted to mice [130, 134].

Contradictory studies have been published concerning the effect of TGF- β 1 on mural cells. For instance, Zonneville and colleagues show that inactivation of TGF- β 1 signaling in breast tumor cells *via* expression of a kinase-inactive TGF- β 1 mutant receptor and subsequent implantation in mice led to decreased association between endothelial cells and pericytes, whilst expression of constitutively active mutant receptor increased the pericyte coverage of blood vessels. They postulate that production of TGF- β 1 by CAFs increases levels of fibronectin, that is a potent inducer of interactions between endothelium and mural cells [136]. Another study showed that TGF- β 1 impairs SMC migration and recruitment to the endothelium in an *in vivo*

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model of angiogenesis where SMC-containing Matrigel is injected subcutaneously to SCID mice [137]. Thus, effect of TGF- β 1 on mural cells is also dual, as it has been shown to both positively and negatively regulate recruitment of mural cells to blood vessels. As for endothelial cells, dual functions of TGF- β 1 on mural cells is representative of differential signaling *via* ALK1 and ALK5. On the one hand, ALK5 signaling promotes vessel maturation by increasing the production of ECM proteins and by inducing differentiation of mesenchymal cells to mural cells. Moreover, PAI-1, induced by ALK5, prevents degradation of the vessel-supporting matrix. On the other hand, ALK1 promotes vessel destabilization to favor sprouting [97, 134].

Thus, the outcome of signaling by TGF- β 1 in endothelial cells and mural cells is highly context-dependent and it seems difficult to decipher a common function of TGF- β 1 in the context of cancer, because there is a lot of variability between cancer types and even in patients bearing the same cancer type. This is well illustrated by the presence of studies showing opposite effects of TGF- β 1 on angiogenesis and vessel maturation in similar murine tumor models such as breast carcinoma [136, 138].

2.3 Overview of existing TGF- β 1 blockade therapies

The diverse pro-tumorigenic effects that TGF- β 1 exerts on cells of the tumor microenvironment support its possible contribution to the mechanisms of resistance to PD-1/PD-L1 blockade. For this reason, TGF- β 1 has become a coveted target in order to increase the efficacy of ICB therapies. A wealth of publications has reported that TGF- β 1 blockade can induce rejection of tumors otherwise unresponsive to PD-1/PD-L1 blockade in mouse models. In the clinics, progress in this direction are slower because of concerns that blocking the cytostatic effect that TGF- β 1 exerts on tumor cells at early stages of carcinogenesis could alleviate efficacy of such therapy. Moreover, severe toxicities could arise from the blockade of TGF- β 1 signaling in cells outside of the tumor. So far, the main adverse effect reported for TGF- β 1 blockade in cancer patients is the reversible development of skin lesions such as hyperkeratosis and eruptive keratoacanthomas [139].

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Strategies used to block TGF- β in human and/or mouse tumors are shown in Figure 10 and comprise:

- ***Inhibitors of TGF- β RI kinase activity***, such as LY364947 and galunisertib. Clinical trials revealed that the use of galunisertib led to increased objective response rate and survival when used alone in glioblastoma and when combined to chemotherapy in pancreatic cancer [140, 141]. It is currently being tested in combination with anti-PD-1 mAbs. Galunisertib has been shown to increase survival of tumor-bearing mice and prevent metastasis formation. Combination with an anti-PD-L1 mAb further potentiates this therapeutic efficacy [63].
- ***TGF- β RII traps***, composed of Fc-coupled versions of the extracellular (EC) domain of TGF- β RII. They thus act as traps by capturing circulating mature TGF- β 1 and preventing binding to its receptor. TGF- β RII trap has been shown to inhibit metastases formation from 4T1 and EMT6 mammary tumors in mouse [142] and is now being tested in early phases of clinical trials. Other studies have highlighted the therapeutic potential of a fusion between an antibody against immune checkpoints and the extracellular domain of TGF- β RII. Notably, anti-PD-L1 mAb (avelumab) is fused to the EC domain of TGF- β RII in a trap called M7824. Lan and colleagues have shown that administration of M7824 suppressed tumor growth and metastasis more efficiently compared to treatment with anti-PD-L1 or TGF- β RII trap alone [143]. Similar results were shown for fusion protein of TGF- β RII EC domain and anti-CTLA-4 mAb as well as two versions of anti-PD-L1 mAb (avelumab and atezolizumab). This time, efficacy was observed on NSG mice that were immune-reconstituted and transplanted with A375 human melanoma cells [144]. M7824 has been tested in a phase I clinical trial on NSCLC patients, and showed encouraging results as 86% of patients harboring high proportion of PD-L1⁺ tumor cells responded to the treatment, whereby the objective response rate to PD-1 blockade in the same category of patients ranged from 29 to 44% in other non-comparative studies [145].
- ***Antibodies directed against mature TGF- β 1, 2 and 3***, such as fresolizumab (monoclonal human IgG4) and 1D11 (monoclonal mouse IgG1). Studies

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performed in mouse tumor models have shown that the administration of 1D11 is not sufficient to induce tumor rejections but that it can overcome resistance to PD-1/PD-L1 blockade [62, 117]. As far as humans are concerned, although a phase II clinical trial has been launched for testing of fresolizumab in malignant pleural mesothelioma patients, no efficacy report was published because the trial was interrupted.

- ***Antibodies directed against TGF- β RII.*** Phase I clinical trial on 14 patients with advanced solid tumors did not show generation of objective response for LY3022859 (monoclonal human IgG1) treatment, probably linked with very short half-life of this antibody [146].
- ***Antibodies directed against latent TGF- β 1.*** Martin *et al* have recently developed an antibody targeting human and mouse latent TGF- β 1 (but not - β 2 or - β 3), called SRK-181. Using crystallographic structure of SRK-181:latent TGF- β 1 complexes, they showed that this antibody binds to LAP and not the mature cytokine, and proposed that it blocks integrin-dependent activation of TGF- β 1 because it binds to the latency lasso of LAP that is thought to be loosened by integrin-mediated pulling forces. Using 3 different murine tumor models, they showed that SRK-181 induces tumor rejections when co-injected with an anti-PD-1 antibody [65].
- ***Antibodies directed against GARP:TGF- β 1 complexes.*** Our group has developed antibodies that specifically target GARP:latent TGF- β 1 complexes (as opposed to free GARP), and not GARP:TGF- β 2 or β 3 complexes. We have recently shown that anti-GARP:TGF- β 1 mAbs increase the number of tumor rejections in 2 murine tumor models (CT26 and MC38) when combined with PD-1 blockade. Using mice that harbor Treg-restricted deletion of GARP (*Foxp3^{Cre} x Garp^{fl/fl}*), we have shown that the presence of GARP⁺ Tregs is indispensable for the anti-tumor efficacy of the combined therapy. In CT26 tumors, combined treatment does not lead to increase in density of T cells, but rather to an enhancement of effector functions of T cells already infiltrating the tumor. These results were recently published and constitute the first part of my thesis' results

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[73]. In the second part of my thesis, I could show that combined anti-GARP:TGF- β 1 and anti-PD-1 treatment mediates different changes in the TME of MC38 tumors compared to that of CT26 tumors. Indeed, combined therapy increase the infiltration of T cells, which include anti-tumor activated T cells, in MC38 tumors and we propose that this is linked with increased density of normalized GARP+ vessels. Phase I clinical trial testing the use of these antibodies in oncology was initiated in 2019.

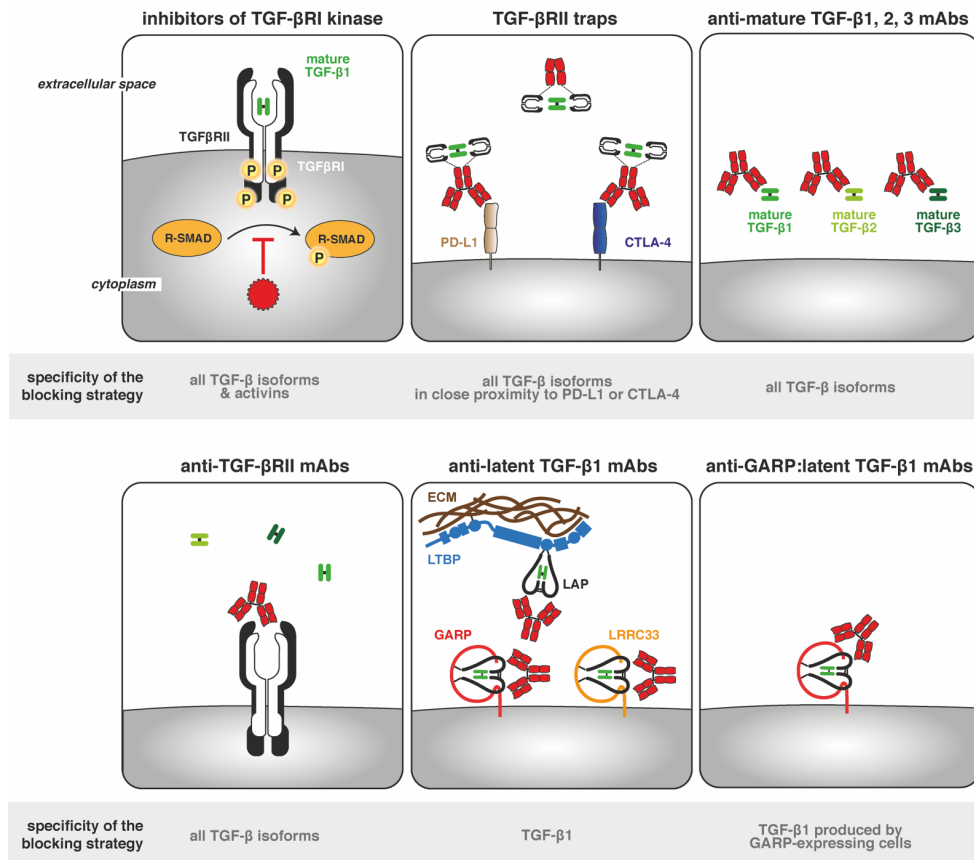


FIGURE 10. Overview of existing strategies to block TGF- β 1 in tumors.

Strategies to block TGF- β 1 in human and/or mouse tumors are depicted in each box. In the upper panel, from left to right: inhibitors of type I TGF- β receptor (TGF- β RI) kinase activity, traps composed of the extracellular domain of type II TGF- β receptor (TGF- β RII) alone or combined with antibodies directed against PD-L1 or CTLA-4 and

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antibodies directed against the 3 isoforms of mature TGF- β . In the lower panel, from left to right: antibodies directed against TGF- β RII, antibodies targeting LAP-1, tethered to LTBP or bound to surface proteins, and antibodies against GARP:latent TGF- β 1 complexes. Specificity of each strategy is indicated in grey, below each box.

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3 Cancer immunotherapy by blocking PD-1/PD-L1 and TGF- β 1 pathways: focus on 3 steps of the cancer-immunity cycle

The Cancer-Immunity Cycle refers to the stepwise events that the immune system must follow in order to properly eliminate the tumor. Figure 11 represents the 7 steps of the Cancer-Immunity Cycle [1]. Briefly, cancer cell death leads to release of tumor-specific antigens (step 1), that are captured and processed by APCs such as DCs. Antigen-presenting cells then present the antigen on MHC molecules to T cells (step 2), leading to priming and activation of anti-tumor effector T cells (step 3). Next, activated T cells traffic to (step 4) and infiltrate the tumor *via* vessels (step 5). Once inside the tumor bed, cytotoxic T cells recognize (step 6) and kill (step 7) cancer cells, and this results in the release of additional tumor antigens leading to subsequent revolutions of the cycle that increase anti-tumor immunity [1]. Obviously, this scenario is idealistic and does not perform optimally in patients whose tumor continue to grow. In the following sections, I will discuss the factors that can favor tumor growth focusing on 3 crucial pivot points of the Cancer-Immunity Cycle, that are priming and activation of T cells (step 3), T cell trafficking and infiltration in the tumor bed (steps 4-5) and cancer cell recognition and killing by T cells (steps 6-7). As my thesis focuses on the combined blockades of PD-1 and GARP:TGF- β 1, I will also illustrate how PD-1/PD-L1 blockade and TGF- β blockade have been shown to act on these steps to create a self-sustaining cycle of cancer immunity. These effects are also summarized in Figure 11.

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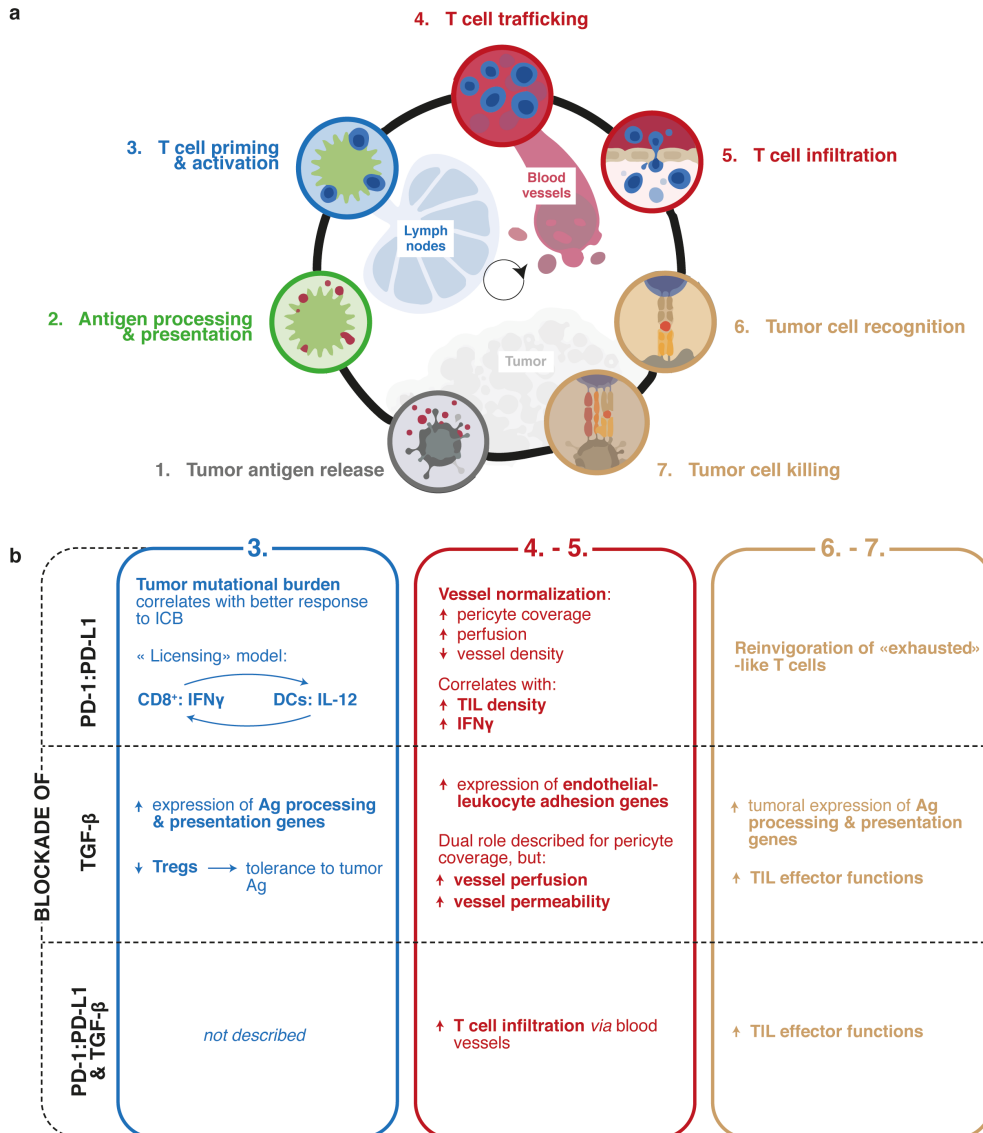


FIGURE 11. Cancer-Immunity Cycle (modified from [1]) **and overview of effects of PD-1/PD-L1 and/or TGF- β blockade**

(a) Schematic representation of the Cancer-Immunity Cycle and **(b)** how PD-1/PD-L1 and/or TGF- β blockade have been shown to act on, from left to right, T cell priming and activation (3, blue), T cell trafficking and infiltration (4 – 5, red) and tumor cell recognition and killing (6 – 7, light brown).

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3.1 Priming and activation of T cells

The Cancer-Immunity cycle is initiated by the release of tumor-associated antigens following destruction of cancer cells. Such antigens are taken up by APCs, which then migrate to the draining lymph node, where they process and present them to T cells *via* MHC molecules. The nature of the immune response is determined at this stage, based on thymic induction of tolerance for self-antigens. Tumoral antigens have been regrouped into 5 categories, that yield different magnitude of immunogenicity, and that are reviewed in [147]. Antigens encoded by genes that are overexpressed in the context of cancer (“overexpressed antigens”) and antigens encoded by genes involved in cell differentiation (“differentiation antigens”) lead to less potent immune responses because they lack tumoral specificity, as they are also expressed in healthy tissues, and tolerance against such antigens was induced in the thymus. Tumor antigens that yield immune responses of higher quality are cancer-germline antigens (expressed only in cancer cells thanks to demethylation of regulatory sequences), viral antigens (expressed in the context of virally-induced tumors and thus absent from normal cells), and mutated antigens or “neoepitopes” that are generally generated by SNP (single-nucleotide polymorphism) mutations [147]. These 3 categories of antigens are present only on tumor cells, and not on normal cells, which allows T cells to target tumor cells more specifically.

Primary resistance to immune checkpoint blockers such as anti-PD-1 or anti-PD-L1 mAbs can thus be explained by a lack of immunogenic tumor antigens. Accordingly, the number of non-synonymous nucleotide variant (nsSNV) mutations in the tumor genome, referred to as tumor mutational burden (TMB), has been proposed by several as a biomarker to predict response to ICB therapies. Based on extensive search of the literature, Yarchoan, Hopkins and Jaffee plotted the objective response rate for anti-PD-1 or anti-PD-L1 therapy against the corresponding median TMB across 27 tumor types [148]. They observed a significant correlation between TMB and the objective response rate to PD-1/PD-L1 blockade. Nevertheless, this analysis revealed some outliers such as Merkel-cell carcinoma that has high response rate but modest number of mutations [148]. TMB was also correlated with higher overall

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survival following ICB within a given cancer type, as it was shown for melanoma by Van Allen *et al* and Snyder *et al* [149, 150] and for NSCLC by Rizvi and colleagues [151]. However, TMB is not a perfect predictor of response to ICB therapies, as some patients with low TMB respond to ICB and some patients with high TMB do not. The latter observation could be explained by the fact that only up to 5% of mutations give rise to an antigen that can be processed, presented on MHC molecules and recognized by T cells [152]. Cancers develop mechanisms that allow them to escape the immune system and that can lead to secondary resistance to ICB therapies. Escape mechanisms notably comprise elimination of tumor cell clones that express highly immunogenic antigens towards selection of those that express less immunogenic antigens [116].

There has been a lot of speculation as to whether PD-1/PD-L1 blockade could act on priming of T cells by antigen-presenting cells such as DCs, because they respectively express PD-1 and PD-L1. The importance of a crosstalk between T cells and dendritic cells, for response to PD-1 blockade has been established by Garriss and colleagues in murine MC38 tumors. The authors proposed a “licensing” model, in which anti-PD-1 mAb would activate T cells in a more indirect manner. Following binding of anti-PD-1 mAb on CD8 T lymphocytes, IFN γ production is increased. Upon IFN γ sensing, DCs produce IL-12 which reinforces the activation and expansion of anti-tumor T cells. This interplay takes place in the tumor bed, and not in the draining lymph node [153].

For antigen presentation to yield a robust anti-tumor response, it must be accompanied by additional immunogenic signals that skew the balance towards reactivity rather than tolerance against the given antigen. Co-stimulatory signaling and secretion of pro-inflammatory cytokines reinforce T cell activation during the priming process [1]. As previously mentioned, expression of MHC molecules and co-stimulatory ligands by DCs, that is induced during DC maturation, is dampened by TGF- β [116, 117]. During T cell priming and activation, the ratio of effector and regulatory T cells is crucial to determine the reactivity against the antigen [1]. As TGF- β promotes the generation of peripheral Tregs from naïve T cells [118], it could

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thus contribute to the induction of partial tolerance against some tumor antigens. Such mechanisms can lead to suboptimal T cell activation, therefore explaining resistance to ICB therapies to some extent. TGF- β 1 blockade could improve the efficacy of immune checkpoint blockers by improving antigen presentation by professional APCs.

3.2 T cell trafficking and infiltration in tumors

Once T cells have been activated in the lymph node, they travel through the blood stream to reach and infiltrate the tumor. Blood vessels have been suggested to be an important physical barrier restricting immune cell infiltration in tumors, thereby generating the immune desert or immune excluded phenotypes described in a previous section [65]. Indeed, intratumoral blood vessels are structurally abnormal as they harbor loose cellular junctions, high number of fenestrations and discontinuous basement membrane. Coverage of vessels by pericytes (mural cells that surround the endothelial cells to ensure stability of blood vessel structure and regulate vascular permeability) is also decreased in tumors [154]. This creates poor blood vessel perfusion, that leads to dysfunctional flow characteristics and that generally limit tumor infiltration of effector T cells [155].

3.2.1 Mechanism of leukocyte recruitment and diapedesis

Leukocyte transmigration or diapedesis is the physiological phenomenon that allows immune cells to breach the inner line of blood vessels, composed of endothelial cells. Endothelial cells provide guidance for leukocytes to travel from lymphoid organs to places where their afflux is necessary, for instance to sites of inflammation. Although the endothelium can drive diapedesis in a cell type-specific manner, the global mechanism is fundamentally the same for all immune cells and includes rolling, crawling, firm adhesion, arrest, docking structure formation and trans-endothelial migration (reviewed in [156]). During this process, pro-inflammatory cytokines locally induce the expression of adhesion molecules at the surface of the endothelium. Binding of endothelial selectins (E- and P-selectins) to its leukocyte ligand PSGL-1 (P-Selectin Glycoprotein Ligand 1) promotes the recruitment of leukocytes to the site

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of inflammation, by inducing a slow-rolling behavior. Chemokines such as CXCL9 and CXCL10, presented by the endothelium through binding with heparan sulfate, bind to chemokine receptors on leukocytes (CXCR3 in this case), which increases the expression of integrin subsets like LFA-1 (Lymphocyte Function-associated Antigen 1) on leukocytes. This leads to stable adhesion to endothelial cells *via* binding to CAMs (Cytokine-induced Adhesion Molecules) such as ICAM-1 and VCAM-1, thus supporting crawling, firm adhesion and arrest of leukocytes on endothelial cells. Finally, transmigration can occur paracellularly (between two adjacent endothelial cells) or transcellularly (through the cell body of an individual endothelial cell) [156].

3.2.2 How tumors escape the immune system due to blood vessel properties

Evasion of tumor cells from the immune system can occur through inhibition of cytotoxic T cell recruitment, mediated by diverse mechanisms that include tampering with expression of endothelial-leukocyte adhesion molecules, of chemokines or chemokine receptors and induction of immune inhibitory molecules at the surface of endothelial cells. This section was mainly inspired by review [156].

Firstly, as a result of genetic instability of tumor endothelial cells, expression of molecules involved in leukocyte homing such as ICAM-1 can be decreased, as shown by Lambrechts and colleagues in lung cancer [157]. Inherently to its abnormal structure and lack of functionality, the tumor vasculature creates areas of hypoxia, that leads to the expression of HIFs (Hypoxia-Inducible Factors), further leading to expression of pro-angiogenic factors such as VEGF-A. This creates a vicious cycle because formation of new aberrant tumor vasculature further increases hypoxia in the tumor bed. Angiogenic factors dampen expression of CAMs [158, 159]. Moreover, hypoxia-inducible and pro-angiogenic factors lead to selective immune trafficking that favors entry of immunosuppressive immune cells such as Tregs, monocyte-derived suppressive cells (MDSCs) or tumor-associated macrophages (TAMs) and dampens trafficking of CTLs. Notably, VEGF, bFGF and Egfl7 increase the expression of endothelin B receptor (ETBR) which has been associated with

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lower numbers of CD8 T cells and higher infiltration by Tregs in glioblastoma [156, 160]. Similarly, VEGF-A upregulates Stabilin-1 (or Clever-1) that repels cytotoxic T cells and allows entry of Tregs in the tumor bed [156]. Binding of VEGF to Treg-expressed Neuropilin-1 also favors their homing in tumors [161].

Secondly, chemokine signaling can be tampered with. VEGF-A decreases the levels of chemokines CXCL9, 10 and 11 that are crucial for specific recruitment of CD8 T cells. Tumor blood vessels can also express chemokine decoy receptors, that are generally described as “silent” receptors because they are unable to transmit signals that lead to cell chemoattraction. For example, the chemokine decoy receptor DARC expressed on tumor endothelium blocks recruitment of CD8 T cells by interfering with CCL2 and CCL5 signaling. Nitrosylation of chemokine receptors can also play a role in blocking CD8 T cell trafficking to tumors. CCL2 can be nitrosylated by tumor cells following exposure to reactive nitrogen species, which impairs cytotoxic T cells recruitment and favors attraction of MDSCs. Moreover, in breast cancer, Tregs exposed to hypoxia have increased levels of CXCR4 at their surface. This favors their entry into the tumor bed *via* chemokine CXCL12 [156].

Thirdly, immunosuppressive barriers are increased by hypoxia and pro-angiogenic factors. HIF1 α induces T cell anergy by increasing the expression of PD-L1 on MDSCs, tumor cells, DCs and macrophages. As a result of VEGF-A signaling, tumoral endothelium expresses higher levels of PD-L1 and PD-L2, which can mediate a loss of T cell function *via* PD-1 engagement. VEGF also increases the expression of FasL on tumor endothelial cells themselves, which has been suggested to increase T cell apoptosis [156].

3.2.3 How PD-1/PD-L1 and/or TGF- β 1 blockade act on tumor vasculature

Several studies have evidenced that immune checkpoint blockade therapies can act on tumor vasculature in cancer patients or in murine tumor models. More precisely, CTLA-4 and PD-1/PD-L1 blockade have been shown to induce vascular normalization [154, 155, 162], a process by which the tumor vessels are remodeled to increase their stability and functionality, in such a way that they more closely

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resemble vessels of healthy tissues. Decreased vessel density, increased pericyte coverage, vessel perfusion and expression of endothelial adhesion molecules are often used as hallmarks of vascular normalization.

Zheng *et al* have shown that vascular normalization can be used as a biomarker of response to ICB. Anti-CTLA-4 mAb enhanced pericyte coverage and vessel perfusion only in treatment-sensitive murine EO771 and MMTV-PyVT breast tumors and CT26 colorectal tumors. Anti-PD-1 mAb exerts similar effects in murine MC38 colorectal tumors. Hallmarks of vessel normalization were correlated with increased density of CTLs inside the tumor and with increased expression of *Ifng*. Depletion of CD8 T cells, but not of CD4 T cells, as well as neutralization of IFN γ *in vivo*, abrogated the anti-tumor effect and reversed the vascular normalization phenotype in anti-CTLA-4- or anti-PD-1-treated mice. This strongly suggests that immune checkpoint blockade induces vascular normalization by reactivation of anti-tumor CD8 T cells [155]. Thus, this study shows evidences of crosstalk between immune and endothelial cells. Another publication by Tian *et al* showed that gene signatures associated with vessel normalization positively correlated with TCR signaling pathway gene signature in human breast and liver cancer [162]. After implantation of E0771 mammary tumors, they showed that mouse strains lacking pericytes (*Ng2^{CreER}* or *Ng2^{CreDTR}*), and thus unable to proceed with vessel normalization, presented particularly decreased densities of tumor-infiltrating T lymphocytes. This indicates that vessel normalization is crucial to promote entry of T cells. Reciprocally, E0771, 4T1 and AT3 tumors transplanted to mice lacking CD4 T cells (*Cd4^{tm1Mak}*), but not those lacking CD8 T cells (*Cd8^{tm1Mak}*), exhibited pericyte deficiency and enhanced hypoxia. In mice harboring deletion of MHC-II in professional APCs (*Tie2^{Cre} x H2Ab^{fl/fl}*), IFN γ -producing Th1 CD4 T cells are less abundant and pericyte coverage is decreased. IFN γ increases expression of ICAM-1 and E-selectin, but also of chemokines CXCL9, 10 and 11 that allow recruitment of Th1 cells and pericytes. Based on these results, the authors conclude that activated antigen-specific CD4 T cells play crucial roles in vessel normalization. However, it should be noted that Tregs, that are increased in the context of hypoxia, decrease vascular

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normalization [154]. Results obtained by Tian and colleagues might thus also be partially explained by alleviation of the inhibitory effect of Tregs on vascular normalization.

Both studies highlighted that CD8 and CD4 T cells seem to be important in the process of vessel normalization induced by immune checkpoint inhibitors. So far, the precise contribution of both lymphocyte subsets remains unclear. It is likely that this will depend on the highly variable vascular and immunological contexts that take place in tumors. There seems to be a consensus that IFN γ is the immunological molecular driver of vessel normalization. Whatsoever, it seems that robust anti-tumor responses take place in tumors treated with ICB therapies thanks to the establishment of a feedback loop involving both vascular normalization and immune reprogramming. Immune checkpoint blockers would increase the proliferation and activity of tumor-infiltrating T lymphocytes, leading to the release of pro-inflammatory cytokines that will further support normalization of tumoral blood vessels. As endothelial cells notably express immune checkpoint ligands such as PD-L1, we cannot exclude that some ICB therapies could primarily induce vascular normalization in an immune-independent manner, and that subsequent entry of activated T cells further reinforces vascular normalization. The lack of such positive reinforcement loop might explain the fact that some cancer patients fail to demonstrate long-term tumor rejection [154, 163]. A new line of cancer treatment consists in the combination of ICB and anti-angiogenic agents, because such agents have been shown to potentiate the effect of immunotherapy by inducing vascular normalization when appropriate low doses are used. Current studies focus on the disruption of the VEGF-VEGFR axis and/or blocking of the Ang1-Tie2 pathway [154].

Several papers that use genetic and pharmacologic approaches to block TGF- β have shown that this is associated with vessel normalization and increased vessel perfusion. Liu *et al* showed that blocking TGF- β with soluble TGF- β RII (sT β RII) transfection or treatment with an antibody targeting the 3 isoforms of active TGF- β (clone 1D11) increased the proportion of blood vessels covered by pericytes. Vessel perfusion, assessed by intravenous injection of lectin coupled to FITC, was also

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increased in murine 4T1 and human MDA-MB-231 tumors, two models of breast cancer implanted in nude mice [138]. In another study focusing on lymphatic vessels, Liao and colleagues showed that the orthotopic implantation of sT β RII-transfected human ovarian cell line SKOV3ip1 in nude mice or the treatment with sT β RII protein leads to less development of ascites, because lymphatic drainage is improved. After TGF- β blockade, lymphatic vessels of the diaphragm displayed characteristics of normalization, such as intact valve structure, lower density of strips and fewer branchings [164]. The observations made by Liu, Liao and others [165] are concomitant with the previous observations that TGF- β act as a negative regulator of pericyte recruitment during blood vessel stabilization. In contrast, TGF- β blockade has also been described to decrease vascular integrity. Three studies have shown that LY364947, an inhibitor of TGF- β type I receptor, decreased pericyte differentiation and attachment to endothelial cells, leading to higher vessel permeability [166-168]. Opposite effects described for TGF- β blockade reflect the dual role of TGF- β 1 on angiogenesis and pericyte recruitment that I described in a previous section. These effects thus appear to depend on the tumor type as well as the cellular context, or the method used for TGF- β 1 blockade and underlying mechanisms are still poorly understood. Whether TGF- β blockade up- or downregulates pericyte recruitment, it has been shown to increase the efficiency of drug delivery (shown for chemotherapy and nanotherapeutics) by increasing blood vessel perfusion or blood vessel openings, respectively [166-168]. The use of TGF- β blockade alone is still debated in the field, notably because increasing vessel perfusion or permeability could also support tumor growth by better supplying nutrients and oxygen to tumor cells. For that reason, preclinical and clinical studies generally combine TGF- β blockade strategies with other therapeutic agents, with perspective that TGF- β blockade will potentiate the effect of such drugs by increasing their penetration into the tumor bed [169].

TGF- β has been described to regulate expression of endothelial-leukocyte adhesion molecules [170-175], notably the expression of E-selectin (encoded by gene *SELE*) on endothelial cells [170-172]. Gamble *et al* and DiChiara *et al* have shown that *in*

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vitro treatment of human umbilical cord endothelial cells (HUVECs) with TGF- β decreases basal and TNF α - or IL-1 β -induced expression of E-selectin, both at the mRNA and the protein levels [170, 171]. Walshe and colleagues have shown that adenovirus-mediated expression of soluble endoglin *in vivo* in BALB/c mice increases the expression level of P- and E-selectin, as well as expression of VCAM-1. This leads to a significant increase in the number of leukocytes rolling on the mesenteric endothelium and to decreased velocity of leukocyte rolling, indicative of higher stable adhesion preceding extravasation through the vascular wall [172]. Other studies describe the inhibitory role that TGF- β plays on LFA-1 expression and function. LFA-1 is both a marker of T cell extravasation, because it binds to CAMs on the endothelium prior to transmigration, and of T cell activation. In splenocytes of *Tgfb1*^{-/-} mice, LFA-1 protein levels are increased by 2-fold [173]. Anti-LFA-1 antibody rescues the severe inflammatory phenotype of *Tgfb1*^{-/-} mice, suggesting that extravasation and/or activation of T cells could be exacerbated by increased LFA-1 expression and functionality in these mice [82]. In human lung tumors, Boutet *et al* showed that TGF- β dampens LFA-1 protein level at the surface of tumor-specific tumor-infiltrating T cells. They isolated an LFA-1⁺ T cell clone, named Heu171, from TILs of a patient with large cell carcinoma (LCC) and tested adhesion and migration on immobilized recombinant ICAM-1-Fc (rICAM-1-Fc). Treatment with TGF- β inhibited adhesion of LFA-1⁺ Heu171 cells to rICAM-1-Fc and decreased the motility of LFA-1⁺ Heu171 cells attached to rICAM-1-Fc in response to CXCL12. This suggests that TGF- β decreases T cell adhesion and cytoskeleton rearrangement by decreasing LFA-1 expression and functionality [174]. Thus, TGF- β 1 tampers with adhesion molecules that are crucial in the process of leukocyte recruitment to the endothelium surface and transmigration across the blood vessel wall. Accordingly, Bessa and colleagues have observed that TGF- β 1 immunoblockade increased leukocyte rolling in venules of human colon adenocarcinoma HT29 injected in nude mice [175].

Four studies combining PD-1/PD-L1 blockade and TGF- β blockade in mouse have shown that this combination leads to increased infiltration of T cells inside the tumor

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bed [62, 63, 65, 117]. More precisely, Martin and colleagues used the combination of an anti-PD-1 mAb (clone RMP1-14) and an antibody specifically directed against the LAP-1 (clone SRK-181). Using 3 different models of transplantable tumors (MBT-2, Cloudman S91 and EMT-6), they showed that latent TGF- β 1 blockade, which did not exert any anti-tumoral effect when injected alone, could induce tumor rejection in mice resistant to PD-1 blockade. Monotherapy treatments did not have any effect on CD8 T cell density in MBT-2 tumors. The authors suggested that increased CD8 T cell density could be the result of entry *via* blood vessels, because CD8 T cells clustered around blood vessels, regardless of the treatment [65]. This illustrates that blood vessels are a route for T cells to infiltrate the tumor and it suggests that combined PD-1 and TGF- β 1 blockade could modulate such route to increase entry of T cells inside the tumor bed.

3.3 Cancer cell recognition and killing

The final step of the Cancer-Immunity cycle consists in the recognition and killing of the tumor cells by cytotoxic CD8 T cells. TCR recognition of cognate peptide/MHC-I complexes expressed on tumor cells triggers the cytolysis of tumor cells by CD8 T cells, that can occur *via* two mechanisms. On the one hand, the granule-exocytosis pathway can take place, during which perforin proteins form pores in the target cell, allowing for the entry of granzymes, a family of serine proteases that induce programmed cell death of the target cell. On the other hand, expression of FasL on CD8 T lymphocytes can trigger apoptosis of Fas-expressing tumor cells, by activating Caspase 3 in the tumor cells [116].

Abundance of TILs is not always positively correlated with better control of tumor growth. Indeed, a significant number of patients with inflamed (or “hot”) tumors, that contain a high density of T lymphocytes, have progressing tumors and fail to respond to ICB. Generation of inflamed tumors is the result of successful progression through the first 5 steps of the Cancer-Immunity cycle. However, at some point, T cells that have been efficiently primed and that traffic from the dLN to the tumor site fail to

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recognize and/or eliminate the tumor cells. This is due to the presence of mechanisms that allow the cancer cells to evade the immune system.

Cancer recognition can be decreased by the loss, downregulation or alteration of MHC I at the surface of tumor cells [116]. This can be the result of downregulation of expression of MHC I itself, but also to altered expression of genes coding for proteins of the antigen processing machinery. Dodagatta-Marri and colleagues have shown that TGF- β decreases the expression of *Mhc1*, *B2m* (coding for β 2-microglobulin), *Tap1* and *Tap2* (coding for two transporters that allow the entry of peptides in the endoplasmic reticulum during antigen processing) *in vitro* in a murine skin tumor cell line (CCK168) and that these effects are reversed by a treatment with anti-TGF- β . They showed that TGF- β -induced loss of MHC was correlated with a transition from epithelial to fibroblastoid phenotype of CCK168 cells, illustrative of EMT [117]. Although no study has directly reported the effect of *in vivo* TGF- β 1 blockade on antigen presentation by tumor cells to my knowledge, it is possible that it could increase cancer cell recognition by tumor-specific CD8 T cells.

Inflamed tumors that continue to grow are generally characterized by the abundance of dysfunctional T lymphocytes. As a result of chronic exposure to the same antigen, TCR activation is altered. Dysfunctional or so called “exhausted” CD8 T cells display impaired proliferation and effector functions like decreased production of pro-inflammatory cytokines and cytolytic molecules. They are also characterized by higher expression levels of inhibitory receptors such as TIM-3, LAG-3, TIGIT and PD-1. In mouse tumors, PD-1 or PD-L1 blockade restores the function of T cells that previously exhibited an exhausted-like phenotype [176, 177]. LAG-3, TIM-3 or TIGIT blockade displayed an anti-tumor effect only when combined with anti-PD-1 or anti-PD-L1 therapies, illustrating that the PD-1/PD-L1 axis seems to play a critical role in T cell exhaustion compared to other inhibitory receptors [116]. In humans, some studies suggest that PD-1 blockade could target exhausted-like T cells. Notably, patients bearing tumors highly infiltrated with T cells that express markers of exhaustion such as PD-1 before treatment respond better to anti-PD-1 mAbs [178]. However, PD-1 expression on CD8 T cells is not always correlated with response to

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anti-PD-1 or anti-PD-L1. Indeed, some patients with PD-1⁺ CD8 TILs can develop resistance to such therapies, because these T cells also co-express multiple inhibitory receptors and this generates a more severely dysfunctional state that renders them less sensitive to PD-1/PD-L1 blockade [116, 176].

Using CAR (Chimeric Antigen Receptor) T cells that are specific of human prostate cancer (anti-PSMA) and that express a dnT β RII, Kloos *et al* have shown that TGF- β inhibition leads to increased proliferation of these T cells and increased production of Th1 and Th2 cytokine. They were also characterized by increased expression of transcription factors associated with robust effector functions and long-term persistence *in vivo* [179]. Moreover, using a model in which they transplant Kras-mutant mouse tumor organoids to C57BL/6 mice, Tauriello and colleagues showed that treatment with galunisertib increased the proportion of CD8 T cells that were positive for granzyme B (GZMB) staining by flow cytometry of liver metastases [63]. Taken together, these results suggest that blocking the TGF- β 1 pathway could overcome T cell exhaustion and enhance the response to PD-1/PD-L1 blockade. Several studies using mouse tumor models have confirmed that combined blockade of TGF- β and of the PD-1/PD-L1 axis increases the effector functions of CD8 T cells. For instance, Tauriello *et al* combined galunisertib with an anti-PD-L1 mAb, which significantly improved tumor control, and led to an increase in the proportion of GZMB⁺ CD8 TILs, of IFN γ ⁺ and of T-bet⁺ CD4 TILs in liver metastases, as well as to an enhanced expression of genes involved in T lymphocyte effector functions and proliferation [63]. Mariathasan and colleagues have shown similar results with the combination of 1D11 and an anti-PD-L1 mAb in EMT6 tumors [62].

To sum up, numerous studies have highlighted the action of PD-1/PD-L1 and/or TGF- β 1 blockade on the Cancer-Immunity cycle in models of mouse tumor. In some studies, PD-1/PD-L1 or TGF- β 1 blockade applied alone decreases tumor burden, by increasing antigen presentation, T cell infiltration or by reinvigorating exhausted T cells, or by a combination of these factors. The diversity of effects identified for both types of therapy are likely to depend on the tumor context, as well as on the dose, mode and frequency of administration of the treatment. Although few studies that

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decipher the mode of action of anti-PD-1 or anti-PD-L1 mAbs in cancer patients have been published, they suggest that such therapies increase T cell proliferation and activity in humans [180-182]. Nevertheless, studies performed in mouse highlight that TGF- β 1 blockade potentiates response to PD-1/PD-L1 blockade, because it overcomes mechanisms that otherwise allow the tumor to evade the immune system. This opens an interesting doorway to further increase clinical benefit of immunotherapies and overcome resistance to anti-PD-1 mAbs in cancer patients.

AIM OF THE STUDY

AIM OF THE STUDY

Our team previously developed monoclonal antibodies directed against GARP:(latent)TGF- β 1 complexes that block TGF- β 1 activation and immunosuppression by human Tregs *in vitro* and *in vivo*, in a model of xenogeneic graft-versus-host disease in NSG mice. Because Tregs and TGF- β 1 exert deleterious activities in the tumor microenvironment, we wished to examine the potential of anti-GARP:TGF- β 1 mAbs as a novel approach for the immunotherapy of cancer.

More precisely, my project aimed at assessing anti-GARP:TGF- β 1 mAbs in mouse models of cancer to evaluate their potential relevance for future use in the clinics.

Our specific objectives were to:

- Determine whether anti-GARP:TGF- β 1 mAbs, used alone or in combination with anti-PD-1, exert anti-tumor effects in mice transplanted with syngeneic tumor cells;
- Define the mode of action by which anti-GARP:TGF- β 1 mAbs exert anti-tumor effects, if any.

Results of my work are presented in two research articles comprised in the “Results” section of this thesis manuscript. The first article, by de Streel, Bertrand *et al*, was published in *Nature Communications* in September 2020. Together with Grégoire de Streel, we demonstrated the superior anti-tumor efficacy of combined GARP:TGF- β 1/PD-1 blockade by comparison to PD-1 blockade alone in mice bearing CT26 and MC38 tumors. We identified Tregs as the main target of anti-GARP:TGF- β 1 in MC38 tumor-bearing mice, and showed in CT26 tumors that the combined treatment did not increase infiltration of T cells but increased the effector functions of already present anti-tumor T cells. In the second article by Bertrand *et al*, recently published

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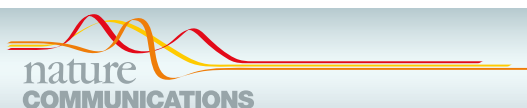
in *Frontiers in Immunology*, we identify an additional mode of action of combined GARP:TGF- β 1/PD-1 blockade in MC38 tumor-bearing mice, which implies densification and normalization of intratumoral blood vasculature and increase of T cell infiltration into the tumors. Our observations that the combined blockades of GARP:TGF- β 1 and PD-1 can exert anti-tumor activity *via* multiple mechanisms may prove important for the selection of cancer patients who could benefit from combined GARP:TGF- β 1/PD-1 blockade in the clinics.

RESULTS

RESULTS

- 1 Selective inhibition of TGF- β 1 produced by GARP-expressing Tregs overcomes resistance to PD-1/PD-L1 blockade in cancer (de Streel, Bertrand *et al*, Nature Communications 2020)**

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<https://doi.org/10.1038/s41467-020-17811-3>

OPEN

Selective inhibition of TGF- β 1 produced by GARP-expressing Tregs overcomes resistance to PD-1/PD-L1 blockade in cancer

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TGF- β 1, β 2 and β 3 bind a common receptor to exert vastly diverse effects in cancer, supporting either tumor progression by favoring metastases and inhibiting anti-tumor immunity, or tumor suppression by inhibiting malignant cell proliferation. Global TGF- β inhibition thus bears the risk of undesired tumor-promoting effects. We show that selective blockade of TGF- β 1 production by Tregs with antibodies against GARP:TGF- β 1 complexes induces regressions of mouse tumors otherwise resistant to anti-PD-1 immunotherapy. Effects of combined GARP:TGF- β 1/PD-1 blockade are immune-mediated, do not require Fc γ R-dependent functions and increase effector functions of anti-tumor CD8⁺ T cells without augmenting immune cell infiltration or depleting Tregs within tumors. We find GARP-expressing Tregs and evidence that they produce TGF- β 1 in one third of human melanoma metastases. Our results suggest that anti-GARP:TGF- β 1 mAbs, by selectively blocking a single TGF- β isoform emanating from a restricted cellular source exerting tumor-promoting activity, may overcome resistance to PD-1/PD-L1 blockade in patients with cancer.

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Immunosuppression by regulatory T cells (Tregs) is indispensable to maintain peripheral immune tolerance, but is detrimental in cancer or chronic infections. Targeting Tregs or their functions in cancer patients has remained a coveted, but challenging and unmet therapeutic approach. Coveted, because notwithstanding the remarkable progress in cancer treatment achieved with monoclonal antibodies (mAbs) blocking the CTLA-4 or PD-1 inhibitory pathways, a vast majority of patients do not respond to immunotherapy due to primary or acquired resistance to T-cell-mediated anti-tumor immunity^{1,2}, and Tregs appear deleterious to anti-tumor immunity in most patients and cancer types^{3–12}. Very recently, Tregs were even suggested to be amplified and contribute to disease hyperprogression in response to PD-1 blockade in a small subset of cancer patients¹³. Nevertheless, whereas mouse Tregs were shown to suppress immune responses by a variety of context-dependent mechanisms, which mechanism, if any, should be targeted to block suppression of anti-tumor immunity by Tregs in cancer patients is not known. None of the current cancer immunotherapies allows to specifically block Treg immunosuppression without killing these cells in the tumor microenvironment.

We recently identified a mechanism of immunosuppression by human Tregs that can be blocked by mAbs. This mechanism implicates production of the potentially immunosuppressive TGF- β 1 cytokine. Like most other immune cells, Tregs produce TGF- β 1 in a latent, inactive form, in which the mature TGF- β 1 dimer is non-covalently associated to the latency associated peptide (LAP). LAP forms a ring around mature TGF- β 1, masking the interaction sites with the TGF- β 1 receptor chains^{14,15}. Only a few cell types are able to activate the cytokine by releasing mature TGF- β 1 from LAP, through cell-type-specific mechanisms¹⁶. Upon T-cell receptor (TCR) stimulation, Tregs present latent TGF- β 1 on their surface via disulfide linkage of LAP to a transmembrane protein called GARP^{15,17,18}. Integrin α V β 8 interacts with GARP:(latent)TGF- β 1 complexes, leading to release of active TGF- β 1 close to the surface of stimulated Tregs^{19,20}. Treg-derived active TGF- β 1 exerts paracrine, short-distance immunosuppressive effects on immune cells, including T cells¹⁶. We derived anti-GARP:TGF- β 1 mAbs that block the TGF- β 1 activation by TCR-stimulated human Tregs, through a molecular mechanism elucidated via X-ray crystallography^{15,21}. These mAbs do not bind complexes of GARP and latent TGF- β 2 or β 3, which are produced by non-Treg cells and once activated, signal via the same receptor as TGF- β 1¹⁵. Blocking anti-GARP:TGF- β 1 mAbs inhibited the immunosuppression by human Tregs of a xenogeneic graft-versus-host disease induced by transfer of human PBMCs into immunodeficient NSG mice²¹.

Here, we derive an anti-mouse GARP:TGF- β 1 mAb that blocks release of active TGF- β 1 by mouse Tregs, allowing to examine the therapeutic benefit of blocking Treg function in tumor-bearing individuals. We show that this mAb increases the effector functions of anti-tumor T cells and induces immune-mediated rejections of tumors otherwise resistant to anti-PD-1 therapy. We also show that GARP-expressing Tregs are present in a sizeable subset of human melanoma samples, warranting trials to test anti-GARP:TGF- β 1 mAbs in the clinics.

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Anti-GARP:TGF- β 1 mAb blocks TGF- β 1 activation by mouse Tregs. Previously described blocking antibodies against human GARP:TGF- β 1 complexes do not recognize mouse GARP:TGF- β 1²¹. To derive mAbs that block TGF- β 1 activation from GARP:TGF- β 1 complexes on murine Tregs, we immunized llamas with plasmids encoding mouse GARP and TGF- β 1, and constructed V_H/V_k and V_H/V _{λ} cDNA libraries to select Fab clones binding

mouse GARP:TGF- β 1 by phage display. Fab-encoding regions from selected clones were sequenced to construct >50 full-length mAbs by subcloning into a murine immunoglobulin G2a (mIgG2a) backbone. Clone 58A2 bound GARP:TGF- β 1 complexes but not free GARP or free latent TGF- β 1 (Fig. 1a). It bound the surface of mouse Tregs, both resting and even more so after TCR stimulation (Fig. 1b). It also blocked the release of active TGF- β 1 induced by TCR stimulation of mouse Tregs in vitro (Fig. 1c), whether the mAb was used as a wild-type (WT) or an Fc-dead (FcD) mIgG2a subclass antibody. The FcD mIgG2a contains two amino-acid substitutions in the Fc region (D₂₆₅A/N₂₉₇A) that preclude binding to all mouse Fc γ Rs^{22,23} (Supplementary Fig. 1). Binding and blocking activities of clone 58A2 closely resembled those of blocking anti-human GARP:TGF- β 1 mAbs^{15,21}. Clone 58A2 was thus further selected to test whether treatment with blocking anti-GARP:TGF- β 1 mAbs could improve anti-tumor immune responses and favor tumor rejections in mice.

Anti-GARP:TGF- β 1 overcomes resistance to anti-PD-1. We injected CT26 colon carcinoma cells subcutaneously (s.c.) in BALB/c mice and started antibody treatments after 6 days, when tumors were well established in all mice (Fig. 2a). Tumors grew uniformly and no rejection (complete response or CR: 0/9) was observed upon injection of an isotype control mIgG2a antibody (Fig. 2b). No rejection was observed either after treatment with the blocking anti-GARP:TGF- β 1 clone 58A2, either as a WT or an FcD mIgG2a. A single tumor rejection (CR: 1/10) was observed in mice treated with mAb 1D11, which neutralizes all three TGF- β isoforms²⁴. Thus, antibodies that neutralize TGF- β 1 or block TGF- β 1 activation from GARP:TGF- β 1 complexes do not display anti-tumor activity when administered as monotherapies in CT26 tumor-bearing mice (Fig. 2b).

Anti-PD-1 mAbs displayed very limited anti-tumor activity in this model (Fig. 2b). No rejection (CR: 0/10) occurred when anti-PD-1 clone RMP1-14 was administered as a rat IgG2a subclass mAb (WT), and only a minority of the mice (CR: 2/10) rejected their tumors after treatment with an anti-PD-1 comprising the RMP1-14 variable regions in an Fc-Silent (FcS) mIgG2a backbone (Absolute Antibodies[®]). Although minor, the increased anti-tumor activity of anti-PD-1 FcS compared to WT was expected because the FcS mAb contains amino-acid substitutions precluding binding to Fc γ Rs, a feature known to enhance the anti-tumor activity of anti-PD-1 mAbs. In the case of clone RMP1-14, this has been suggested to result from abrogation of an Fc γ RIIb-dependent agonistic activity on PD-1 expressed by CD8⁺ T cells²⁵.

As shown in Fig. 2b, combination with anti-GARP:TGF- β 1 WT improved the anti-tumor activity of anti-PD-1 in both WT and FcS formats (CR: 2/10 and 5/10 mice, respectively). Interestingly, the anti-tumor effect of anti-GARP:TGF- β 1 did not require its binding to Fc γ Rs: tumor rejection was also more frequent (CR: 4/10) when anti-GARP:TGF- β 1 FcD was combined with anti-PD-1 FcS. Anti-TGF- β clone 1D11 modestly increased the frequency of tumor rejections (CR: 3/10) when combined with anti-PD-1 FcS. By comparison to treatment with anti-PD-1 FcS alone, reductions in mean tumor volumes were statistically significant in mice receiving anti-PD-1 FcS combined with anti-GARP:TGF- β 1 (WT or FcD), but not with anti-TGF- β (Fig. 2c). This indicates that in CT26-bearing mice, blocking the activity of TGF- β 1 emanating from GARP:TGF- β 1-expressing cells only was at least as efficient as blocking the activity of the three TGF- β isoforms, whichever their cellular source. Anti-GARP:TGF- β 1 significantly increased the anti-tumor activity of anti-PD-1 against established CT26 tumors in seven independent

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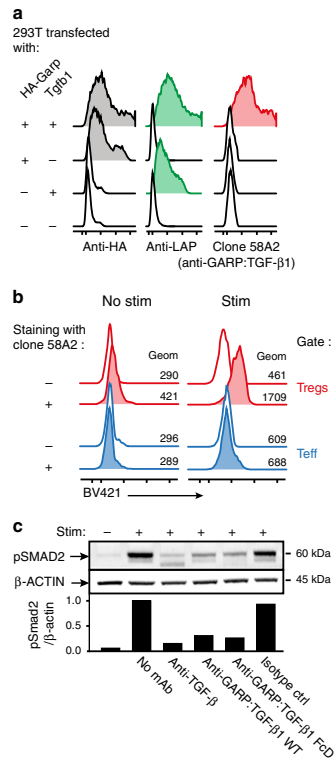


Fig. 1 An anti-GARP:TGF- β 1 mAb that blocks TGF- β 1 activation by mouse Tregs in vitro. **a** 293T cells were transfected with constructs encoding HA-tagged mouse GARP, mouse TGF- β 1 (i.e. LAP + mature TGF- β 1), or both, to induce surface expression of free GARP, free latent TGF- β 1, or GARP:TGF- β 1 complexes, respectively (Cuende et al.²¹). Transfected cells were stained with the indicated antibodies and analyzed by flow cytometry. Histograms are gated on live cells. **b** Mouse splenocytes were stimulated (stim) or not with anti-CD3/CD28 coated beads during 24 h, then analyzed by flow cytometry after staining with anti-CD4 and anti-FOXP3 antibodies in the presence (+) or absence (–) of the biotinylated anti-GARP:TGF- β 1 clone 58A2, followed by streptavidin coupled to BV421. Histograms in red are gated on CD4⁺FOXP3⁺ cells (Tregs), in blue on CD4⁺FOXP3[–] cells (Teff). **c** Magnetically-sorted CD25⁺ mouse splenocytes were stimulated during 24 h with anti-CD3/CD28 coated beads in the presence or absence of blocking antibodies against active TGF- β (clone 1D11), GARP:TGF- β 1 complexes (clone 58A2, mlgG2a WT or FcD), or an isotype control (mlgG2a WT), then analyzed by Western blot with antibodies against β -ACTIN and pSMAD2, as a read-out for active TGF- β 1 production. Bar graphs on the bottom show quantification of ECL signals (ratio of pSMAD2/ β -ACTIN signals relative to that in cells stimulated in the absence of blocking mAb). Full scans are shown in Supplementary Fig. 14.

experiments, allowing for a 2.8 to 5-fold increase of the proportion of mice surviving until the end of the experiment after having completely rejected their tumor (proportions of CR in meta-analyses shown in Fig. 3).

We verified whether our observations could be generalized to another tumor model, another genetic background, or yet other blocking anti-GARP:TGF- β 1 mAbs. First, we injected MC38 colon carcinoma cells in WT C57BL/6 mice. We observed that anti-GARP:TGF- β 1 increased the anti-tumor activity of anti-PD-1 in this model also (Supplementary Fig. 2). Second, we injected MC38 cells in *Garp*^{YSG/YSG} C57BL/6 mice. These mice carry a homozygous knock-in mutation that replaces three contiguous HGN amino acids of mouse GARP by the YSG amino acids found at the corresponding positions of human GARP. Mutated GARP:TGF- β 1 complexes can be bound by MHG-8 and LHG-10, two previously described blocking anti-human GARP:TGF- β 1 antibodies²¹. Treatment of MC38-bearing *Garp*^{YSG/YSG} mice with anti-PD-1 combined with MHG-8 or LHG-10 increased the frequency of tumor rejections by comparison to treatment with anti-PD-1 alone (Supplementary Fig. 3).

Altogether, these data indicate that antibody-mediated blockade of TGF- β 1 activation from GARP:TGF- β 1 complexes induces rejection of tumors resistant to anti-PD-1 monotherapy. This anti-tumor activity does not require Fc γ R-dependent effector functions of the blocking anti-GARP:TGF- β 1 mAbs.

Anti-GARP:TGF- β 1 protects against re-challenge with tumor.

Four mice that had rejected a CT26 tumor after treatment with a combination of anti-PD-1 WT and anti-GARP:TGF- β 1 WT were injected 47 days after the last mAb administration with live CT26 cells in the right flank, and live RENCA or EMT6 syngeneic tumor cells in the left flank. No tumor grew in the right flanks whereas all grew in the left flanks (Fig. 4a). Control tumors grew readily in naive mice (Fig. 4b). This suggests that anti-GARP:TGF- β 1 combined with anti-PD-1 induces protective T-cell-mediated immunity against CT26-specific tumor antigens.

Anti-GARP:TGF- β 1 does not modify numbers of TILs.

We examined whether treatment with anti-GARP:TGF- β 1, alone or combined with anti-PD-1, modified the quantity or quality of the immune cells infiltrating CT26 tumors. Mice were injected subcutaneously with CT26 cells, treated with mAbs as indicated in Fig. 2a, and euthanized 1 day after the third mAb injection to collect spleens and tumors for flow cytometry, RNA analyses, or multiplexed immunofluorescence microscopy (Fig. 5, Supplementary Figs. 4–7). Tumor volumes and weights were measured the day before or immediately after dissection on day 13, respectively. On that early time point, tumor weights were already significantly reduced in mice treated with anti-GARP:TGF- β 1 combined with anti-PD-1 by comparison to mice receiving the isotype control antibody (Fig. 5a and Supplementary Fig. 7a).

No difference in numbers and proportions of any leukocyte subset was observed in the spleens (Supplementary Fig. 4). Regardless of the treatment, all tumors contained $\sim 9 \times 10^4$ leukocytes (CD45⁺ cells) per mm³ (Fig. 5b). Tumor-infiltrating leukocytes (TILs) comprised $16 \pm 2\%$ (mean $\pm$ sem) and $17 \pm 2\%$ of CD4⁺ and CD8⁺ T cells, respectively, and these proportions were not significantly different between the various treatment groups (Supplementary Fig. 5a). Likewise, numbers of these cells per mm³ of tumor (i.e. densities) were not significantly different between the various treatment groups (Fig. 5b). This was also observed in two similar independent experiments (Supplementary Fig. 6a). We also examined CD8⁺ T cells specific for a tumor

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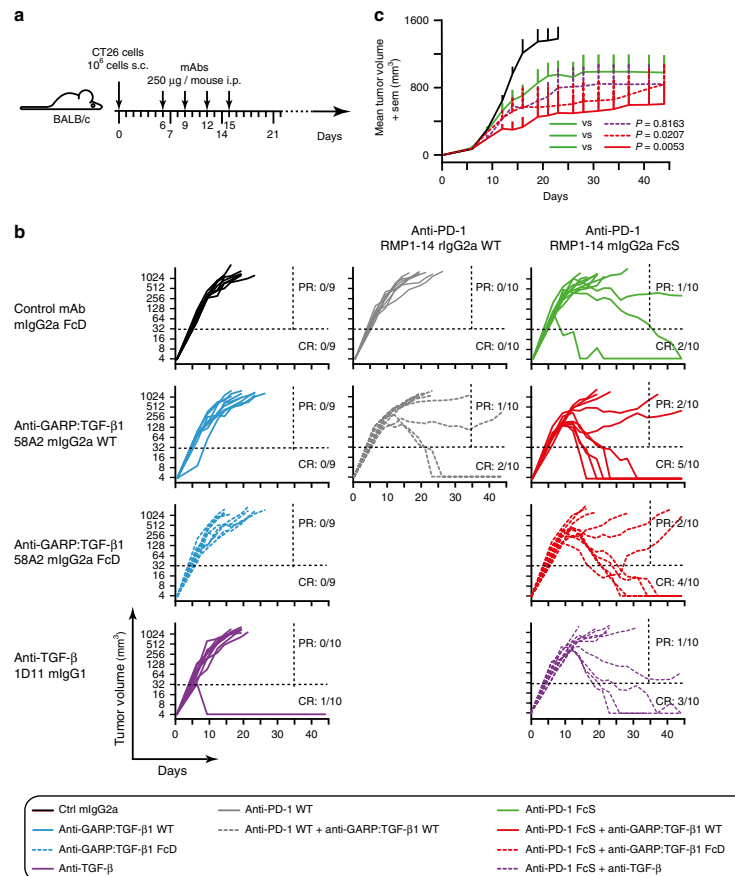


Fig. 2 Combined blockade of GARP:TGF- $\beta 1$ and PD-1 shows anti-tumor efficacy. **a** Schematic representation of the experimental design. BALB/c mice were injected s.c. with live CT26 cells on day 0. Tumor diameters were measured twice a week. Mice were randomized in 10 groups ($n = 9-10$ mice per group) on day 6, and mAbs were injected i.p. every 3 days from day 6 to 15. Mice were euthanized when the tumor surface was $\geq 200 \text{ mm}^2$. **b** Evolution of tumor volumes in the 10 treatment groups (each line represents one mouse). Ratios indicate the proportions of complete responders (CR: mice alive on day 45 with no detectable tumor) and partial responders (PR: mice alive on day 35 that carry a tumor $>32 \text{ mm}^3$ at the time of euthanasia). Dotted horizontal and vertical lines indicate the two arbitrary limits (tumor volume of 32 mm^3 and day 35) used to identify PR and CR. **c** Evolution of mean tumor volume $\pm \text{sem}$ in five treatment groups. P values for relevant comparisons are indicated and were calculated with a mixed effects model, as recommended for analyses of longitudinal data (Liu et al.⁴⁶; Sugar et al.⁴⁵), with a post-hoc Tukey's test for multiple comparisons. Similar results were observed in five other independent experiments (Fig. 3 shows meta-analyses of all pooled experiments).

antigen, using an AH1/H-2L^d tetramer. AH1 is an immunodominant peptide presented by H-2L^d on CT26 tumor cells. It is encoded by gene *Gp70*, an endogenous mouse retrovirus gene that is silent in normal mouse tissues and reactivated in CT26 cells²⁶. Anti-AH1 CD8⁺ T cells represented $31 \pm 2\%$ of the total CD8⁺ T cells infiltrating tumors on day 13 (Supplementary Fig. 5a). Again, proportions and numbers of anti-AH1 CD8⁺ T cells per mm^3 of tumor were not significantly different between

the various treatment groups (Fig. 5b and Supplementary Fig. 5a). Densities of total leukocytes or leukocyte subsets, including B, NK, or myeloid cells, were not modified in response to any treatment (Supplementary Fig. 6b).

We also estimated densities and distribution of CD4 and CD8 T cells in formalin-fixed paraffin-embedded (FFPE) tumor sections by immunofluorescence microscopy and quantitative digital imaging. Here again, we observed no significant difference

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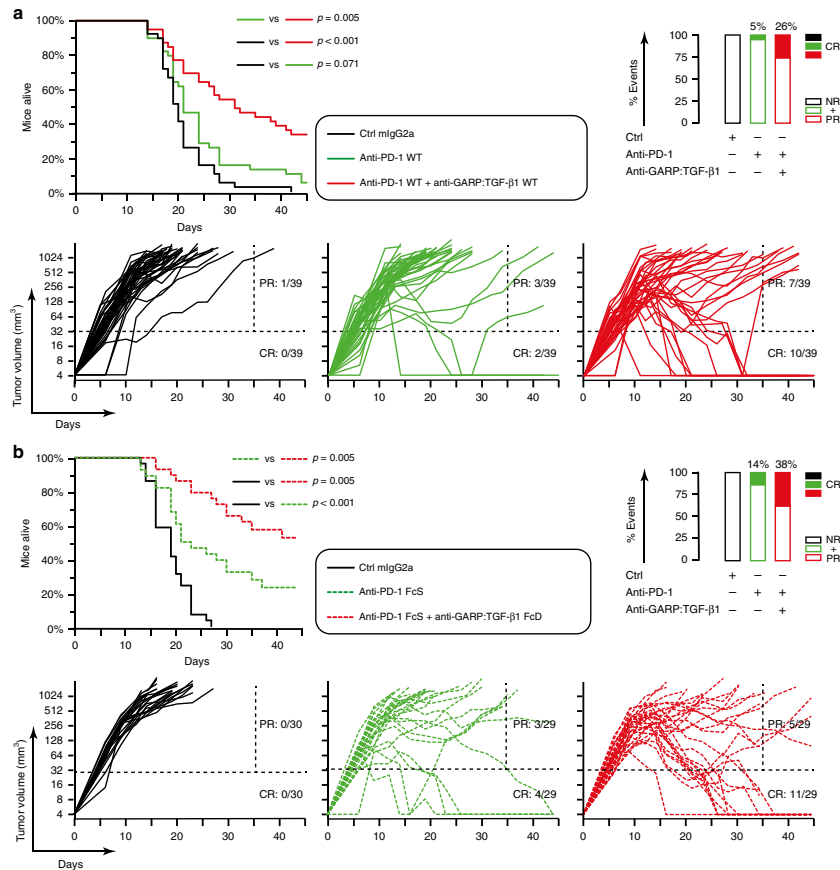


Fig. 3 Combined blockade of GARP:TGF-β1 and PD-1 shows anti-tumor efficacy against established CT26 tumors. BALB/c mice were injected s.c. with live CT26 cells on day 0. Tumor diameters were measured two to three times a week. On day 6, mice were randomized in various experimental groups and received the first of 3–4 mAb injections. Mice were euthanized when the tumor surface was ≥ 200 mm². **a** Meta-analysis of four pooled independent experiments in which mice received anti-PD-1 WT alone, or in combination with anti-GARP:TGF-β1 WT ($n = 39$). **b** Meta-analysis of three pooled independent experiments in which mice received anti-PD-1 FcS alone, or in combination with anti-GARP:TGF-β1 FcD ($n = 29$ –30). **a, b** Kaplan Meier plots on top represent the proportions of mice alive during the experiment, with P values calculated using a one-tailed Wilcoxon test. Graphs on bottom represent evolution of tumor volumes in various groups (each line represents one mouse). Ratios indicate the proportions of CR and PR, as defined in Fig. 2. Rectangles on the right represent proportions (%) of CR (filled rectangles), and partial + non-responders (PR + NR, empty rectangles).

between the various treatment groups (Supplementary Fig. 7a–c). A statistically non-significant trend toward increased densities of T cells, apparent in both the periphery and center of tumors, was observed in mice treated with anti-PD-1, whether it was combined or not with anti-GARP:TGF-β1 (Supplementary Fig. 7c).

Altogether, our results show that CT26 tumors are heavily infiltrated with leukocytes, including tumor-specific CD8⁺ T cells, which are nonetheless unable to control tumor growth in non-treated mice. Importantly, it also shows that the anti-tumor activity of the anti-GARP:TGF-β1 and anti-PD-1

combination does not result from an increased recruitment of anti-tumor T cells within these already inflamed tumors.

Intra-tumoral Tregs are not depleted by anti-GARP:TGF-β1.

Flow cytometry analyses of cells isolated from CT26 tumors on day 13 indicated that $45 \pm 3\%$ of the CD4⁺ TILs were Tregs (FOXP3⁺), and $54 \pm 2\%$ of the tumor-infiltrating TILs expressed GARP, regardless of the treatment (Supplementary Fig. 5a). We did not detect GARP on non-Treg TIL subsets, suggesting that the main target of anti-GARP:TGF-β1 mAbs within CT26 tumors

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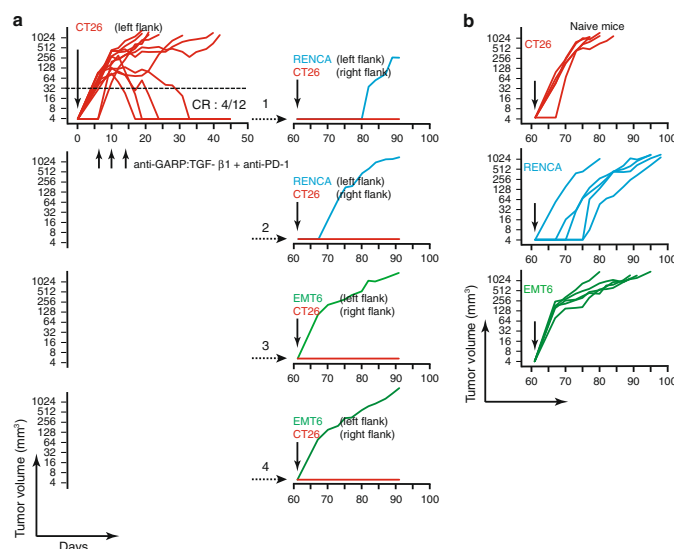
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Fig. 4 Combined blockade of GARP: TGF- β 1 and PD-1 induces protective, CT26-specific immunity. **a** On day 0, live CT26 cells were injected in the left flank of BALB/c mice ($n = 12$; 10^6 cells/mouse). Anti-GARP: TGF- β 1 WT combined with anti-PD-1 WT were injected i.p. on days 6, 10, and 14. Graph on the left shows evolution of tumor volumes until day 50 in individual mice, and the ratio (CR) indicates the proportion of mice that completely rejected their tumor. On day 61, the four mice that had rejected their tumor were re-injected in the right flank with live CT26 cells (10^6 cells/mouse, red lines), and in the left flank with syngeneic RENCA (10^6 cells/mouse, blue lines) or EMT6 cells (3×10^5 cells/mouse, green lines). Each graph on the right shows evolution of tumor volumes in the left and right flanks in one of the four re-challenged mice. In all cases, mice were euthanized when the surface of any tumor was ≥ 200 mm 2 . **b** On day 61, control naive BALB/c mice ($n = 5$ per group) were injected in the left flank with live CT26, RENCA or EMT6 tumor cells (cell numbers as in **a**). Graphs show evolution of tumor volumes in individual mice.

are Tregs. Nevertheless, numbers of total Tregs and GARP⁺ Tregs per mm³ of tumor were not decreased in mice that had received an anti-GARP:TGF- β 1 mAb, alone or in combination with anti-PD-1 (Fig. 5c and Supplementary Fig. 6a). If anything, an increase in Treg and GARP⁺ Treg numbers was observed in mice treated with the anti-GARP:TGF- β 1 FcD + anti-PD-1 combination in one experiment (Fig. 5c), but this was not confirmed in two others (Supplementary Fig. 6a). This indicates that anti-GARP:TGF- β 1 antibodies, either in a WT or an FcD format, did not deplete intra-tumoral Tregs in this model.

GARP:TGF- β 1/PD-1 blockade augments anti-tumor T cell functions. Anti-tumor CD8⁺ T lymphocytes promote anti-tumor immunity via production of pro-inflammatory cytokines such as TNF α and IFN γ and direct perforin/granzyme-dependent killing of tumor cells. Signaling induced in CD8⁺ T cells by binding of TGF- β 1 to its receptor or PD-L1/L2 to PD-1 were both shown to inhibit these effector functions^{27–29}. By comparison to mice injected with an isotype control mAb, significantly increased levels of *Prfl*, *Gzmb*, *Tnf*, and *Ifng* mRNAs were observed in tumors from mice treated with a combination of anti-PD-1 and anti-GARP:TGF- β 1 WT or FeD mAbs, but not with either mAb alone (Fig. 5d). This suggested increased production of cytokines and cytolytic molecules by intra-tumoral T cells in response to the combination mAb treatment. We performed RNAseq and Gene Set Enrichment Analyses (GSEA^{30–32}) to examine gene

expression signatures of response to pro-inflammatory cytokines within the tumor samples. By comparison to mice treated with the isotype control mAb, increased expression and significant enrichment of genes from the hallmark response signatures to IFN γ , TNF α , IL-2, and inflammation were observed in mice treated with the anti-GARP:TGF- β 1 (WT or FcD) + anti-PD-1 combinations, but not with either monotherapy (Fig. 5e and Supplementary Fig. 8). This confirmed that combination therapy with anti-GARP:TGF- β 1 and anti-PD-1 increased production of effector, pro-inflammatory cytokines within the tumors.

We next verified which immune cells produced more cytokines and cytolytic molecules in mice treated with the mAb combination. Cells isolated from CT26 tumors were left resting or briefly re-stimulated with peptide AH1 or PMA/Ionomycin *in vitro*, then stained for intracellular IFN γ and TNF α , and surface CD107a as a read out for degranulation and cytolytic activity. No significant difference in the proportions of cells expressing a single effector molecule, or a combination of these, was observed among total TILs, CD8 $^{+}$, or CD4 $^{+}$ T cells between the various treatment groups (Supplementary Fig. 9). In contrast, we observed significant increases in the proportions of cells expressing IFN γ , TNF α , or CD107a, and even more strikingly all three effector molecules, among the subset of CD8 $^{+}$ T cells directed against the tumor-specific AH1 antigen (AH1/H-2L d tetramer positive cells) in tumors from mice treated with a combination of anti-PD-1 and anti-GARP:TGF- β 1 WT or Fc μ mAbs, but not with either mAb alone (Fig. 5f and Supplementary

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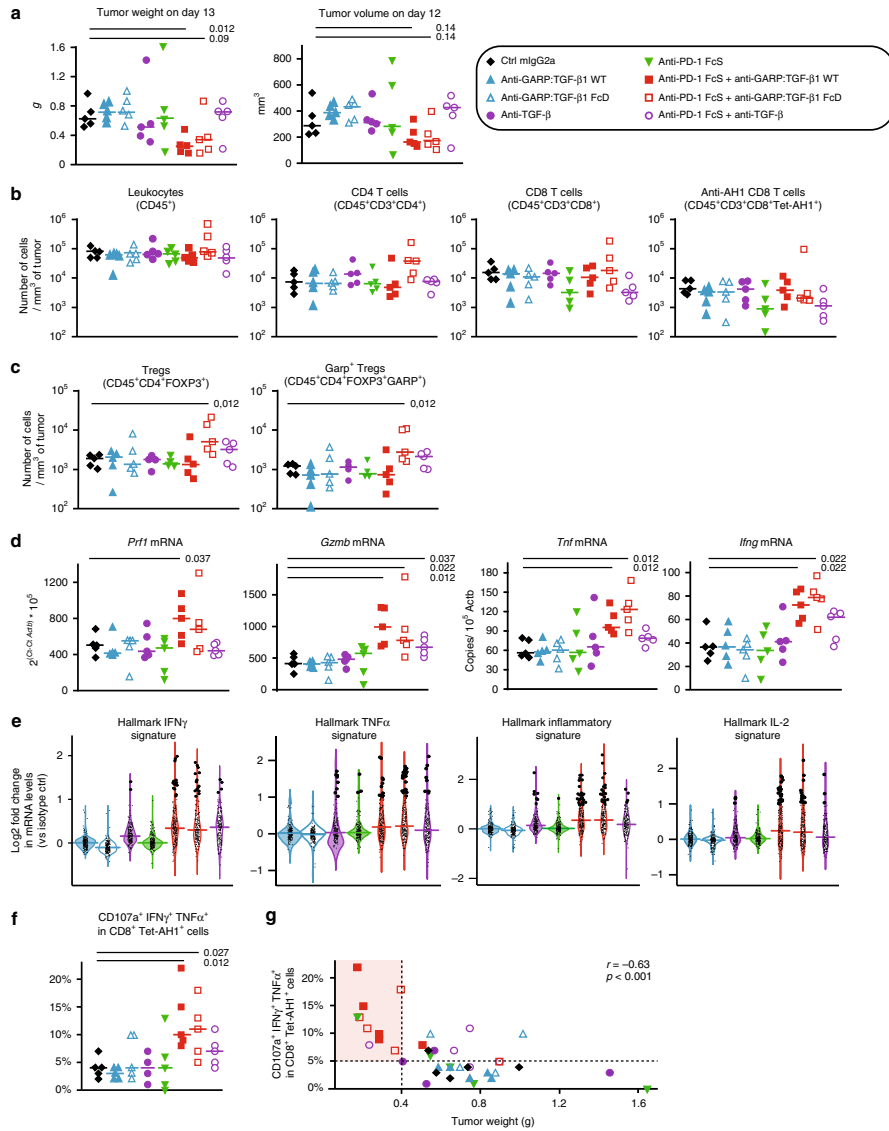


Fig. 9). This increase was observed whether or not cells had been re-stimulated in vitro with peptide AH1 or PMA/Ionomycin. Similar trends were observed when anti-PD-1 was combined with anti-TGF-β, although in that case, differences were in most cases not statistically significant (Fig. 5d–g and Supplementary Fig. 9).

Interestingly, tumor weights inversely correlated with proportions of anti-AH1 CD8⁺ T cells with multiple effector functions (Fig. 5g). However, and as expected from above, tumor weights did not inversely correlate with densities of total leukocytes or any leukocyte subset (Supplementary Fig. 5b). Eight of the 10 mice

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Fig. 5 Combined blockade of GARP:TGF- β 1 and PD-1 increases effector functions of anti-tumor CD8⁺ T cells. BALB/c mice ($n = 5/\text{group}$) were injected with CT26 cells on day 0 and treated with mAbs on days 6, 9, and 12, as illustrated in Fig. 2. Tumors were collected on day 13. **a** Weight of tumors collected after euthanasia on day 13 and volume of tumors measured the day before. Each data point represents the value measured in one mouse, and horizontal bars the median per group. **b, c** Numbers of various subsets of cells infiltrating 1 mm³ of tumor, as determined by flow cytometry. Data points and horizontal bars as in **a**. **d** Expression of genes as determined by RT-qPCR. Data points and horizontal bars as in **a**. Numbers in italics show P values < 0.05 for the comparisons with the control group (isotype ctrl mlgG2a), as calculated with a two-sided Wilcoxon test. **e** Violin plots representing fold change in expression of genes from hallmark signatures (MSigDB database) in each treatment group by comparison to the isotype control group, as determined by RNAseq. Within a group, each dot represents one gene of the indicated signature, with its position on the Y axis representing the ratio between mean expression in the treated versus control group. Genes with a fold change ≥ 2 are represented by larger dots. Horizontal bars are median fold change for all genes of the signature. Violin contours show the kernel density distributions of fold changes. Numbers of genes in the indicated signatures: IFN $\gamma = 179$; TNF $\alpha = 184$; inflammatory = 164; and IL-2 = 175. **f** Cells isolated from tumors were stimulated in vitro with the AHI peptide, and analyzed by flow cytometry for surface markers and intracellular cytokines. Data points, horizontal bars, and P values as in **d**. **g** Correlation between proportions of anti-tumor CD8⁺ T cells displaying multiple effector functions (shown in **f**) with tumor weight in the corresponding mouse at day 13. $r =$ Pearson's correlation coefficient with corresponding P value calculated with one-tailed F -test. Results shown here are representative of at least three independent experiments.

with the smallest tumors (< 0.4 g) had $\geq 5\%$ of anti-AHI CD8⁺ T cells with multiple effector functions and received the combination of anti-PD-1 and anti-GARP:TGF- β 1 WT or FcD mAbs (Fig. 5g).

Altogether, these data indicate that treatment with a combination of anti-GARP:TGF- β 1 and anti-PD-1 increases the effector functions of tumor-specific CD8⁺ TILs without increasing their number or penetration within the tumors.

Anti-tumor activity requires CD8⁺ cells and IFN γ signals. We next sought to determine whether increased effector functions of anti-tumor CD8⁺ TILs contributed to the anti-tumor activity of the anti-GARP:TGF- β 1 and anti-PD-1 combination. First, we administered a monoclonal antibody that depletes CD8⁺ cells (Supplementary Fig. 10) into tumor-bearing mice, 2 days before starting the treatment with anti-GARP:TGF- β 1 WT and anti-PD-1 mAbs. Depletion of CD8⁺ T cells abrogated the anti-tumor efficacy of the combination treatment (Fig. 6a). Second, we administered a neutralizing anti-IFN γ mAb into tumor-bearing mice on the same days as treatments with anti-GARP:TGF- β 1 and anti-PD-1. Neutralization of IFN γ also abrogated the anti-tumor efficacy of the combination treatment (Fig. 6b). Together, these results suggest that increased IFN γ production by CD8⁺ T cells contributes to the anti-tumor activity of anti-GARP:TGF- β 1 combined with anti-PD-1.

Anti-GARP:TGF- β 1 acts by blocking TGF- β 1 activation on Tregs. In CT26, GARP expression is detected on a majority of Tregs (Fig. 5) but not on other TIL subsets. This suggests that when combined with anti-PD-1, anti-GARP:TGF- β 1 exerts anti-tumor activity by blocking TGF- β 1 activation by Tregs but not by other GARP-expressing cell types. However, TGF- β 1 activation by GARP⁺ platelets was also suggested to suppress anti-tumor immunity³³. We thus derived two C57BL/6 mouse strains carrying a Treg- or a platelet-specific deletion of the *Garp* gene, respectively (Supplementary Fig. 11). We injected MC38 cells in Treg- and platelet-specific *Garp* KO mice and their WT littermates, and treated tumor-bearing mice with anti-PD-1 combined or not with anti-GARP:TGF- β 1 (Fig. 7a). As shown in Fig. 7b, the proportions of complete responses to anti-GARP:TGF- β 1 + anti-PD-1 were superior to anti-PD-1 alone in platelet-specific *Garp* KO mice, as well as in their WT littermates (42% vs 23%, and 46% vs 13%, respectively). These results were in line with our previous experiments but did not reach statistical significance. In Treg-specific *Garp* KO mice, this difference was not observed (Fig. 7c), and if anything, the combination was modestly inferior to anti-PD-1 alone (not statistically significant). These results indicate that targeting GARP on Tregs, but not platelets, with a

blocking anti-GARP:TGF- β 1 mAb is necessary to overcome resistance to PD-1 blockade in tumor-bearing mice. Our observation that the anti-tumor activity of anti-PD-1 alone is only very modestly increased in Treg-specific *Garp* KO mice by comparison to WT littermates (CR: 28% vs 20%) suggests that GARP-deficient Tregs may acquire compensatory immunosuppressive mechanisms during differentiation that inhibit immune responses against experimentally transplanted tumors.

GARP⁺ Tregs are present in human melanoma metastases. The results above suggest that anti-GARP:TGF- β 1 mAbs could exert anti-tumor effects and increase response rates to PD-1/PD-L1 blockade, particularly in patients with tumors containing GARP-expressing Tregs. We thus resorted to multiplex immunofluorescence (mIF) staining to assess the presence and abundance of GARP⁺FOXP3⁺ cells in a series of 19 melanoma samples (Fig. 8a–c). Double positive cells correspond to activated Tregs in human tissues: even though FOXP3 can be expressed in non-Treg T cells, GARP expression is induced by TCR stimulation in Tregs, but not in other T cells¹⁸. We tested many anti-GARP mAbs but we could not detect specific GARP staining on sections from FFPE tissue samples. In contrast, we observed specific staining on sections of frozen cells or tissues, using three different anti-GARP mAbs (clones Plato-1, MHG-6 and LHG-10; Supplementary Fig. 12). In frozen tonsil tissue, GARP immunoreactivity was observed in a subset of FOXP3⁺ cells, but also mainly in the blood vessel wall including the endothelium, as indicated by dual CD34 staining. Tumors displayed a similar staining pattern, including GARP⁺ blood vessels and GARP⁺FOXP3⁺ Tregs. FOXP3⁺ cells (further referred to as Tregs, even though these cells may also include some activated, non-Treg T cells) and GARP⁺FOXP3⁺ Tregs were enumerated by quantitative digital imaging (Fig. 8b, c). Intra-tumoral Treg abundance varied greatly, ranging from almost zero per 10⁵ nuclei, to similar counts to tonsil tissue (± 5000 per 10⁵ nuclei). Altogether, a third (6/19) of cutaneous melanoma metastases contained ≥ 1000 Tregs and ≥ 50 GARP-expressing Tregs per 10⁵ nuclei (Fig. 8c). RNAseq analysis was performed on frozen fragments derived from the same 19 metastasis samples (Fig. 8a). Proportions of Tregs and GARP⁺ Tregs as determined by mIF correlated strongly with *FOXP3* gene expression (Fig. 8d), thereby validating the observations from staining and counting. They also correlated strongly with T-cell genes such as *CD3G* (Fig. 8d), and IFN γ responsive genes, indicating that the more T cells and activated IFN γ -producing T cells, the more Tregs and GARP⁺ Tregs in the melanoma samples (Supplementary Data 1). We used GSEA to determine whether specific gene signatures were enriched in genes whose expression correlated most closely with proportions of GARP⁺ Tregs in the melanoma samples. The gene signatures were obtained either

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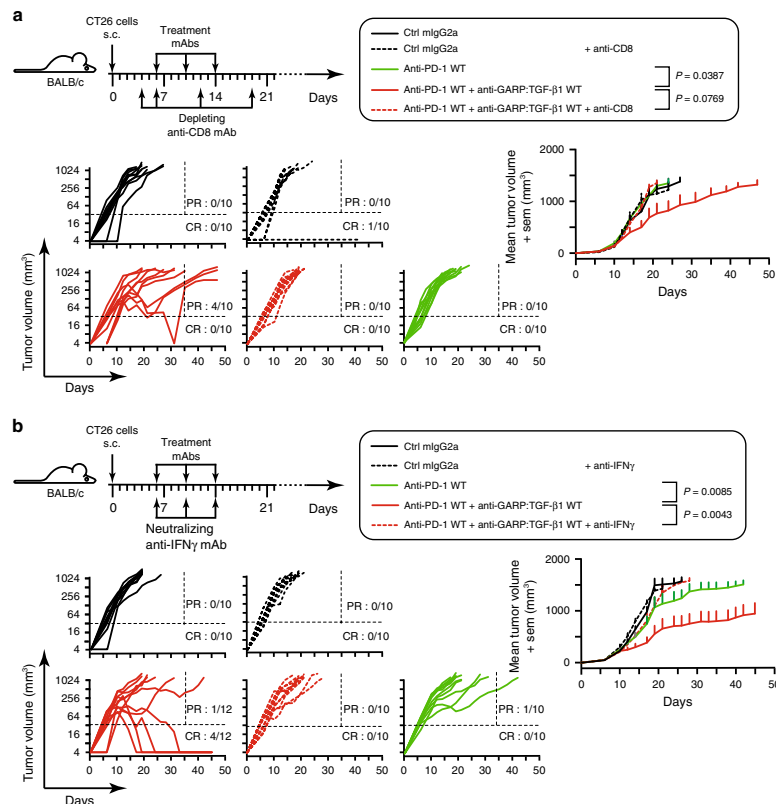


Fig. 6 Anti-tumor efficacy of combined blockade of GARP:TGF- β 1 and PD-1 requires CD8⁺ cells and IFN γ . **a** Experimental design as in Fig. 2, except that mice ($n = 10$ – 12 /group) also received injections of a depleting anti-CD8 antibody at the indicated time points. Graphs on the left show evolution of tumor volumes in individual mice. Ratios indicate the proportions of CR, (mice alive at the end of the experiment with no detectable tumor) and PR (mice alive on day 35 that carry a tumor >32 mm³ at the time of euthanasia). Dotted horizontal and vertical lines indicate the two arbitrary limits (tumor volume of 32 mm³ and day 35) used to identify PR and CR. Graph on the right shows evolution of mean tumor volumes $\pm$ sem in the five treatment groups. P values for relevant comparisons are indicated in the graphical legend and were calculated with a mixed effects model (Liu et al.⁴⁶; Sugar et al.⁴⁵), with a post-hoc Tukey's test for multiple comparisons. **b** As in **a**, except that mice received a neutralizing anti-IFN γ .

from the MSigDB public database (hallmark gene sets, <http://software.broadinstitute.org/gsea/msigdb/>) or from our own experimental data (experimental gene sets, Supplementary Data 2). Hallmark signatures of response to inflammation, IFN γ , TNF α , or IL-2 were significantly enriched at the left end of the gene list ordered by correlation with proportions of GARP⁺ Tregs (Enrichment Score >0.5 and False Discovery Rate $<0.1\%$; Fig. 8e). Similar enrichments were observed for experimental gene sets of IL-1 β , TNF α , or TGF- β 1-response induced in human melanoma cell lines, primary endothelial cells, fibroblasts, melanocytes, or CD4⁺ T cell clones (Fig. 8f). Altogether, this indicates that GARP⁺ Tregs were mostly found in inflamed melanoma metastases infiltrated by activated T cells, and were associated with higher levels of TGF- β signaling, including TGF- β signaling within the T-cell compartment.

Discussion

Our observations in tumor-bearing mice and human cutaneous melanoma metastases suggest that anti-GARP:TGF- β 1 mAbs could serve as an immunotherapeutic approach to inhibit TGF- β 1-dependent immunosuppression by intra-tumoral Tregs in patients with cancer.

In mice bearing CT26 or MC38 tumors, blocking anti-GARP:TGF- β 1 mAbs did not induce tumor regression when administered alone, in line with observations in mice carrying a Treg-specific deletion of the *Garp* gene, which did not show reduced growth of MC38 or GL261 tumors by comparison to WT (Fig. 7 and Vermeersch et al.³⁴). But when combined with anti-PD-1 mAbs, anti-GARP:TGF- β 1 mAbs significantly increased the frequency of tumor rejection relative to anti-PD-1 alone. This does not necessarily imply that anti-GARP:TGF- β 1 mAbs will have no

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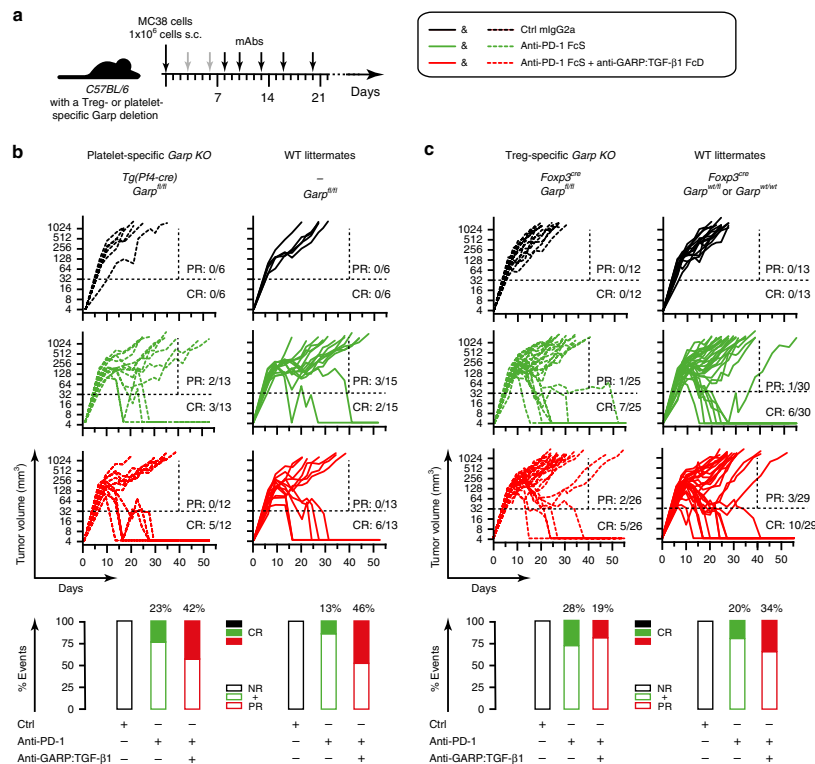


Fig. 7 Targeting GARP on Tregs is sufficient to increase the anti-tumor activity of anti-PD-1 blockade. **a** Schematic representation of the experimental design. Genetically modified C57BL/6 mice were injected s.c. with live MC38 cells on day 0. Anti-GARP:TGF- β 1 and isotype control mAbs (250 μ g/mouse) were injected i.p. on day 3, 6, 8, 10, 13, 16, and 20 (gray and black arrows). Anti-PD-1 mAb (25 μ g/mouse) was injected on day 8, 10, 13, 16, and 20 (black arrows). Mice were euthanized when the tumor surface was ≥ 200 mm². **b** Evolution of individual tumor volumes in groups of mice carrying a platelet-specific deletion of GARP (left) and their littermate controls (right). **c** Same as **b**, for mice carrying a Treg-specific deletion of GARP (left) and their littermate controls (right). Results pooled from two independent experiments. **b**, **c** Each line represents one mouse. Ratios indicate the proportions of CR (live mice with no detectable tumor at the end of the experiment) and PR (mice alive on day 40 that carry a tumor > 32 mm³ at the time of euthanasia). Dotted horizontal and vertical lines indicate the two arbitrary limits (tumor volume of 32 mm³ and day 40) used to identify PR and CR. Rectangles on the bottom represent proportions (%) of CR (filled rectangles), and partial + non-responders (PR + NR, empty rectangles).

anti-tumor activity as monotherapy in patients with cancer, but it does suggest that they are able to overcome primary or acquired resistance to PD-1/PD-L1 blockade.

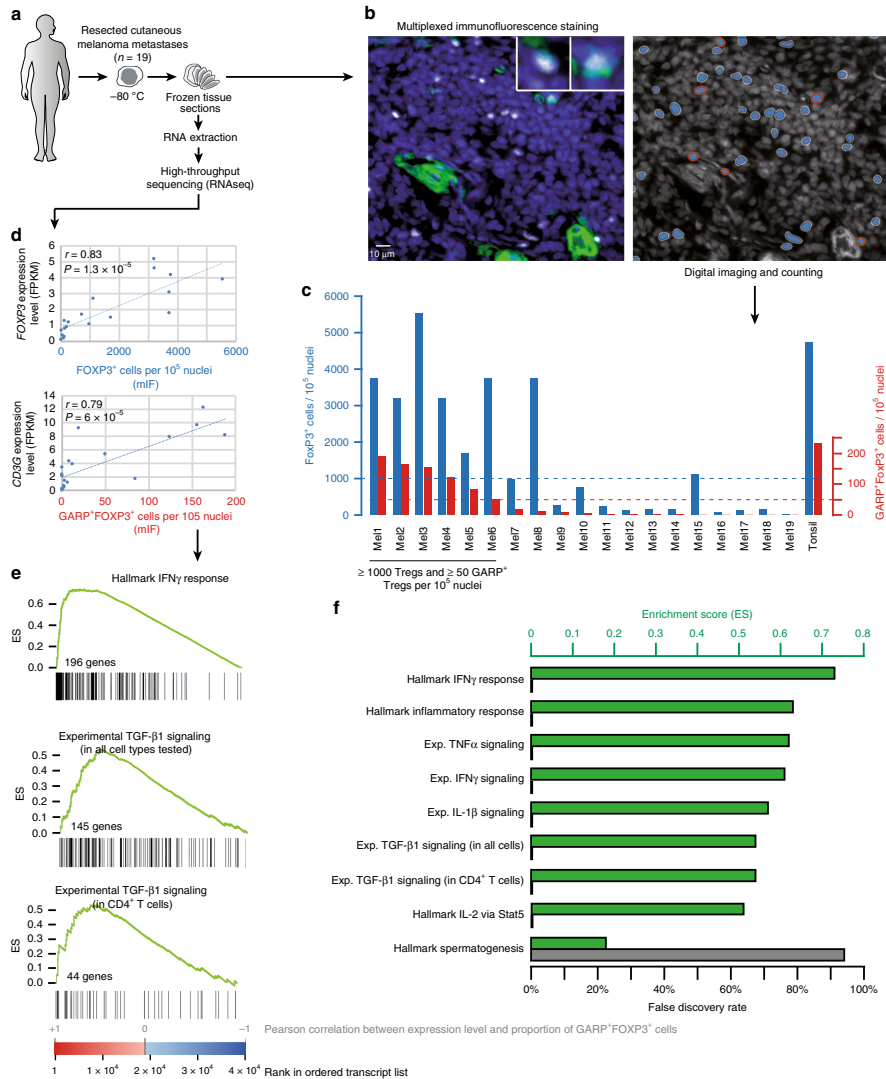
Our experiments in cell-specific *Garp* KO mice suggest that blocking the activity of TGF- β 1 emanating from GARP-expressing Tregs is required for anti-GARP:TGF- β 1 to exert anti-tumor activity. They suggest also that blocking the activity of TGF- β 1 emanating from GARP-expressing platelets or endothelial cells is neither necessary nor sufficient, although this requires further investigation.

In WT mice, combination therapy required CD8⁺ cells and IFN γ signals for efficacy, and increased the expression of multiple effector molecules by anti-tumor CD8⁺ TILs. Notably, tumors from untreated mice were already heavily infiltrated by immune cells, and densities of TILs or TIL subsets (including anti-tumor

CD8⁺ T cells) were not modified by anti-GARP:TGF- β 1, anti-PD-1, or a combination of the two. This suggests that in MC38 or CT26 models at least, blocking the activity of TGF- β 1 emanating from GARP-expressing Tregs triggers tumor regression by inducing or re-invigorating inflammatory and cytolytic activities of anti-tumor CD8⁺ T cells that are already present in the tumor.

This mode of action strikingly differs from those reported by Mariathasan et al.³⁵ and Dodagatta-Marri et al.³⁶ for neutralizing anti-TGF- β mAbs combined with PD-1/PD-L1 blockade. In the former report, therapeutic co-administration of anti-TGF- β and anti-PD-L1 mAbs was found to reduce TGF- β signaling in stromal cells such as fibroblasts and increase penetration of CD8⁺ T cells into EMT6 tumors, thus provoking anti-tumor immunity and tumor regression³⁵. In the latter report, anti-TGF- β combined with anti-PD-1 was suggested to act by blocking TGF- β

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activity on CCK168 tumor cells and intra-tumoral Tregs. This was based on observations that anti-TGF- β reduced the tumor cell SMAD3 phosphorylation and Treg/Th ratios, which were increased by treatment with anti-PD-1 alone³⁶. Variation in the proposed modes of action of anti-GARP:TGF- β 1 and anti-TGF- β may result from different tumor models being used in our laboratories. It could result also from the different sources of TGF- β activity blocked by the various mAbs: anti-GARP:TGF- β 1

only blocks TGF- β 1 emanating from GARP-expressing cells such as Tregs, whereas anti-TGF- β neutralizes activity of all three TGF- β 1, β 2, and β 3 isoforms, regardless of their cellular source. Our data suggest that blocking only the TGF- β 1 produced on Treg surfaces with anti-GARP:TGF- β 1 mAbs is sufficient to increase the anti-tumor activity of PD-1/PD-L1 targeting, while exerting less undesired effects on the tumor microenvironment than that induced by broad blockade of all TGF- β activity. In

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Fig. 8 Association between inflammatory gene signatures and activated Tregs in a series of 19 human melanoma metastases. **a** Experimental setting. See text for details. **b** FOXP3 and GARP-expressing cells were stained by mIF on frozen tumor sections. Left panel: representative view from tumor sample Mel1. Nuclei appear in blue, FOXP3 in white and GARP in green. Upper right: detail of two FOXP3⁺GARP⁺ cells. Right panel: automated detection of FOXP3⁺GARP⁺ (blue) and FOXP3⁺GARP⁺ cells (blue ringed red). **c** Diagram representing the proportion of FOXP3⁺ and FOXP3⁺GARP⁺ cells in each tumor sample, calculated from the automated counts of nuclei, FOXP3⁺ and FOXP3⁺GARP⁺ cells. **d** Top: graph showing the Pearson correlation between the proportion of FOXP3⁺ cells and the level of expression of the FOXP3 gene, obtained by RNAseq analysis of tissue sections from the same tumors. Bottom: idem with FOXP3⁺GARP⁺ cells and CD3G. Indicated *P*-values for Pearson correlation were calculated with a two-sided *t*-test. **e** Gene set enrichment analysis (GSEA). The 38,602 transcripts measured by RNAseq were ordered by decreasing Pearson correlation between their expression level and the proportion of FOXP3⁺GARP⁺ cells in the 19 tumor samples. Black vertical bars indicate positions of genes from various gene sets in the ordered transcript list. Gene set enrichment in the ordered transcript lists are plotted as green curves, with the Enrichment Score (ES) corresponding to the maximum value. Upper panel: gene set of the hallmark IFN γ signature (MSigDB). Middle and bottom panels: gene sets induced by TGF- β 1 as determined in expression microarray experiments in which human melanoma cell lines, primary endothelial cells, fibroblasts, melanocytes, or a CD4⁺ T cell clone were exposed to the recombinant cytokine (middle panel: genes induced by TGF- β 1 in at least one of the five cells types; bottom panel: genes induced by TGF- β 1 in CD4⁺ T cells; See also Supplementary Data 2). **f** ES (green rectangles) and false discovery rate (gray rectangles) measured by GSEA for the indicated gene sets. The false discovery rate was obtained by calculating ES for 1000 gene-set permutations.

human melanoma samples infiltrated by T cells and GARP-expressing Tregs, we found evidence of TGF- β signaling within the T cell compartment, suggesting that blockade of Treg-derived TGF- β 1 activity with anti-GARP:TGF- β 1 mAbs may be sufficient to increase CD8⁺ T-cell-mediated anti-tumor immunity, while avoiding potential toxicity associated with a more global inhibition of TGF- β signaling.

Fc γ R-dependent functions of immunostimulatory mAbs affect their anti-tumor activity in different ways. Anti-CTLA-4 and anti-PD-L1 mAbs must bind activating Fc γ Rs to exert potent anti-tumor activity by ADCC- or ADPC-mediated depletion of Tregs, or myeloid and tumor cells, respectively^{10,25,37–39}. On the contrary, anti-PD-1 mAbs are more potent in formats that do not bind Fc γ Rs, because this prevents cross-linking and agonistic activity or depletion of PD-1-expressing anti-tumor CD8⁺ T cells^{25,40}. Here, we establish that anti-GARP:TGF- β 1 mAbs do not require Fc γ R-dependent functions to exert anti-tumor activity in mice, supporting a mode of action by which they block TGF- β 1 activation and downstream signaling without depleting GARP-expressing cells. This suggests that in cancer patients, blocking anti-GARP:TGF- β 1 mAbs that are unable to bind Fc γ Rs would be as efficient and probably safer than effector-competent formats, because they would not cause Treg depletion and potential subsequent auto-immune adverse events, nor kill any other GARP-expressing cells in cutaneous melanoma metastases and non-cancerous tissues.

Taken together, our results support the clinical evaluation of blocking anti-GARP:TGF- β 1 mAbs, administered alone or in combination with other therapeutic strategies, to treat patients with cancer resistant to currently available immunotherapies. A phase I trial was recently initiated to test such antibodies in the clinics (ClinicalTrials.gov: NCT03821935).

Methods

Mice. BALB/c, *Garp*^{YSG/YSG} C57BL/6, Treg-specific-, and platelet-specific-*Garp* KO mice were bred at the SPF animal facility of the UCLouvain. Cell type specific *Garp* KO and WT littermates were obtained by crossing *Lrrc32*^{tm1.1Hsfj} with *B6.129* (*Gg*)-*Foxp3*^{tm1.1Hsfj} or *Tg(Pf4-icre)Q3Rsko* mice. WT C57BL/6 mice used in Fig. S3 mice were bred at the conventional animal facility of the KULeuven. The facility is controlled to maintain temperature between 20 and 24 °C; HR between 40 and 65% and day-night cycles of 12h–12h. All animal studies were performed in accordance with national and institutional guidelines for animal care, under permit numbers 2015/UCL/MD/19 and 2019/UCL/MD/032 at the UCLouvain, and 263–2014 at the KULeuven.

Cell lines. CT26 colon carcinoma, EMT6 breast carcinoma, RENCA renal carcinoma, MC38 colon adenocarcinoma and 293T cells were maintained in vitro as a monolayer culture in Iscove's Modified Dulbecco Medium (CT26), RPMI (EMT6 and RENCA), or Dulbecco's Modified Eagle Medium (293T and MC38), supplemented with 10% fetal calf serum (FCS) and 10 mM Hepes, 1 mM sodium

pyruvate, and 0.1 mM non-essential amino acids, at 37 °C in an atmosphere of 8% or 5% CO₂ in air. Murine tumor cells in exponential growth phase were harvested, washed in PBS, and resuspended in endotoxin-free Dulbecco's PBS (Millipore) prior to s.c. inoculation into mice.

Anti-GARP:TGF- β 1 mAb 58A2. To determine whether clone 58A2 bound free GARP, GARP:TGF- β 1 complexes or both, 293T cells were transiently transfected with plasmids encoding HA-tagged mouse GARP, mouse TGF- β 1, or both, and analyzed by flow cytometry 24 h after transfection with anti-HA, anti-LAP, or mAb 58A2. Binding of 58A2 to murine Tregs was evaluated by performing flow cytometry on mouse splenocytes that had been stimulated in vitro or not during 24 h with anti-CD3/28 coated beads (ThermoFisher). Antibodies used for staining were: anti-CD4, anti-FOXP3, biotinylated anti-GARP:TGF- β 1 58A2, and streptavidin coupled to BV421. The ability of 58A2 to block active TGF- β 1 production by murine Tregs was evaluated using CD25⁺ cells isolated from mouse spleens with anti-CD25 microbeads and an AUTOMACS sorter (Miltenyi) as a source of murine Tregs. Sorted CD25⁺ cells were highly enriched in Tregs and contained $\pm$ 70% of CD4⁺FOXP3⁺ cells as determined by intracellular FACS analyses. Sorted CD25⁺ cells were stimulated in vitro with anti-CD3/CD28 coated beads during 24 h in presence or absence of 20 μ g/ml of clone 58A2 (mIgG2a WT or FcD), or control antibodies (mIgG2a isotype control Motavizumab, or anti-TGF- β), and were analyzed by Western blot with antibodies against β -ACTIN (Sigma) or phosphorylated SMAD2 (Cell Signaling Technology) as a read-out for active TGF- β 1 production²¹. ECL signals were quantified using the BioID software. Full scans of Western blots are shown in Supplementary Fig. 14.

Binding of mAbs to Fc γ receptors. Recombinant biotinylated extracellular domains of immunoglobulin Fc γ receptors (Fc γ Rs) were purchased from Sino-Biologicals. Interactions of mAbs with Fc γ Rs were measured using the Biacore T200 (GE Healthcare). Briefly, the biotin CAPture kit was used to immobilize biotinylated Fc γ Rs to the sensor chip. Prior to measurements, one conditioning cycle consisting of three serial regeneration steps (1 min, flow rate 10 μ L/min) was performed. In each measurement cycle, Biotin CAPture reagent was applied to the reference and measurement channel (5 min, flow rate 2 μ L/min). Biotinylated Fc γ Rs at a concentration of 1 μ g/mL were injected (2 min, flow rate 10 μ L/min) in the active flow cell only. For each kinetics experiment, five dilutions of purified mAb were applied at concentrations in the range of 6.2–500 nM for binding to Fc γ RI, 12.8–8000 nM for Fc γ RII/III, and 0.8–8000 nM for Fc γ RIV. Interaction analysis was performed in the single-cycle kinetic mode (2 min at 30 μ L/min) followed by 10 min of dissociation. Flow cells were regenerated (2 min, 20 μ L/min) with a 6-M guanidine-HCl/0.25 M NaOH solution. Data were collected using dual detection at 10 Hz and analyzed using the Biacore T200 Evaluation Software.

Animal experiments. On day 0, live CT26 cells (10⁶ cells/mouse) or MC38 cells (1.5 or 0.5 $\times$ 10⁶ cells/mouse in Supplementary Figs. 3 and 4, respectively) were injected s.c. into 6- to 12-week-old syngeneic mice. Large (D) and small (d) tumor diameters were measured with a caliper every 2 or 3 days starting on day 6. Mice were euthanized for ethical reasons when the tumor surface (D $\times$ d) reached 200 mm² (Figs. 2, 3, 4, 6, 7 and Supplementary Fig. 4), or when the tumor volume reached 1300 mm³ or the surface was ulcerated (Supplementary Fig. 3). Tumor volumes were calculated as follows: $V = \pi \times D \times d^2/6$. When tumors were not palpable or too small to be measured (i.e. prior to day 6, or after complete tumor rejection), volumes were arbitrarily set to 4 mm³. On days indicated in the figure legends, mice received intraperitoneal (i.p.) injections of the following mAbs, administered alone or combined as indicated in the figure legends: isotype control (motavizumab), anti-GARP:TGF- β 1 (clone 58A2, mIgG2a WT or FcD), anti-PD-1 (WT or FcSilent), or anti-TGF- β . Once a tumor reaches the maximum tolerated size (pre-defined as an ethical humane endpoint in our protocol), the

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corresponding mouse was euthanized. The last tumor size measured prior to euthanasia (i.e. maximum size) is carried forward in the data series for later time points. Average tumor size per group is calculated for all tumors, including the largest tumors, at all time points. As soon as all mice in a given group have been euthanized, the average size is not computed anymore for that group, and the average growth curve is interrupted on the graphs.

For re-challenge experiments, BALB/c mice that had rejected CT26 tumors injected in their left flank on day 0 were re-injected s.c. with live tumor cells in both flanks on day 61. CT26 cells (10^6 cells/mouse) were re-injected in the right flank, and RENCA cells (3×10^5 cells/mouse) or EMT6 cells (10^6 cells/mouse) were injected in the left flank.

In some experiments, mice received i.p. injections of a depleting anti-CD8 mAb (0.5 or 0.25 mg/mouse, as indicated in the legend of Fig. 6) on days 4, 6, 12, and 19 after tumor cell inoculation. In other experiments, mice received i.p. injections of a neutralizing anti-IFN γ mAb (clone FX4F3⁴¹, 0.25 or 0.1 mg/mouse) on days 6, 10, and 14.

Monitoring of immune responses in tumor-bearing mice. On day 13 after inoculation of tumor cells performed as indicated above, tumors were harvested and mechanically dissociated in the presence of enzymes (Collagenase I 100 mg/ml, Life Tech; Collagenase II 100 mg/ml, Life Tech; Dispase 1 mg/ml, Life Tech; and DNase I 0.4 U/ml, Roche), using two cycles in the GentleMac disruptor (Miltenyi) separated by 30 min of incubation at 37 °C. Tumor cell homogenates were clarified through 70 μ m and 40 μ m filters. Single-cell suspensions were counted on a Luna[®] cell counter with a live-dead cell marker, then pelleted, and resuspended either in PBS containing 2 mM EDTA and 1% FCS for immediate staining, or in X-Vivo 10 medium (Invitrogen) to shortly stimulate cells prior to staining.

Cells used for immediate staining were incubated with antibodies against surface markers (CD45, CD4, CD8 α , NKp46, and GARP) in the presence of a viability dye (eBioscience) and anti-CD16/32 to block Fc γ Rs, fixed and permeabilized using FOXP3/Transcription Factor Staining Buffer set (eBioscience) during 20 h at 4 °C, then stained with anti-FOXP3 in the presence of anti-CD16/32.

Cells used for ex vivo stimulations were incubated at 37 °C during 4 h in the presence or absence (no stimulation controls) of AH1 peptide (SPSYVYHQF, 10 μ M), or PMA and Ionomycin (both from Sigma and at 500 ng/ml). Brefeldin A (Sigma, 5 μ g/ml), anti-CD107a coupled to BV421 and the H2-L^I/AH1 tetramer coupled to PE (synthesized in-house) were added to the stimulation mix. After stimulation, cells were stained with antibodies against surface markers (CD16/CD32, CD45, CD4, and CD8 α) in the presence of a viability dye (eBioscience) and anti-CD16/32 to block Fc γ Rs, fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences), then stained with antibodies against intracellular cytokines (IFN γ and TNF α) in the presence of anti-CD16/32. Analyses were performed on a FACs LSR Fortessa flow cytometer (DIVA, BD Biosciences) and data were computed using the FlowJo software (Tree Star). Representative examples of gating strategies are illustrated in Supplementary Fig. 13.

RT-qPCR and RNAseq of mouse tissue samples. Mouse spleen and tumor fragments were collected and stored at –80 °C until processing. After tissue disruption with the Tissue Lysar (Qiagen), total RNA was extracted using Nucleospin Mini Columns (Macherey Nagel).

For RT-qPCR, RNA was reverse transcribed with Maxima First Strand cDNA Synthesis Kit (ThermoFisher). qPCR was performed in a StepOnePlus device (ThermoFisher) in reaction volumes of 20 μ l containing 0.025 U/ μ l of Takyon Master Mix (Eurogentec), 300 nM of each primer, and 100 nM of Takyon probe under either standard conditions (95 °C for 3'; 45 cycles of 95 °C for 10' and 60 °C for 30') or fast conditions (95 °C for 3'; 95 °C for 3' and 60 °C for 30') depending on amplicon size. Sequences of primers and probes are listed in Supplementary Table 1.

For RNAseq, RNA quality was verified with an Agilent Bioanalyzer. Sequencing was outsourced to Macrogen and performed on an Illumina HiSeq2000 with the following requirement: 100 pb of read length, paired-end reads, and 30 M reads/sample. Sequences were aligned to the GRChm38 mouse genome using the STAR software, and read counts were determined with the HTSeqCount software. Raw read counts were normalized using the DESeq2 package⁴² and the R software.

Human samples for RNAseq and multiplexed immunofluorescence. Tumors and healthy tissues were obtained as surgical discard samples, or as research-aimed surgery or biopsy, after informed consent and under approval of the Commission d'Ethique Biomédicale Hospitalo-Facultaire, Brussels, Belgium (reference CEHF 2014/457).

RNA extraction and RNAseq of human tissue samples. Total RNA was extracted from sequential 10–30 μ m cryosections cut from frozen tumor tissue, using the guanidinium isothiocyanate/cesium chloride procedure⁴³. RNA quality was verified with an Agilent Bioanalyzer. A short-insert cDNA library was prepared with the TruSeq RNA-seq library kit (Illumina), and sequenced as 100 nucleotide-long paired-end reads using a HiSeq2000 sequencer (Illumina, ≥ 108 reads per sample). The read sequences were aligned to the GRCh38 human genome

sequence using Hisat2, and gene expression levels, normalized according to the FPKM method, were obtained with StringTie⁴⁴.

Gene set enrichment analyses. Hallmark gene signatures of response to IFN γ , TNF α , IL-2, or inflammation were downloaded from the MSigData base^{30,32}. Experimental gene signatures were established in our laboratory using expression microarray data from various human primary or tumor cell lines exposed to cytokines in vitro. Melanoma cell lines LB2259-MEL.A and LB2667-MEL were established from the resected metastasis of two melanoma patients, and grown in IMDM medium (Invitrogen Life technologies) supplemented with 10% FCS and amino acid mix; 100 U/ml penicillin and 100 μ g/ml streptomycin. Primary keratinocytes were derived from the detached epidermis layer of a healthy skin sample and were cultured in selective CnT-02 growth medium (Celltech). Dermal fibroblasts were obtained by mincing the dermal part of the skin sample and growing the cells in the same medium as the melanoma cell lines. Primary melanocytes and dermal endothelial cells (Promocell) were amplified in Medium 254 + HMG2 (ThermoFischer Scientific) and Endothelial Cell Growth Medium (Promocell), respectively. The cultured cells were treated with either IFN γ (100 U/ml), IL-1 β (10 ng/ml), TGF- β 1 (5 ng/ml), TNF α (10 ng/ml), or no cytokine for 24 h. Total RNA was extracted and microarray assays were performed according to the Affymetrix Genechip Expression Analysis manual. Data acquisition and processing were conducted with Affymetrix Genechips Operating Software and Microsoft Excel. An additional normalization step was carried out by setting the mean expression level of each sample to 10 (arbitrary units). Expression data from a human CD4⁺ T cell clone treated or not with TGF- β 1 were obtained from GEO Series accession number GSE14330. A gene was considered induced by a given cytokine and included in the corresponding experimental gene signature if its level after cytokine treatment was >4 and was at least five times the level in the untreated condition in one of the five cell lines (experimental TGF- β 1 signature in all cells), or in the CD4⁺ T clone (experimental TGF- β 1 signature in CD4⁺ T cells). Genes of the experimental signatures are listed in Supplementary Data 2.

Multiplexed immunofluorescence on mouse tumor sections. Paraffin-embedded CT26 tumors were cut in 5- μ m-thick sections then stained with anti-CD3 (CD3: Abcam #ab16669, clone SP7, diluted 1:500) and anti-CD8 (CD8: Cell signaling #98941, clone D4W2Z, diluted 1:400) primary antibodies. Ready-to-use Dako EnVision+ System-HRP Labelled Polymer Anti-Rabbit was used as detection reagent. Nuclei were stained with Hoechst reagent.

Multiplexed immunofluorescence on human tumor sections. Two adjacent 7- μ m-thick cryosections freshly cut from frozen tonsil or tumor tissue were mounted on a microscope slide and immediately fixed in formaldehyde 4% during 5 min, washed with demineralized water (dH₂O), and Tris-buffered saline (TBS), incubated with Peroxidase Blocking Reagent (Dako) for 10 min, washed with TBS supplemented with Tween20 (TBS-T), blocked with TBS + human immunoglobulins 1%, milk powder 2%, bovine serum albumin (BSA) 5% and Tween20 0.1% for 30 min, incubated with 5 μ g/ml of mouse monoclonal anti-GARP antibody (clone MHG-6, in-house), in Dako Antibody Diluent or no primary antibody (negative control) for 90 min, washed twice with TBS-T, incubated with secondary antibody (Dako EnVision + HRP Labelled Polymer Anti-mouse) for 60 min, washed twice with TBS-T, incubated with TSA Working Solution (PerkinElmer) + fluorescein dye 1:200 + H₂O₂ 0.003% for 10 min, washed thrice with TBS-T, heated in citrate buffer pH6 in a microwave oven (3 min at 900 W and 15 min at 90 W) and washed twice with dH₂O. The sections were kept immersed in dH₂O until the next day. The slides were washed with TBS and underwent the same steps as above starting from the blocking step, with mouse monoclonal anti-CD34 antibody (clone Qbend 10, Abcam) diluted 1:200 as primary antibody, and BDP FL dye 1:200 instead of fluorescein. This second staining was omitted for the staining of the tumor samples. The slides were washed with TBS and underwent the same steps as above starting from the blocking step, with rabbit monoclonal anti-FOXP3 antibody (clone D608R, Cell Signaling Technology) diluted 1:100 as primary antibody, and Cy5 dye 1:200 instead of fluorescein. Finally, the slides were incubated with nuclear fluorescent dye Hoechst33258 1:1000 in TBS-T+10% BSA for 5 min, washed once in TBS-T and once in dH₂O, mounted with Fluorescent Mounting Medium (Dako) and covered.

Slide scanning and image analysis. Digital 3 or 4-color images of the stained tissue sections were acquired with a Panoramic P250 Flash III scanner (3DHistech) equipped with a Plan-Apochromat $\times 20$ /N.A. $\times 0.8$ objective (Carl Zeiss) and a Point Grey Grasshopper 5MP camera, using DAPI1, FITC, SpRed, and Cy5 filter sets (Semrock). Images were analyzed with the Halo software and its Highplex FL, Area Quantification FL, and Cytonuclear FL modules (Indicalabs). The total number of cells was determined by dividing the total surface of nuclei by the mean nucleus size. The total number of FOXP3⁺ and FOXP3⁺GARP⁺ cells was obtained by automated counting of FOXP3⁺ nuclei, and FOXP3⁺ nuclei surrounded by GARP stain, respectively. Adequate selection of FOXP3⁺ cells was verified manually over a range (minimum 3) of fields of view. Because automated selection of FOXP3⁺GARP⁺ cells was found to comprise many false positives (mainly FOXP3⁺ cells in contact with GARP⁺ stained blood vessels or fibrotic septa), we

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validated all or a random fraction of at least 100 cells by visual inspection. 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⁺ and FOXP3⁺GARP⁺ cells was obtained by dividing the respective validated counts by the total number of cells.

Statistical analyses. With the exception of RNAseq data, all statistical analyses were performed using the JMP[®] Pro 14 software. Comparisons of measurements taken at a single time point were performed using a two-tailed, non-paired, non-parametric Wilcoxon test. Comparisons of measurements repeatedly taken on each mouse over time (i.e. longitudinal data) were made using a mixed effects model, with a repeated covariance structure of the type "compound symmetry with unequal variances", applied on log-transformed tumor volumes. This approach is recommended for repeated measures over time on individual mice^{45,46}. Post-hoc Tukey's test was performed to adjust for multiple comparisons. Statistical analyses of survival data presented in Kaplan Meier plots were performed with a Wilcoxon test. Numbers of mice (*n*) in the various experimental groups are indicated in the corresponding figure legend.

For Gene Set Enrichment analysis of RNAseq data, false discovery rate (FDR) and enrichment score (ES) were computed with the GSEA 3.0 Java software. Pearson correlations shown in Table S1 were calculated with Microsoft Excel V16.

Reporting summary. Further information on research design is available in the Nature Research Life Sciences Reporting Summary linked to this article.

Data availability

The RNAseq data have been deposited in Gene Expression Omnibus repository with the primary accession code GSE153239 and GSE153388. Mouse (https://www.ensembl.org/Mus_musculus/Info/Index) and human (https://www.ensembl.org/Homo_sapiens/Info/Index) genome sequences for analyses of RNAseq data were retrieved from Ensembl. Hallmark genes signatures used in Figs. 5 and 8 were obtained from the Molecular Signatures Database and can be downloaded from: https://www.gsea-msigdb.org/gsea/msigdb/cards/HALLMARK_INTERFERON_GAMMA_RESPONSE.html; https://www.gsea-msigdb.org/gsea/msigdb/cards/HALLMARK_INFLAMMATORY_RESPONSE.html; https://www.gsea-msigdb.org/gsea/msigdb/cards/HALLMARK_IL2_STAT5_SIGNALING.html; https://www.gsea-msigdb.org/gsea/msigdb/cards/HALLMARK_TGF_BETA_SIGNALING.html; https://www.gsea-msigdb.org/gsea/msigdb/cards/HALLMARK_TNFA_SIGNALING_VIA_NFKB.html; https://www.gsea-msigdb.org/gsea/msigdb/cards/HALLMARK_SPERMATOGENESIS.html. Microarray data used to define experimental gene signatures are accessible with the accession codes GSE14330 and GSE154558. All the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request.

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Author contributions

S.Lu. conceived the study. G.d.S., N.C., S.Li., C.B., O.B., S.Le., J.D., M.G., B.v.d.W., M.S., H.d.H., H.D., E.V., W.M., P.G.C., N.v.B., and S.Lu. analyzed the data. S.Lu., G.d.S. and

N.v.B. wrote the manuscript and performed statistical analyses. G.d.S., N.C., C.B., E.V., and W.M. performed the animals studies. N.v.B. performed mIF imaging and human RNAseq. N.v.B. and G.d.S. did the GSEA analyses. G.D.B., L.M., I.V.D.W., B.v.d.W., H.d.H., M.S., S.Li., and C.B. derived, produced, and characterized the blocking anti-murine GARP/TGF-β1 antibody.

Competing interests

G.D.B., L.M., I.V.D.W., B.v.d.W., H.d.H., and M.S. are full-time employees of argenx BVBA. Patents pertaining to the results presented in the paper have been filed under the Patent Cooperation Treaty (International application Number PCT/IB2019/053753), with S.Lu., G.d.S., S.Li., B.v.d.W., H.d.H., P.G.C., and M.S. as inventors and UCLouvain and argenx as applicants. S.Lu. received research support from argenx and owns stock options in argenx. The remaining authors declare no competing interests.

Additional information

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RESULTS

Supplementary Information

Selective inhibition of TGF- β 1 produced by GARP-expressing Tregs overcomes resistance to PD-1/PD-L1 blockade in cancer

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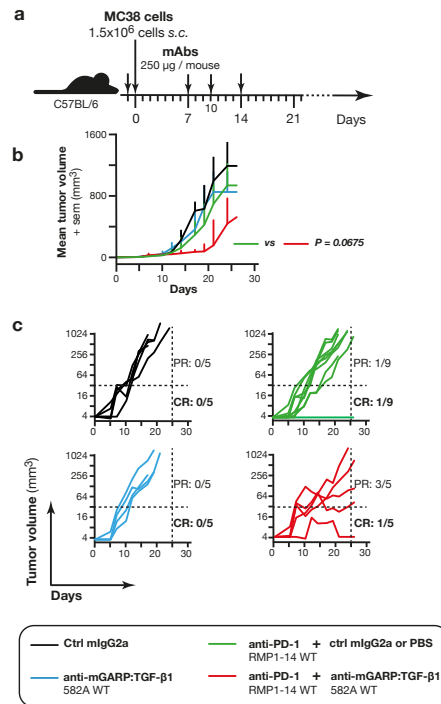
RESULTS

	anti-GARP:TGF- β 1 Clone 58A2		Isotype control Motavisumab	
	mIgG2a WT	mIgG2a FcD	mIgG2a WT	mIgG2a FcD
FcγRI	525	<i>nb</i>	533	<i>nb</i>
FcγRII	46	<i>nb</i>	91	<i>nb</i>
FcγRIII	<i>nb</i>	<i>nb</i>	16	<i>nb</i>
FcγRIV	227	<i>nb</i>	199	<i>nb</i>

Supplementary Figure 1 Binding of anti-GARP:TGF- β 1 and isotype control mAbs to mouse Fc γ Rs

Biotinylated recombinant extracellular domains of the indicated mouse Fc γ Rs were immobilized to a sensor chip and binding by anti-GARP:TGF- β 1 clone 58A2 or a control antibody (Motavisumab), in WT or FcD mIgG2a formats, was measured using Surface Plasmon Resonance (Biacore). Due to very fast dissociation, kinetic constants could not be determined. Values indicate binding response units (RU). *nb*: no binding.

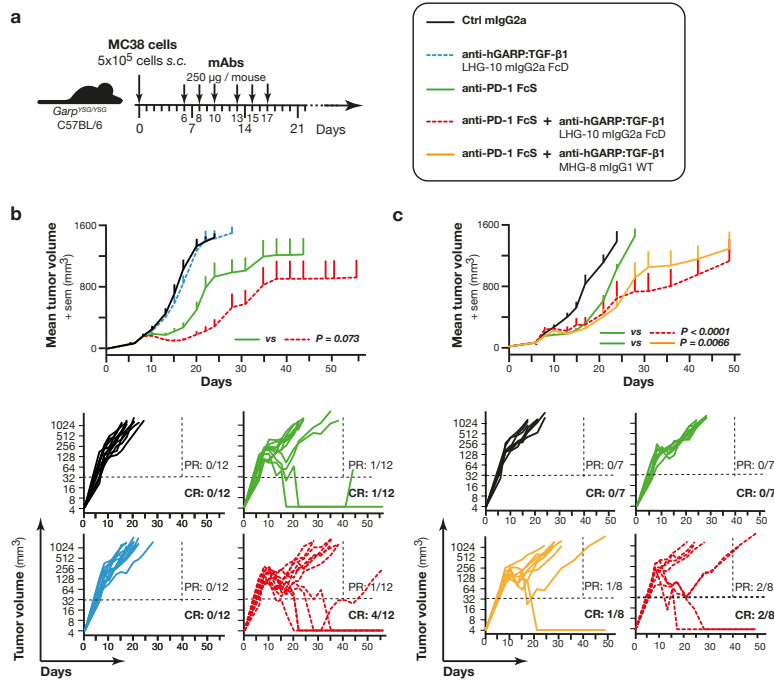
RESULTS



Supplementary Figure 2 Combined blockade of GARP:TGF- β 1 and PD-1 shows anti-tumor efficacy against MC38 tumors in WT C57BL/6 mice

a Experimental design. C57BL/6 mice were injected s.c. with live MC38 cells on day 0. Tumor diameters were measured two to three times a week. mAbs were injected *i.p.* on days -1, 7, 10 and 14. Mice treated with anti-PD-1 alone or anti-PD-1 combined with an isotype control (mlgG2a) were pooled together ($n=9$). Mice were sacrificed when the tumor surface was $\geq 200 \text{ mm}^2$. **b, c** Evolution of mean ($\pm$ s.e.m) and individual tumor volumes in the treatment groups. Ratios indicate the proportions of Complete Responders (CR, *i.e.* live mice with no detectable tumor at the end of the experiment) and Partial Responders (PR, *i.e.* mice alive on day 25 that carry a tumor $>32 \text{ mm}^3$ at the time of euthanasia). Dotted horizontal and vertical lines indicate the two arbitrary limits (tumor volume of 32 mm^3 and day 25) used to identify PR and CR. P value for the indicated relevant comparison was calculated with a mixed effects model, as recommended for analyses of longitudinal data (Liu et al., 2010; Sugar et al., 2012), with a *post-hoc* Tukey's test for multiple comparisons.

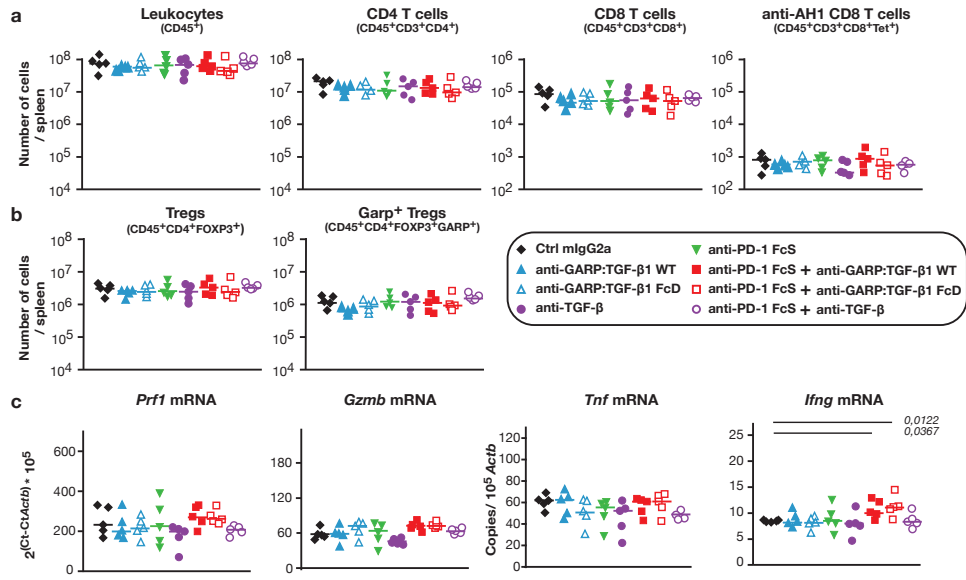
RESULTS



Supplementary Figure 3 Combined blockade of GARP:TGF- β 1 and PD1 shows anti-tumor efficacy against MC38 tumors in *Garp*^{YSG/YSg} C57BL/6 mice

a Schematic representation of the experimental design. *Garp*^{YSG/YSg} C57BL/6 mice were injected s.c. with live MC38 cells on day 0. Tumor diameters were measured twice a week. On day 6, mice were randomized in the indicated experimental groups, and mAbs were injected *i.p.* every 2-3 days from day 6 to 17. Mice were sacrificed when the tumor surface was ≥ 200 mm². **b, c** Evolution of mean and individual tumor volumes in various the treatment groups (b: n=11-12 mice per group; c: n=7-8 mice per group). Ratios indicate the proportions of CR (mice alive at the end of the experiment with no detectable tumor) and PR, (mice alive on day 40 that carry a tumor >32 mm² at the time of euthanasia). Dotted horizontal and vertical lines indicate the two arbitrary limits (tumor volume of 32 mm² and day 40) used to identify PR and CR. *P* values for relevant comparisons are indicated and were calculated with a mixed effects model (Liu et al., 2010; Sugar et al., 2012), with a *post-hoc* Tukey's test for multiple comparisons.

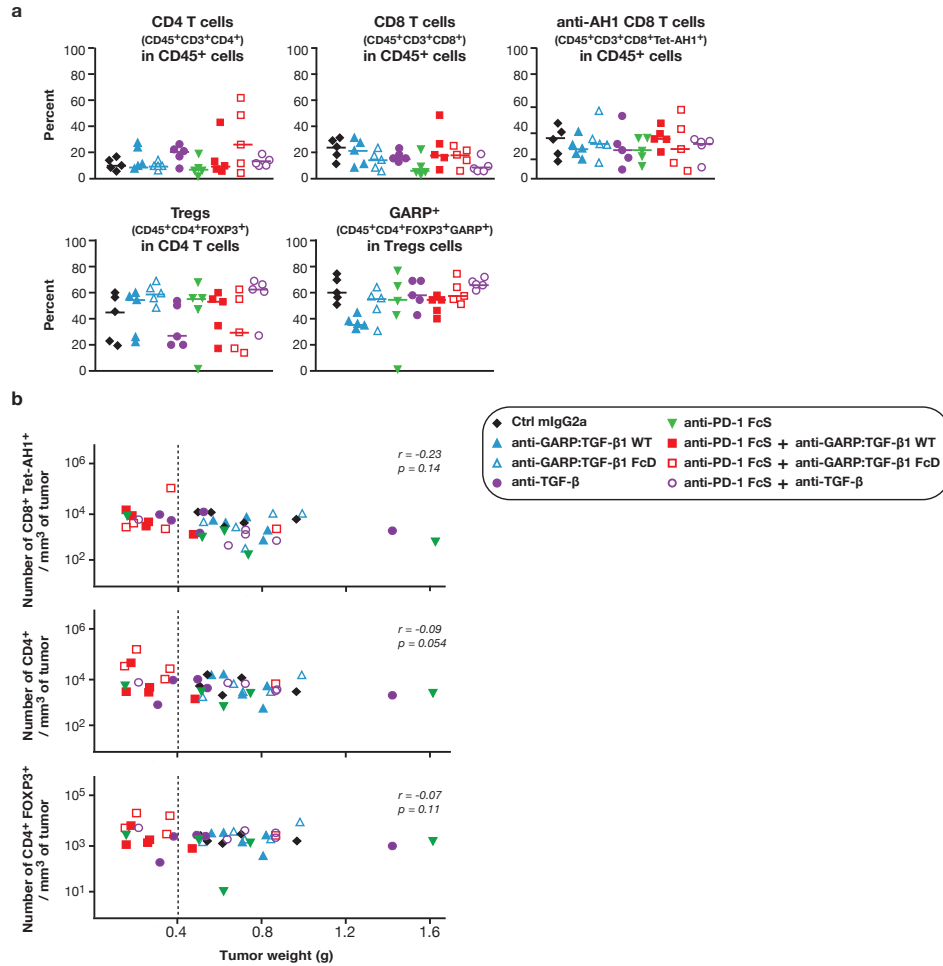
RESULTS



Supplementary Figure 4 Analyses of spleens collected from mice shown in Fig. 5.

a, b Numbers of various subsets of cells as determined by flow cytometry. Each data point represents the value measured in one mouse (n=5 mice/group). Horizontal bars represent medians per group. **c** Expression of genes encoding T cell-effector molecules, as determined by RT-qPCR. Data points and horizontal bars as in **a**. Numbers in italics indicate *P* values < 0.05 by comparison to the control group (isotype mlgG2a), calculated with a two-sided Wilcoxon test. Results shown here are representative of at least 3 independent experiments.

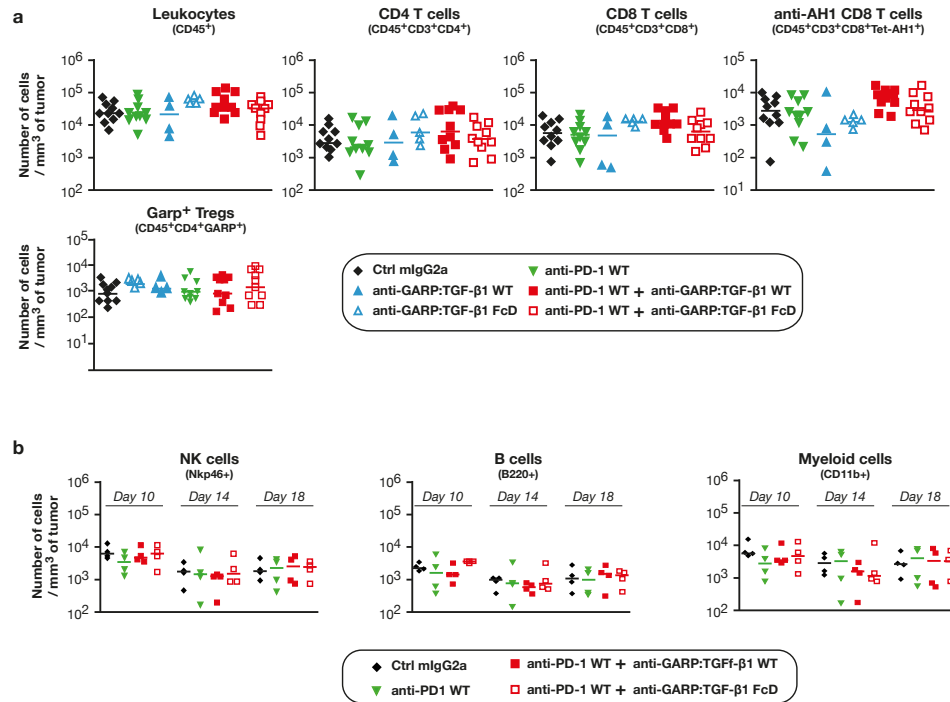
RESULTS



Supplementary Figure 5 Combined blockade of GARP:TGF- β 1 and PD-1 does not modify numbers of cells or proportions of leukocyte subsets infiltrating CT26 tumors

a Proportion of various subsets of immune cell infiltrating the tumor. Each data point represents the value measured in one mouse, and horizontal bars the median per group (n=5 mice/group). **b** Correlation between numbers of various subsets of cells infiltrating one mm³ of tumor with tumor weight in the corresponding mouse at day 13. r = Pearson's correlation coefficient, and corresponding p value as calculated with an F -test. Dotted vertical bars indicate an arbitrary limit set to define smallest tumors as in Fig. 5 (n=5 mice/group).

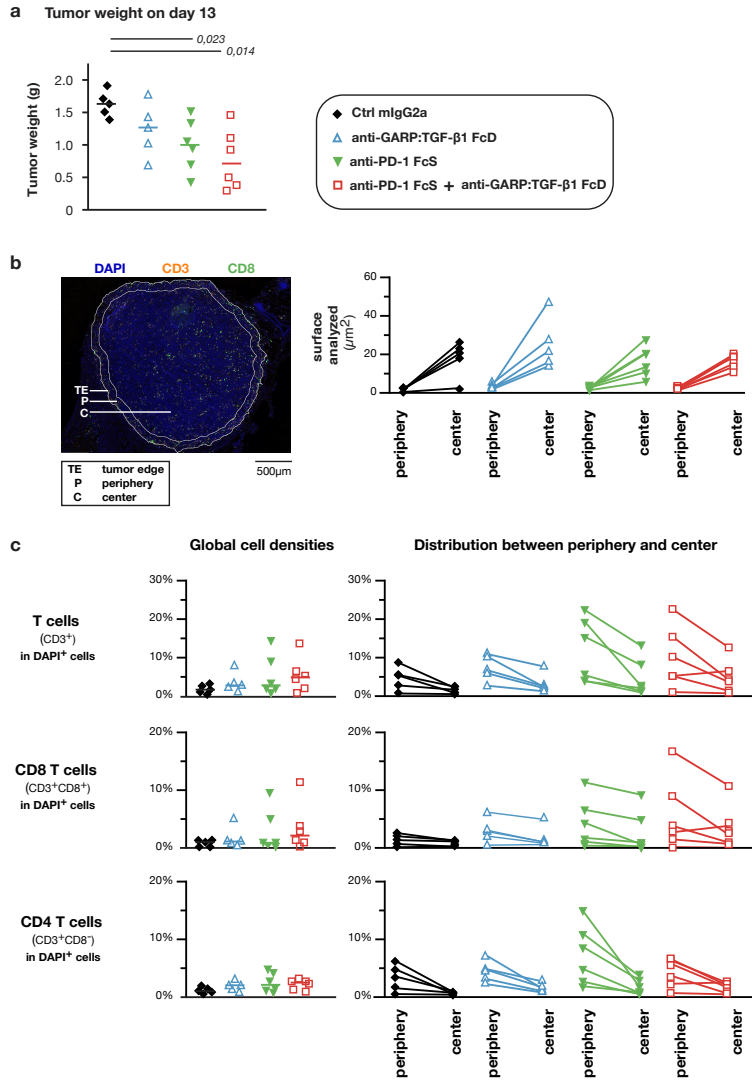
RESULTS



Supplementary Figure 6 Combined blockade of GARP:TGF-β1 and PD-1 does not modify numbers of cells infiltrating CT26 tumors
Independent experiments similar to that shown in Fig. 5.

a Pooled of two independent experiments showing numbers of various subsets of cells infiltrating one mm³ of tumor, as determined by flow cytometry. Each data point represents the value measured in one mouse, and horizontal bars the median per group. **b** BALB/c mice were injected s.c. with live CT26 tumor cells on day 0 and treated *i.p.* with the indicated mAbs on days 6, 10 and 14. Mice were sacrificed on days 10, 14 or 18 (*n* = 4 mice per group and per time point) to collect tumors for flow cytometry analyses. Graphs represent numbers of various subsets of cells infiltrating one mm³ of tumor. Each data point represents the value measured in one mouse, and horizontal bars medians per group.

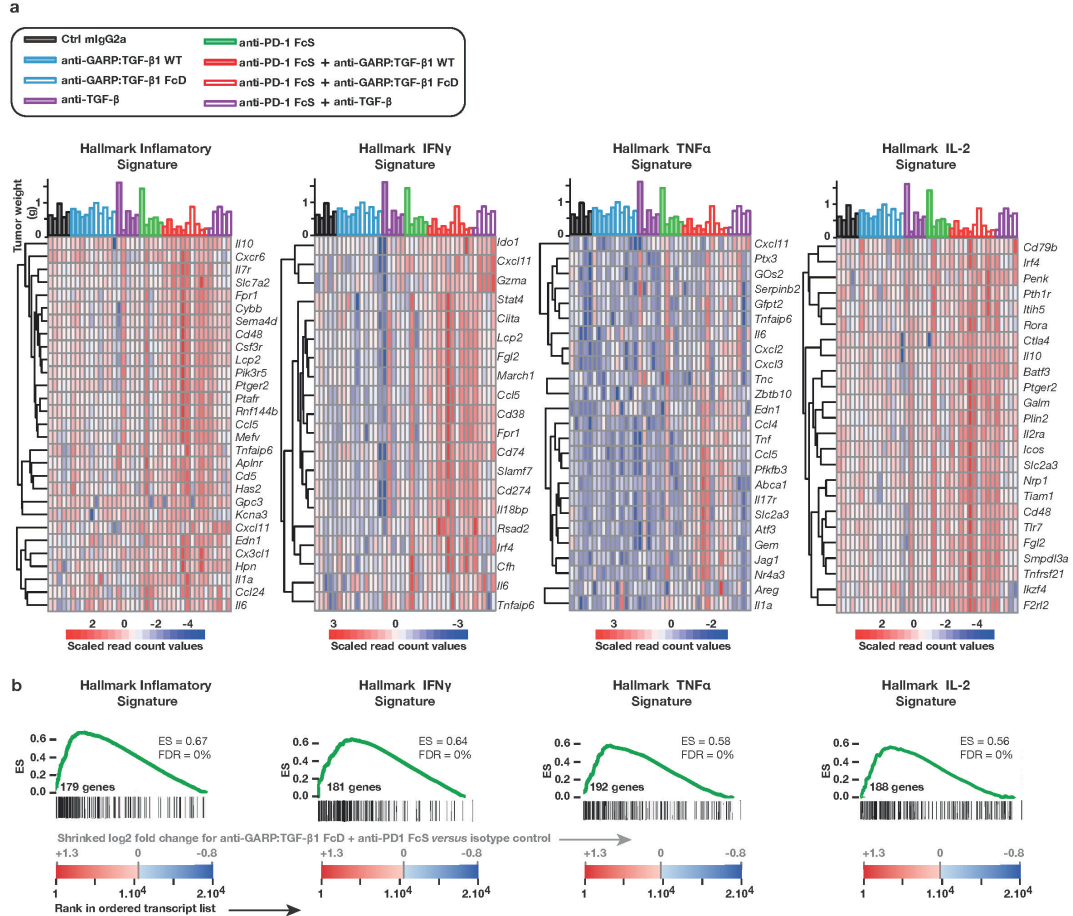
RESULTS



Supplementary Figure 7 Combined blockade of GARP:TGF- β 1 and PD-1 does not increase T cells infiltration and migration towards the tumor center by comparison to anti-PD-1 alone

BALB/c mice ($n=5-6$ /group) were injected with CT26 cells on day 0 and treated with mAbs on days 6, 9 and 12, as illustrated in Fig 2. Tumors were collected on day 13. **a** Weight of tumors on the day of collection. Each data point represents the value in one mouse, and horizontal bars the median per group. Numbers in italics show P values <0.05 for the comparisons with the control group (isotype ctrl mlgG2a), as calculated with a two-sided Wilcoxon test. **b** Left: Representation of zones defined for analysis of T cell densities (one representative tumor section is shown). Periphery is comprised within tumor edge and a concentric line located at $100\mu\text{m}$ from tumor edge. Center is defined as the rest of the tumor. Right: Surface of peripheral and central zones analysed in one tumor section per mouse. Data points from one mouse are connected by a straight line. **c** Densities of total T, CD8 T and CD4 T cells in DAPI $^{+}$ cells in the entire section (left) or in periphery and center (right), as determined by immunofluorescence microscopy followed by digital image analysis and counting. Data points and horizontal bars as in a and b. No P value <0.05 .

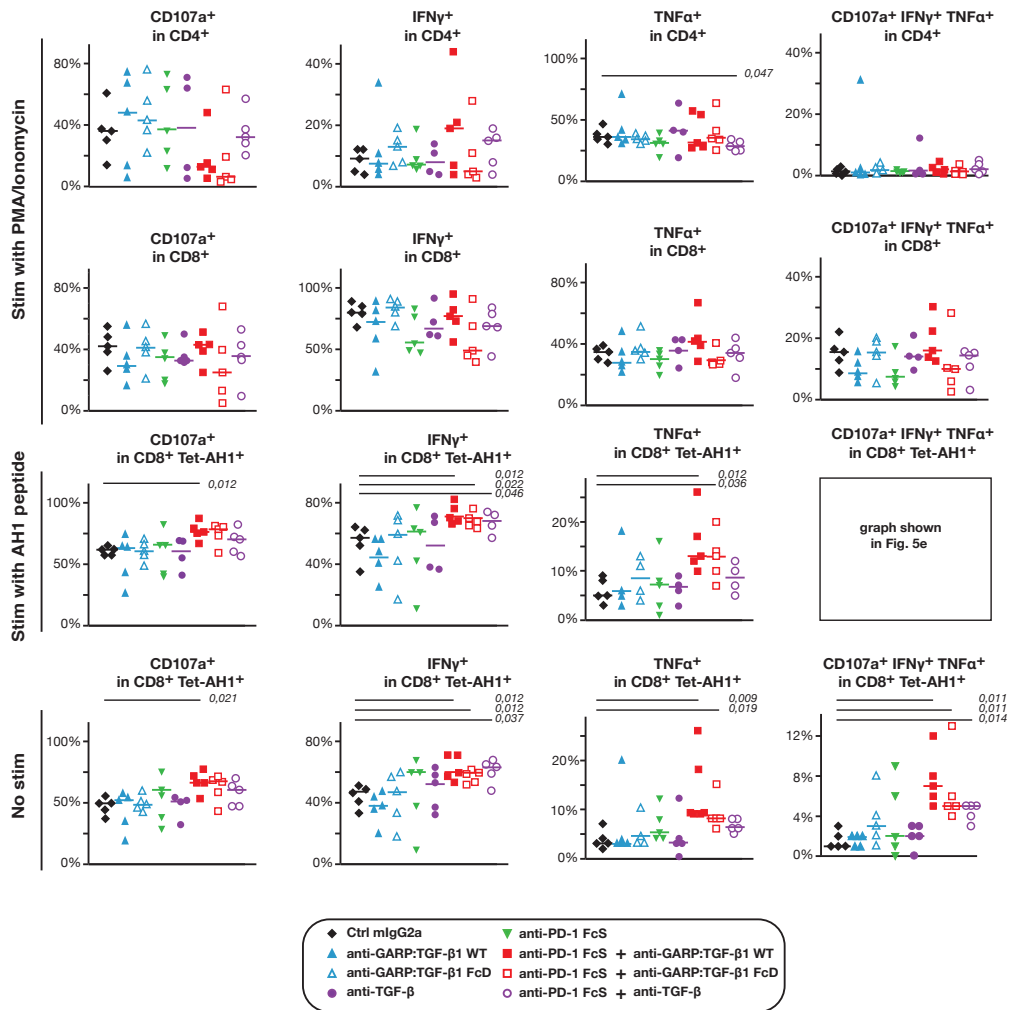
RESULTS



Supplementary Figure 8 Signatures for responses to inflammatory and T cell derived cytokines are enriched in tumors from mice treated with anti-GARP:TGF-β1 combined with anti-PD-1 mAbs

a Bar graphs show weight of tumors collected on day 13 in individual mice from experiment in Fig. 5. Heat maps show scaled transcript read counts in the corresponding mice as determined by RNAseq. Transcripts indicated in each heat map correspond to genes represented by larger dots in the violin plots of Fig. 5e (i.e. genes from the indicated hallmark signatures that show a fold change ≥ 2 for the ratio between mean expression in the treated group versus the isotype control group). **b** GSEA plots for the comparisons between groups treated with the anti-GARP:TGF-β1 FcD + anti-PD-1 FcS combination and isotype control. Transcripts identified by RNAseq are ordered by shrunk log2 fold change between the two groups. Enrichment in the ordered lists are plotted as green curves. Enrichment score (ES) and false discovery rate (FDR) statistics are indicated for each signature. FDR was obtained by calculating ES for 1000 gene-set permutations.

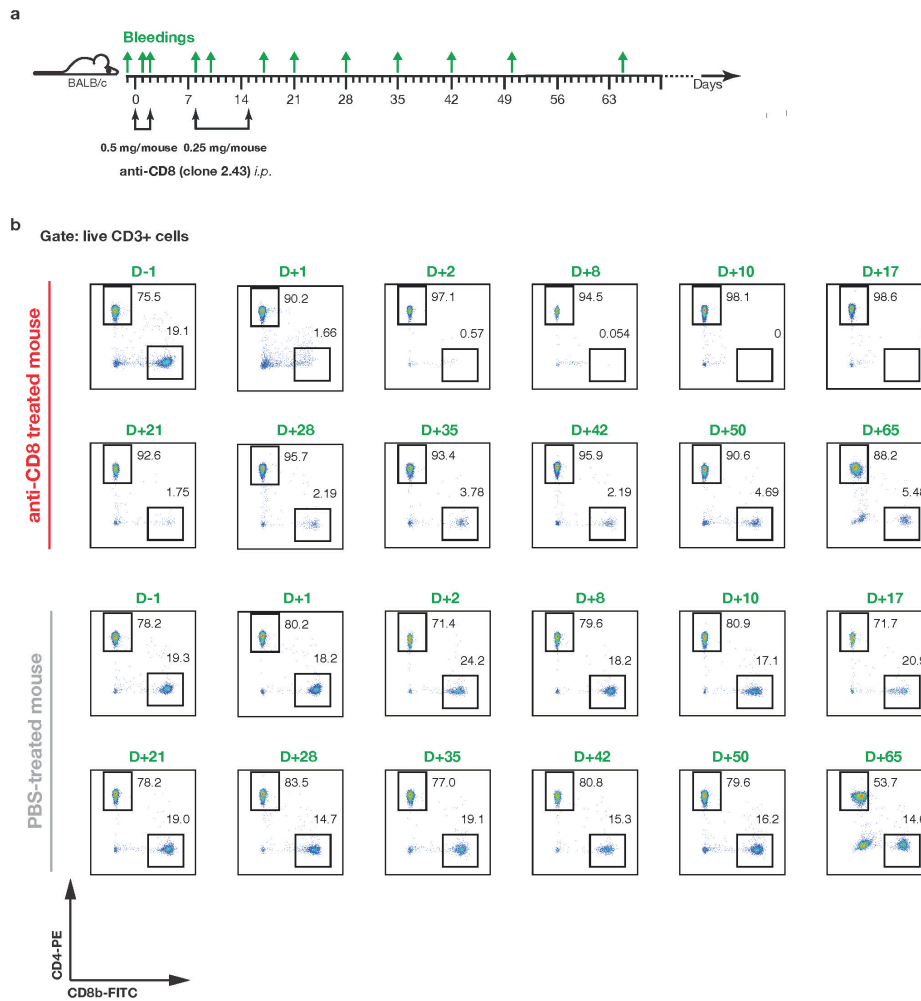
RESULTS



Supplementary Figure 9 Combined blockade of GARP:TGF-β1 and PD-1 increases effector functions of anti-tumor CD8⁺ T cells, but not that of other T cells infiltrating CT26 tumors

Cells collected from tumor samples shown in Fig. 5 were left resting or stimulated during 4 hours in vitro with PMA/Ionomycin or the AH1 peptide, then stained against various surface markers, including surface CD107a, and intracellular IFNγ and TNFα, prior to flow cytometry. Graphs show the proportions of cells expressing the indicated markers within CD4⁺, CD8⁺ or CD8⁺ Tet-AH1⁺ gates. Each data point represents the value in one mouse. Horizontal bars represent medians per group. Numbers in italics show *P* values <0.05 for the comparisons with the control group (isotype ctrl mlgG2a), as calculated with a two-sided Wilcoxon test. Results shown here are representative of at least 3 independent experiments (n=4-5 mice/group).

RESULTS

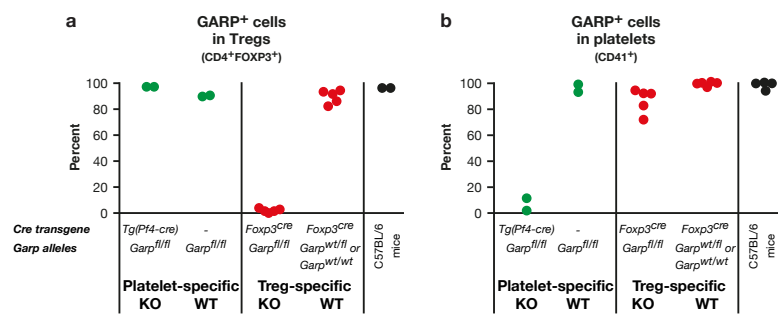


Supplementary Figure 10 Anti-CD8 mAb depletes CD8⁺ T cells in BALB/c mice

a Experimental design. Balb/c mice received *i.p.* injections of depleting anti-CD8 mAb clone 2.43 (n=1) or PBS as control (n=1), as indicated on the scheme, and were bled at various time points to measure proportions of CD8 T cells in the blood by flow cytometry. **b** Results of flow cytometry analyses. Density plots are gated on live CD3⁺ cells. Values indicate percentage of CD3⁺ cells present in the illustrated rectangular gates (CD4⁺ or CD8⁺ cells in blood CD3⁺ cells), anti-CD4-PE (clone RM4-4, Biolegend) and anti-CD8b (clone H35-17.2, eBioscience) which has been verified to not compete with depleting antibody.

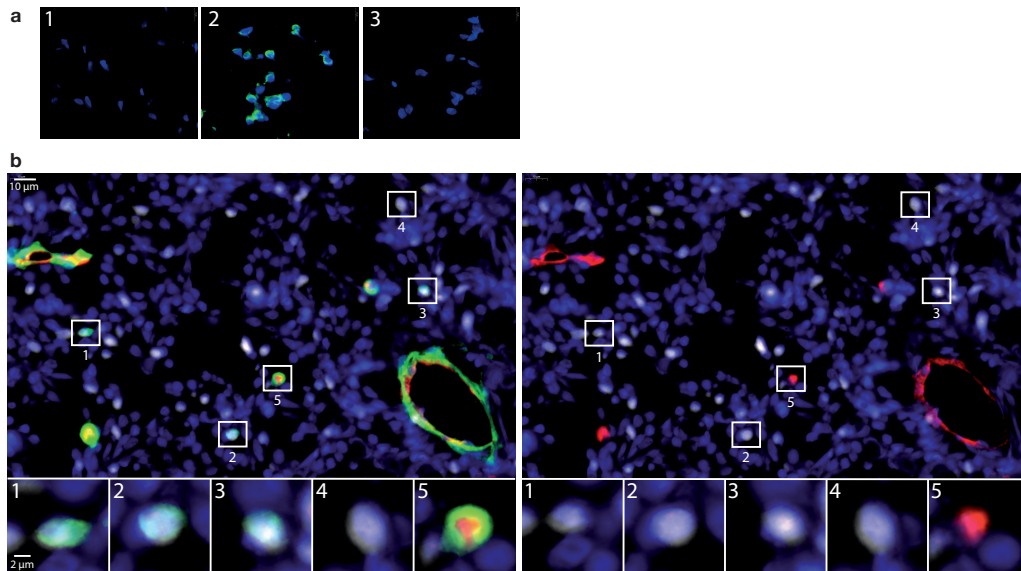
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RESULTS



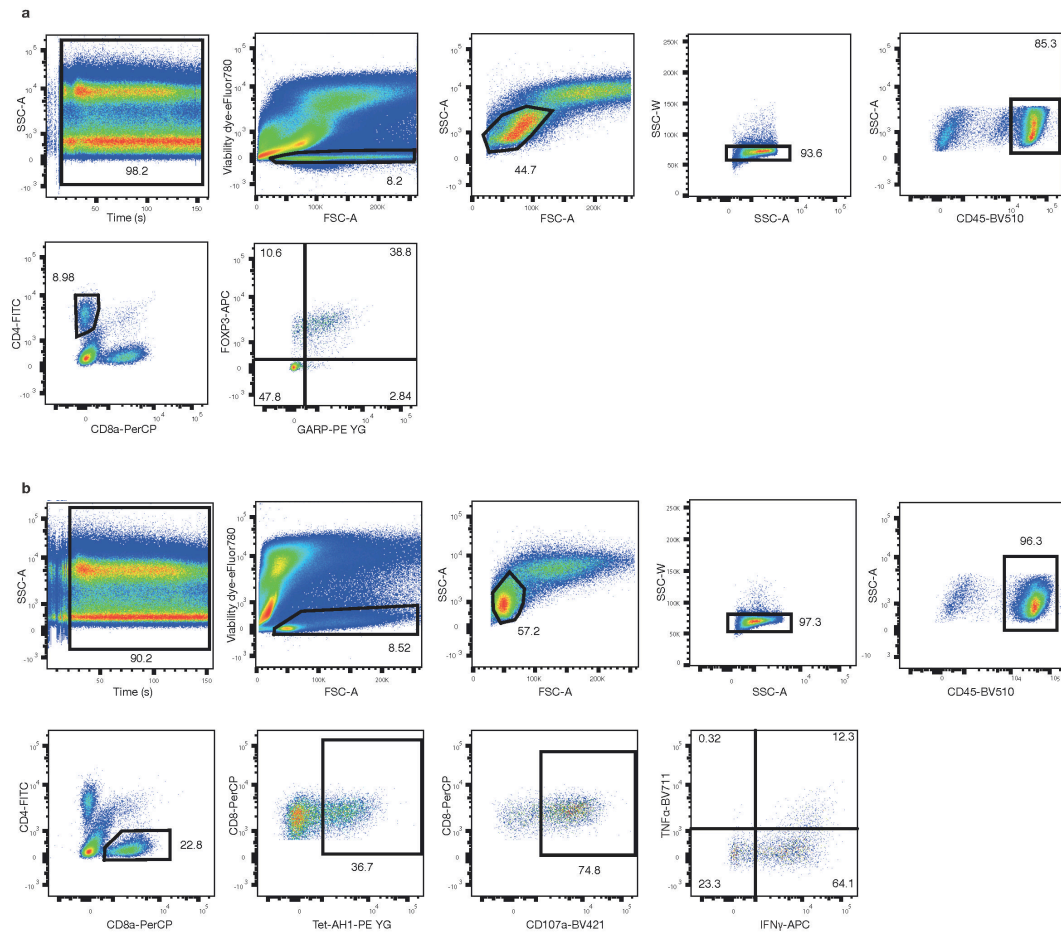
Supplementary Figure 11 Cell type-specific loss of GARP expression in Treg- and platelet- specific *Garp* KO mice.
a Splenocytes from mice of the indicated genotypes were isolated and stimulated *in vitro* with anti-CD3/28 coated beads during 24 hours, then analysed by flow cytometry. Each data point indicates proportions of GARP positive cells in CD4⁺FOXP3⁺ cells (Tregs) for one mouse (n = 2 to 5). **b** Platelets from mice of the indicated genotypes were isolated from fresh blood and analysed by flow cytometry. Each data point indicates proportions of GARP positive cells in CD41⁺ cells (platelets) from one mouse (n = 2 to 5).

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Supplementary Figure 12 Multiplexed IF allows to identify GARP-expressing Tregs and endothelial cells in sections of frozen human tonsils
a Immunofluorescence staining with the anti-GARP MHG-6 mouse monoclonal antibody on sections of frozen cell pellets from cultured Jurkat cells (1) or Jurkat transfected with a GARP-encoding plasmid (2); GARP appears as a green signal, nuclei in blue; 3: same as 2 without MHG-6. **b** Left panel: mIF staining of GARP (green), FOXP3 (white) and CD34 (endothelial cells, red) on a frozen tonsil section. Merged green and red channels appear in yellow. Bottom panels: enlarged view of three FOXP3⁺GARP⁺ cells (1-3), a FOXP3⁺GARP⁻ cell (4) and a GARP⁺CD34⁺ capillary vessel (5). Right panel: idem without the GARP (green) channel. Similar results were obtained with two other anti-GARP antibodies (clones Plato-1 and LHG-10).

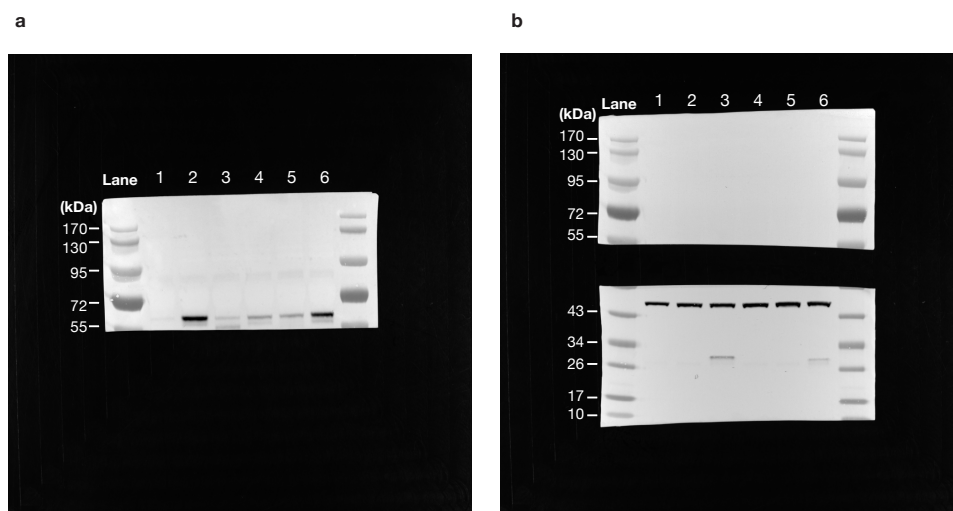
RESULTS



Supplementary Figure 13 Examples of gating strategies used for flow cytometry analyses shown in Fig. 5 and Supplementary Figures 5-6

a Representative example of the gating strategy applied to evaluate the number of Tregs and GARP⁺ Tregs infiltrating the tumor. **b** Same to evaluate infiltration of CD8⁺ Tet-AH1⁺ CD107a⁺ IFNγ⁺ TNFα⁺ cells

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Supplementary Figure 14. Full scans of Western Blot images shown in Fig.1.

a. pSMAD2

b. mouse Actin

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Supplementary Table 1. Primers sequences

Sequence	Definition
TCAAGTGGCATAGATGTGGAAGAA	Sense primer in <i>mlfng</i> mRNA
TGGCTCTGCAGGATTTTCATG	Antisense primer in <i>mlfng</i> mRNA
TCACCATCCTTTTGCCAGTTCCTCCAG	Taqman probe in <i>mlfng</i> mRNA
AAGAGAGCAAGGACAACACTC	Sense primer in <i>mGZMb</i> mRNA
CATGTCCCCCGATGATCTC	Antisense primer in <i>mGZMb</i> mRNA
ACAAGGTCAGCAGTAGCAGGAGGA	Taqman probe in <i>mGZMb</i> mRNA
CAGTAGAGTGTGCGCATGTACAG	Sense primer in <i>mPrf1</i> mRNA
GATGAGCCTGTGGTAAGCAT	Antisense primer in <i>mPrf1</i> mRNA
TCGCCTGGTACAAAAACCTCCACTC	Taqman probe in <i>mPrf1</i> mRNA
CATCTTCTCAAAATTCGAGTGACAA	Sense primer in <i>mTNFa</i> mRNA
GGGTTGTACCTTGTCTACTCCCA	Antisense primer in <i>mTNFa</i> mRNA
CACGTCGTAGCAAACCAAGTGGA	Taqman probe in <i>mTNFa</i> mRNA

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- 2 Combined blockade of GARP:TGF- β 1 and PD-1 increases infiltration of T cells and density of pericyte-covered GARP⁺ blood vessels in MC38 tumors (Bertrand *et al*, Frontiers in Immunology 2021)**

RESULTS



Combined Blockade of GARP:TGF- β 1 and PD-1 Increases Infiltration of T Cells and Density of Pericyte-Covered GARP⁺ Blood Vessels in Mouse MC38 Tumors

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When combined with anti-PD-1, monoclonal antibodies (mAbs) against GARP:TGF- β 1 complexes induced more frequent immune-mediated rejections of CT26 and MC38 murine tumors than anti-PD-1 alone. In both types of tumors, the activity of anti-GARP:TGF- β 1 mAbs resulted from blocking active TGF- β 1 production and immunosuppression by GARP-expressing regulatory T cells. In CT26 tumors, combined GARP:TGF- β 1/PD-1 blockade did not augment the infiltration of T cells, but did increase the effector functions of already present anti-tumor T cells. Here we show that, in contrast, in MC38, combined GARP:TGF- β 1/PD-1 blockade increased infiltration of T cells, as a result of increased extravasation of T cells from blood vessels. Unexpectedly, combined GARP:TGF- β 1/PD-1 blockade also increased the density of GARP⁺ blood vessels covered by pericytes in MC38, but not in CT26 tumors. This appears to occur because anti-GARP:TGF- β 1, by blocking TGF- β 1 signals, favors the proliferation of and expression of adhesion molecules such as E-selectin by blood endothelial cells. The resulting densification of intratumoral blood vasculature probably contributes to increased T cell infiltration and to the therapeutic efficacy of GARP:TGF- β 1/PD-1 blockade in MC38. We conclude from these distinct observations in MC38 and CT26, that the combined blockades of GARP:TGF- β 1 and PD-1 can exert anti-tumor activity via multiple mechanisms, including the densification and normalization of intratumoral blood vasculature, the increase of T cell infiltration into the tumor and the increase of the effector functions of intratumoral tumor-specific T cells. This may prove important for the selection of cancer patients who could benefit from combined GARP:TGF- β 1/PD-1 blockade in the clinics.

Keywords: GARP, TGF- β 1, cancer immunotherapy, immunosuppression, immune checkpoint inhibition

RESULTS

INTRODUCTION

TGF- β 1 is a potent immunosuppressive cytokine produced by most cells in an inactive, latent form. In its latent form, the mature TGF- β 1 dimer is non-covalently associated to the Latency Associated Peptide (LAP), which prevents receptor binding and signaling by the cytokine. Some cell types can activate latent TGF- β 1 in response to various stimuli by releasing mature TGF- β 1 from LAP, *via* cell-type specific mechanisms. Regulatory T cells (Tregs) stimulated through the T cell receptor (TCR) produce latent TGF- β 1 covalently linked to transmembrane protein GARP. Presentation of GARP:(latent)TGF- β 1 complexes, and the interaction with integrin α V β 8 on the surface of TCR-stimulated Tregs, leads to TGF- β 1 activation and autocrine or short-distance paracrine TGF- β 1 activity (1–4). In cancer patients, Tregs can exert detrimental immunosuppression and thus limit the efficacy of immunotherapy (5, 6).

We previously developed monoclonal antibodies (mAbs) that bind GARP:TGF- β 1 complexes and block TGF- β 1 activation and immunosuppression by human or mouse Tregs (4, 7, 8). Anti-GARP:TGF- β 1 mAbs inhibited the immunosuppressive function of human Tregs in a xenogeneic model of graft-versus-host disease induced by the transfer of human PBMCs in NSG mice (7). More recently, we reported that anti-GARP:TGF- β 1 combined with anti-PD-1 induced immune-mediated rejections of CT26 and MC38 tumors resistant to anti-PD-1 alone (8). Blocking TGF- β 1 activation by GARP-expressing Tregs was sufficient for anti-GARP:TGF- β 1 to overcome resistance to anti-PD-1 in these tumor models. Indeed, the anti-tumor activity of combined GARP:TGF- β 1/PD-1 blockade: i) occurred without Treg depletion, ii) was observed using anti-GARP:TGF- β 1 incapable of binding Fc γ receptors, and iii) was lost in MC38 tumor-bearing mice carrying a Treg-specific deletion of the *Garp* gene (8). In contrast, blocking TGF- β 1 activation by GARP-expressing platelets was not required, as anti-tumor activity of combined GARP:TGF- β 1/PD-1 blockade was conserved in MC38 tumor-bearing mice carrying a platelet-specific deletion of the *Garp* gene. Thus, in MC38, the predominant source of active TGF- β 1 that needs to be blocked by anti-GARP:TGF- β 1 to overcome resistance to anti-PD-1 are Tregs, but not platelets (8).

Further characterizing its mode of action, we observed that combined GARP:TGF- β 1/PD-1 blockade increased the effector functions of anti-tumor CD8 T cells already present within CT26 tumors, without augmenting the immune cell infiltration (8). Together with our observation that GARP-expressing Tregs are found mostly in human melanoma metastases that are already infiltrated by activated T cells, this led us to suggest testing anti-GARP:TGF- β 1 to overcome resistance to PD-1/PD-L1 blockade in patients with inflamed tumors.

Different modes of action were proposed to explain the anti-tumor activity of PD-1/PD-L1 blockade combined with mAbs that block latent TGF- β 1 activation (9) or neutralize all three TGF- β 1, β 2 and β 3 isoforms (10, 11). In these reports, infiltration of immune cells in tumors was increased as a consequence of either increased CD8 T cell entry from blood vessels (9), or reduced TGF- β 1 signaling in stromal fibroblasts

with subsequent increased CD8 T cell penetration towards the center of tumors (10, 12). The variations in the mode of action could result from the different tumor models that were used in our laboratories, or from the different cellular sources of TGF- β 1 activity that were blocked by the mAbs. While anti-GARP:TGF- β 1 selectively blocks TGF- β 1 released from GARP-expressing cells, other mAbs block TGF- β 1 activation, or neutralize the activity of all three TGF- β isoforms, whatever their cellular source. It is noteworthy that in addition to Tregs, murine and human endothelial cells and platelets do also express surface GARP:TGF- β 1 complexes. Anti-GARP:TGF- β 1 mAbs could thus exert anti-tumor activities *via* multiple modes of action, by directly or indirectly targeting different cell types depending on the tumor microenvironment (2, 8, 13).

We thus resorted to determine if the anti-tumor activity of combined GARP:TGF- β 1/PD-1 blockade could encompass other mechanisms than increasing the effector functions of anti-tumor CD8 T cells already present within tumors, as observed in CT26.

MATERIALS AND METHODS

Mice

BALB/c and C57BL/6 mice were bred at the SPF animal facility of the UCLouvain. The facility is controlled to maintain the temperature between 20 and 24°C; humidity rate between 40 and 65% and day–night cycles of 12 h–12 h. All animal studies were performed in accordance with national and institutional guidelines for animal care, under permit numbers 2015/UCL/MD/19 and 2019/UCL/MD/032 at the UCLouvain.

Antibodies

Anti-mouse GARP:TGF- β 1 clone 58A2 was described previously (8). Batches of 58A2 under mIgG1 or mIgG2a DANA formats, and corresponding isotype controls (motavizumab) were kindly provided by Dr. Bas van der Woning (argenx BV). mIgG1 and mIgG2a DANA antibodies do not exert Fc γ R-dependent activities (8, 14). Anti-PD-1 clone RMP1-14 (mIgG2a FcSilent format) was purchased from Absolute Antibodies. Anti-active TGF- β 1,2,3 clone 1D11 was purchased at BioXcell.

Cells

CT26 and MC38 colon carcinoma cell lines were maintained *in vitro* as a monolayer culture in Iscove's Modified Dulbecco Medium (CT26), or Roswell Park Memorial Institute Medium (MC38), supplemented with 10% fetal calf serum (FCS) and 10 mM Hepes, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids, at 37°C in an atmosphere of 8% (CT26) or 5% (MC38) CO₂ in air. Murine tumor cells in exponential growth phase were harvested, washed in PBS, and resuspended in endotoxin-free Dulbecco's PBS (Millipore) prior to inoculation into mice.

Endothelial cell lines C166 and MS1 were purchased at ATCC or kindly provided by the group of Agnès Noël (ULiège, Belgium), respectively. Both were maintained *in vitro* as a monolayer culture in Dulbecco's Modified Eagle Medium

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supplemented with 10% FCS, at 37°C in an atmosphere of 5% CO₂ in air.

Animal Experiments

On day 0, live CT26 cells (10⁶ cells/mouse) or MC38 cells (1 × 10⁶/mouse for mIF experiments or 1.5 × 10⁶ cells/mouse for flow cytometry experiments) were injected s.c. into 7- to 8-week-old syngeneic mice. Large (D) and small (d) tumor diameters were measured with a caliper every 2 or 3 days starting on day 6. Tumor volumes were calculated as follows: $V = \pi \times D \times d^2/6$. On days indicated in the figure legends, mice received intraperitoneal (*i.p.*) injections of the following mAbs (250 μ g of each), administered alone or combined as indicated in the figure legends: isotype control (motavizumab), anti-GARP:TGF- β 1 (clone 58A2) and/or anti-PD-1 (clone RMP1-14 FcSilent). Anti-GARP:TGF- β 1 injected in BALB/c or C57BL/6 mice were under mIgG2a DANA or mIgG1 formats, respectively (corresponding isotypes were used for the motavizumab control). All mice were sacrificed on day 13 or 14, and tumors were collected after sacrifice for further analyses.

Multiplexed Immunofluorescence on Mouse Tumor Sections

Immediately after collection, CT26 and MC38 tumors were fixed in formaldehyde (FA) 4% during 24 h, then embedded in paraffin using the Tissue-Tek VIP (Sakura). Formaldehyde-fixed paraffin-embedded (FFPE) tumors were cut in 5 μ m-thick sections and mounted on a microscope slide. Slides were deparaffinized in subsequent baths of HistoClear and decreasing concentrations of ethanol. Antigen retrieval was performed in Tris-EDTA buffer (pH 9) with microwave treatment. Endogenous peroxidases were inactivated using a peroxidase block reagent (Enzo) for 15 min and sections were permeabilized and blocked with Tris Buffered Saline (TBS) supplemented with 0.1% Tween 20, 2% milk, 5% Bovine Serum Albumin (BSA), and 1% human Ig for 30 min. The following primary antibodies were used (incubation time was 90 min) for immunofluorescent staining on these sections, alone or in combination, as indicated in the figure legends: anti-CD3 (Abcam, clone SP7, diluted 1:500), anti-CD8 (Cell signaling, clone D4W2Z, diluted 1:400), anti-CD146 (Abcam, clone EPR3208, diluted 1:250), anti-PDGFR β (Abcam, clone Y92, diluted 1:200). Ready-to-use EnVision+ System-HRP Labelled Polymer Anti-Rabbit (Dako) was used (60 min) as a secondary antibody for all antibodies cited above, followed by incubation with a Tyramide Reagent coupled either to Alexa Fluor 488, Alexa Fluor 555 or Alexa Fluor 647 (Thermo, diluted 1:200), prepared in a buffer containing 0.1 M boric acid, 3 M NaCl, 0.1% Tween 20 (pH 7.8) supplemented with 0.003% H₂O₂, for 10 min. After each staining, an additional step of heat-mediated antibody elution in citrate buffer (pH 6) was performed in a microwave oven. Counterstaining of nuclei was performed with Hoechst33258 reagent (1:1,000) for 5 min. Slides were mounted with Fluorescent Mounting Medium (Dako) and covered.

Another piece of tumor was frozen, embedded in OCT and cut in 7 μ m-thick sections to perform staining, including GARP. Cryosections were fixed for 5 min in FA 4%. Endogenous

peroxidase blocking and permeabilization were performed, as described above. For GARP staining, clone YGIC86 (Thermo, diluted 1:80) was used as the primary antibody and the signal was amplified with ready-to-use ImPRESS HRP Goat Anti-Rat IgG (Vector) and a Tyramide Reagent, coupled to Alexa Fluor 555. Slides were counterstained and mounted as described previously.

Digital 3 or 4-color images of the stained tissue sections were acquired with a Panoramic P250 Flash III scanner (3DHitech) equipped with a Plan-Apochromat 20 $\times$ /N.A. 0.8 $\times$ objective (Carl Zeiss) and with a Point Grey Grasshopper 5MP camera, using DAPI1, FITC, SpRed and Cy5 filter sets (Semrock).

Immunofluorescence Image Analysis

mIF images were analyzed with the Halo software (Indicalabs). The Cytonuclear FL module was used for the quantification of T cell densities as well as for the calculation of distances between the T cells to the tumor periphery (Dp), and to nearest neighbor endothelial cell. To calculate distances, we designed a dedicated R script that uses the position and phenotype of each event identified by Halo. For **Figure 2**, the tumor periphery was defined using the cells that are at the extremities of the tissue section, and the tumor center was calculated as centroid. A mean radius length was calculated for each section based on the lengths of all radii measured in the section (one radius represents the distance between the centroid and one DAPI⁺ nucleus located on the tumor periphery). Five CT26 tumors were excluded from this analysis because their centroid were located outside of the tissue, due to their moon-like shape. The distance of each CD8 T cell to their closest endothelial cell was calculated using the nearest neighbor analysis. For quantification of blood vessels, the Object Quantification FL module of Halo was used, with each object corresponding to an individual blood vessel.

Flow Cytometry Analyses

On the day indicated in the figure legends, tumors were harvested and mechanically dissociated in the presence of enzymes (Collagenase I 100 mg/ml, Life Tech; Collagenase II 100 mg/ml, Life Tech; Dispase 1 mg/ml, Life Tech; and DNase I 0.4 U/ml, Roche), using two cycles in the GentleMacs disruptor (Miltenyi) separated by 30 min of incubation at 37°C under continuous agitation. Tumor cell homogenates were clarified through 70 and 40 μ m filters. Single cell suspensions were counted on a Luna[®] cell counter with a live-dead cell marker, then pelleted and resuspended in PBS containing 2 mM EDTA and 1% FCS for immediate staining, or in X-Vivo 10 medium (Invitrogen) to shortly incubate cells prior to staining.

Cells used for immediate staining were incubated with antibodies against surface markers (CD45, clone 30F-11; CD4, clone RM4-5; CD8a, clone 53-6.7) (Biolegend) in the presence of a viability dye (eBioscience) and anti-CD16/32 to block Fc γ Rs. To identify MC38 tumor-specific T cells, cells were also incubated with an H2-K^b-p15E (KSPWFTTL) pentamer coupled to APC (ProImmune). One million cells per tumor were kept for *ex vivo* incubation with Brefeldin A (Sigma, 5 μ g/ml) to increase the accumulation of cytokines in the Golgi/endoplasmic reticulum. No stimulation reagents were

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applied. Anti-CD107a mAb coupled to BV421 (clone 1D4B, Biolegend) was added to the mix. After 4 h of incubation at 37°C, cells were stained with antibodies against surface markers (CD45, CD4, CD8a) (Biolegend) in the presence of a viability dye (eBioscience) and an anti-CD16/32, fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences), then stained with antibodies against intracellular cytokines (IFN γ , clone XMG1.2; TNF α , clone MP6-XT22) (Biolegend) in the presence of additional anti-CD16/32.

C166 and MS1 cells were incubated with anti-CD16/32 antibody then stained with a biotinylated anti-GARP:TGF- β 1 antibody (clone 58A2) and a streptavidin coupled to PE. Analyses were performed on a FACS LSRFortessa flow cytometer (DIVA, BD Biosciences) and data were computed using the FlowJo software (Tree Star).

RT-qPCR of Mouse Tissue Samples and Cell Lines

Mouse tumor fragments were collected and stored at -80°C until processing. After tissue disruption with the TissueLyser (Qiagen), total RNA was extracted using NucleoSpin Mini Columns (Macherey Nagel). RNA was reverse transcribed with Maxima First Strand cDNA Synthesis Kit (ThermoFisher). For **Figure 6**, the MS1 cell line and splenocytes obtained from BALB/c mice were treated with recombinant TGF- β (rTGF- β) at 1 ng/ml for 24 h, then harvested and pelleted. RNA extraction and reverse transcription were performed as described above. qPCR were performed in the QuantStudio3 device (ThermoFisher) in reaction volumes of 20 μ l containing 0.025 U/ μ l of Taqyon Master Mix (Eurogentec), 300 nM of each primers, 100 nM of Taqyon probe, under either standard conditions (95°C for 3'; 45 cycles of 95°C for 10" and 60°C for 30") or fast conditions (95°C for 3'; 95°C for 3" and 60°C for 30") depending on amplicon size. The sequences of primers and probes are listed in **Table S1**.

Thymidine Incorporation Assay

C166 and MS1 endothelial cells (2,000 cells/well) were incubated in the presence of rTGF- β 1, for the duration indicated in the figure legends. One μ Curie of [3 H]-thymidine (3 H-T) was added during the last 24 h of culture. On the day of revelation, cells were incubated with trypsin and aspirated on a 96-filter plate (Unifilter GF/C) using a harvester (Packard Filtermate 196). The plate was washed several times with water. Radioactivity was measured by a scintillation counter (Packard Microplate Scintillation Counter), which calculates the counts per minute (cpm).

Statistical Analyses

All statistical analyses were performed using the JMP[®] Pro 15 software. Comparisons of measurements taken at a single time point were performed using a two-tailed, non-paired, non-parametric Wilcoxon test. *Post-hoc* Tukey's test was performed to adjust for multiple comparisons. The number of experiments and the number of mice (n) in the various experimental groups are indicated in the corresponding figure legend.

RESULTS

Combined GARP:TGF- β 1/PD-1 Blockade Increases Infiltration of T Cells in MC38 but Not in CT26 Tumors

To compare the mechanism of action of combined GARP:TGF- β 1/PD-1 blockade in two mouse tumor models, we injected MC38 or CT26 cells subcutaneously (s.c.) in syngeneic C57BL/6 or BALB/c mice, respectively. We then started intraperitoneal (*i.p.*) administrations of either isotype control, anti-GARP:TGF- β 1 and/or anti-PD-1 mAbs after 6 days, when the tumors were well established in all mice. A total of three injections of single or combined mAbs were administered on days 6, 9 and 12, and tumors were collected on day 13. This time point was chosen based on our previous study, which showed that similar administration regimens significantly increased the frequency of complete, immune-mediated rejections of MC38 and CT26 tumors in mice treated with anti-GARP:TGF- β 1 + anti-PD-1, as compared with anti-PD-1 alone. Notably, rejections occurred between days 15 and 40 in both MC38- and CT26-tumor bearing mice, leaving no sufficient tumor material for analyses after day 13 (8). Here on day 13, and as expected, volume and weight of tumors were already significantly reduced in mice treated with anti-GARP:TGF- β 1 + anti-PD-1, or anti-PD-1 alone, as compared with controls (**Figure S1**).

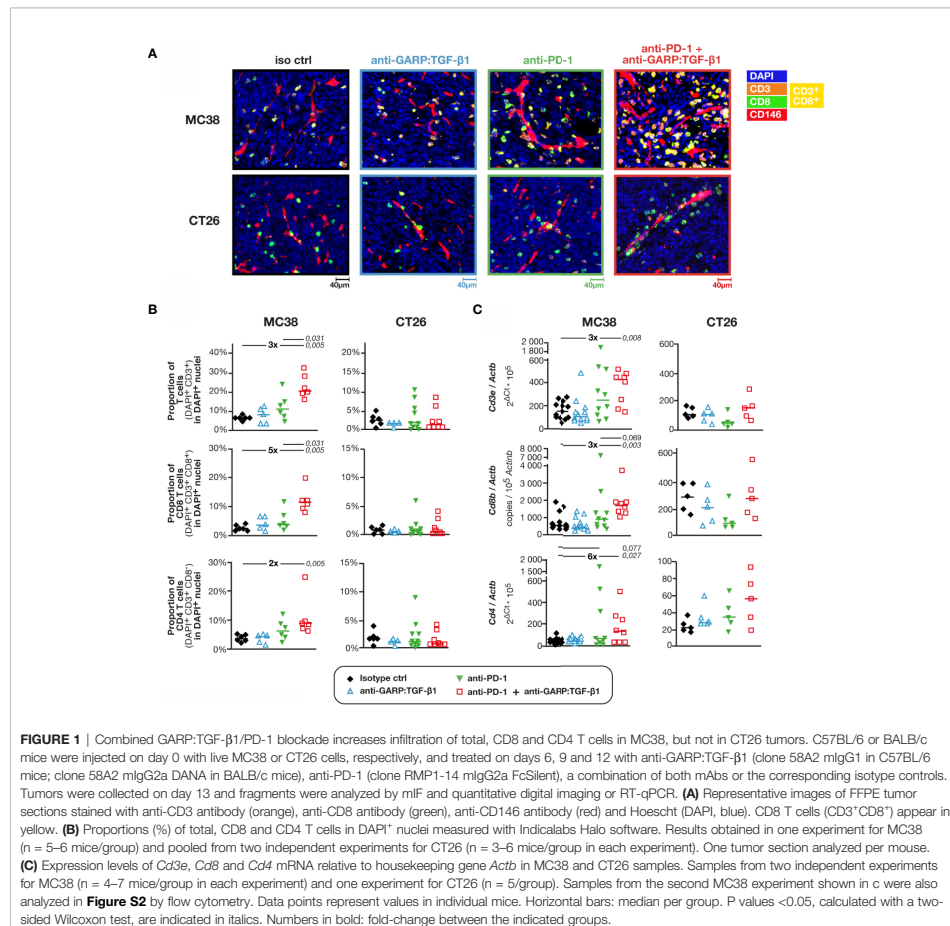
We used digitalized multiplex immunofluorescence stainings (mIF) of formaldehyde-fixed paraffin-embedded (FFPE) sections to quantify T cell infiltration in the tumors (**Figures 1A, B**). In tumors from untreated mice, the proportion of CD3⁺ cells among DAPI⁺ nuclei was approximately 3-fold higher in MC38 (6.8% $\pm$ 0.4%; median $\pm$ interquartile range, or IQR) as compared with CT26 tumors (2.3% $\pm$ 1.4%) (**Figure 1B**). Consistent with our previous observations, administration of anti-GARP:TGF- β 1 or anti-PD-1 alone did not significantly increase T cell infiltration in MC38 or CT26 tumors, and the anti-GARP:TGF- β 1 + anti-PD-1 combination did not increase T cell infiltration in CT26 (**Figure 1B** and (8)). However, the combination caused a significant 3-fold increase of the proportion of T cells infiltrating MC38 tumors in mice treated with the anti-GARP:TGF- β 1 + anti-PD-1 combination (20.9% $\pm$ 7.5%) (**Figure 1B**). T cell infiltration was significantly higher with this combination than with the anti-PD-1 alone. Both CD8 and CD4 T cells contributed to the infiltration, but CD8 T cells more so ($\approx$ 5-fold) than CD4 ($\approx$ 2-fold). RT-qPCR for genes *Cd3e*, *Cd8b* and *Cd4* confirmed increased infiltration of total, CD8 and CD4 T cells in MC38 tumors after combined GARP:TGF- β 1/PD-1 blockade (**Figure 1C**).

We observed with flow cytometry that the proportion of CD8 and CD4 T cells among MC38-infiltrating leukocytes (CD45⁺ cells) were not modified after GARP:TGF- β 1/PD-1 blockade, indicating that the augmented T cell infiltration resulted from an increased infiltration of total leukocytes and not of a particular cell subset (**Figure S2A**). Lymphocytes directed against the tumor-specific antigen p15E were identified by staining with a fluorescent H2-K^b pentamer loaded with the p15E peptide (15). Their proportions among leukocytes (7.6% $\pm$ 4.9%) or CD8

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T cells ($19.1\% \pm 11.6\%$) did not differ between the untreated or treated MC38 tumors (**Figure S2A**). We also did not find differences between the proportions of CD8 T cells or tumor-specific CD8 T cells that produced IFN γ and/or TNF α , or expressed the surface marker of degranulation CD107a (**Figures S2B**). The only difference was the proportions of CD4 T cells producing IFN γ increased after combined GARP:TGF- β 1/PD-1 blockade as compared with PD-1 blockade alone or controls (**Figure S2B**). RT-qPCR analyses confirmed these observations (**Figure S2C**).

We conclude that in MC38 tumors, the combined GARP:TGF- β 1/PD-1 blockade increases the infiltration of T cells,

including activated tumor-specific CD8 T cells. This contrasts with our previously published observations in CT26, where combined GARP:TGF- β 1/PD-1 blockade did not increase T cell infiltration, but did augment the effector functions of the anti-tumor T cells that were already present in the tumors (8).

Combined GARP:TGF- β 1/PD-1 Blockade Does Not Increase the Proliferation or Penetration of Tumor-Infiltrating CD8 T Cells in MC38 or CT26 Tumors

The increased T cell infiltration into MC38 following GARP:TGF- β 1/PD-1 blockade could result from a local T cell

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proliferation within MC38, but not CT26 tumors. However, we observed no change in levels of Ki-67 in CD8 T cells or in the proportion of proliferating Ki-67⁺ cells among CD8 T cells in any treatment group in MC38 or CT26 tumors (Figures S3A, B).

Increased T cell infiltration could also result from an increased penetration of T cells from the periphery towards the center of MC38 tumors, as shown in EMT6 treated with a combination of anti-active TGF- β 1, β 2 and β 3 (clone 1D11) and anti-PD-L1 (clone 6E11) (10). We measured the distance separating each CD8 T cell from the closest point on the tumor periphery in MC38 and CT26 FFPE sections, stained by mIF (Figure 2A), then examined the distribution of all CD8 T cells at various distances from the periphery relative to mean radius length (Figure 2B). In control MC38 tumors, the vast majority of CD8 T cells were closer to the tumor periphery than its center (79% of CD8 T cells at <50% mean radius length). Treatment with anti-GARP:TGF- β 1, combined or not with anti-PD-1, did not modify this distribution (Figure 2B). In control CT26, a majority of CD8 T cells were even closer to the periphery (89% CD8 T cells at <30% mean radius length), but here also, their distribution was not modified by any treatment (Figure 2B).

Combined GARP:TGF- β 1/PD-1 Blockade Increases Entry of T Cells *via* Intratumoral Vessels in MC38

We then examined whether combined GARP:TGF- β 1/PD-1 blockade increased T cell entry *via* intratumoral blood vessels, as reported by Martin et al. for combined latent TGF- β 1/PD-1 blockade in MBT-2 tumors (9). Here we used mIF and quantitative imaging of FFPE tumor sections, this time to measure distances between each CD8 T cell and the nearest blood endothelial cell (DAPI⁺CD146⁺ cell, or BEC) and determine the distribution of all CD8 T cells at various distances from their nearest BEC (Figure 2C). In murine tissues, CD146 is expressed by blood but not lymphatic endothelial cells (16). In control MC38 tumors, the vast majority of CD8 T cells were <100 μ m away from the nearest BEC, with 48% at <30 μ m. In mice treated with anti-GARP:TGF- β 1, anti-PD-1, or both, the proportions of CD8 T cells at <30 μ m from the nearest BEC rose to 55, 61 or 69%, respectively. This suggests that increased infiltration of CD8 T cells in MC38 tumors, after combined GARP:TGF- β 1/PD-1 blockade (Figure 1), results from increased entry of T cells *via* blood vessels, but that T cells do not migrate further in the tumor bed after extravasation. In control CT26 tumors, 75% of CD8 T cells were at <30 μ m from the nearest BEC, and this proportion did not vary with any treatment (Figure 2C).

Combined GARP:TGF- β 1/PD-1 Blockade Increases the Density of Blood Vessels Containing GARP⁺ Endothelial Cells Covered by PDGFR β ⁺ Pericytes in MC38, but Not in CT26 Tumors

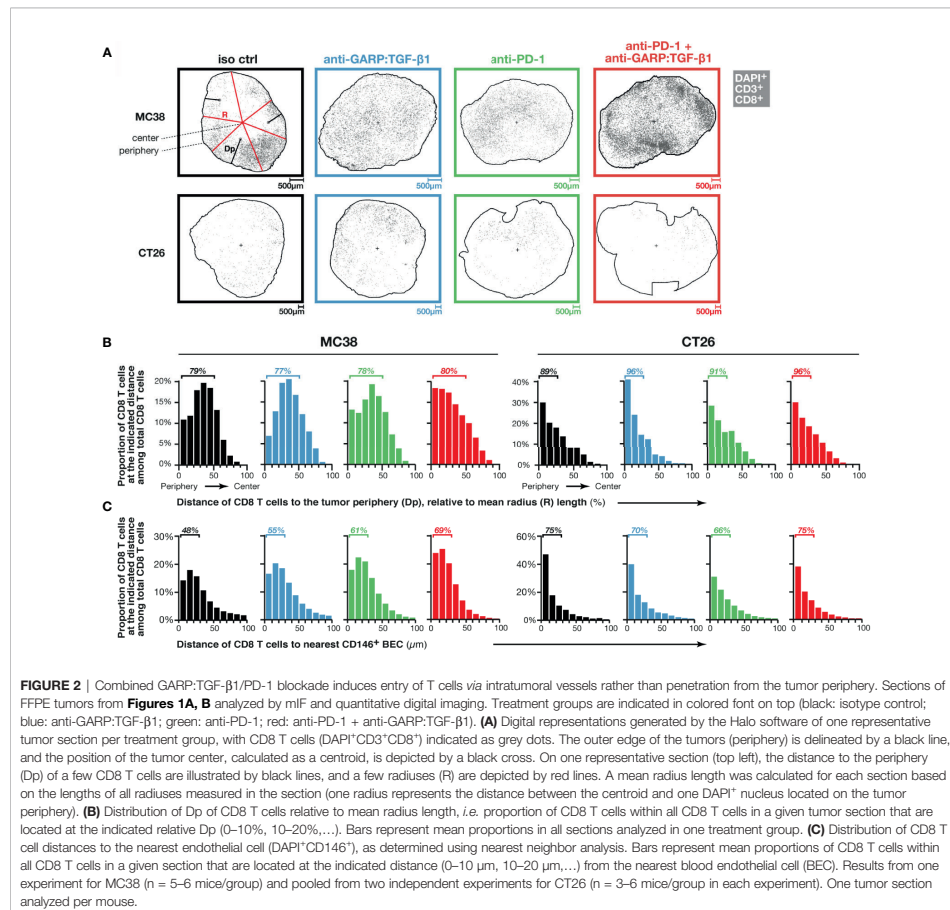
Next, we examined if increased infiltration of T cells was associated with a higher density of blood vessels in MC38 tumors. Blood vessel-like objects (*i.e.* structures defined based

on CD146⁺ surface segmentation) were identified and counted (Figure 3A). Median areas of blood vessels were similar in MC38 and CT26 tumors, regardless of treatment (Figure S4). Densities of blood vessel-like objects in control MC38 and CT26 tumors were also similar, with 161 ± 54 (median $\pm$ IQR) blood vessels per mm² in MC38, and 196 ± 132 in CT26 tumors (Figure 3B). Unexpectedly, we observed that combined GARP:TGF- β 1/PD-1 blockade increased blood vessel density about 2-fold in MC38 (272 ± 91), but not in CT26 tumors (Figure 3B). Blood vessel densities correlated with proportions of tumor-infiltrating CD8 T cells in MC38, but not in CT26 tumors. Three of the four MC38 tumors with the highest CD8 T cell infiltration (>10% CD8 T cells in DAPI⁺ cells) and the highest blood vessel density (>250 vessels/mm²) were from mice treated with anti-GARP:TGF- β 1 + anti-PD-1 (Figure 3C). We counted a median of about 1.8 and 1.2 CD8 T cell in every 20 vessels of untreated MC38 and CT26 tumors, respectively, and this number increased 4-fold after combined GARP:TGF- β 1/PD-1 blockade in MC38, but not in CT26 tumors (Figure 3D). Thus, increased T cell infiltration in MC38 tumors upon combined GARP:TGF- β 1/PD-1 blockade results from both increased blood vessel density and increased CD8 T cell extravasation into the tumors.

Intratumoral blood vessels are often described as structurally abnormal, displaying loose cellular junctions, high number of fenestrations and discontinuous basement membrane, which can impair lymphocyte extravasation (17). To measure the functionality and stability of blood vessels, we used anti-PDGFR β antibodies to identify blood vessels covered by pericytes (*i.e.* CD146⁺PDGFR β ⁺ objects) (Figure 4A). We observed a 2-fold increase in the density of PDGFR β ⁺ blood vessels in MC38, but not in CT26 tumors, upon GARP:TGF- β 1/PD-1 blockade as compared with controls or anti-PD-1 alone (Figure 4B). No increase in the density of PDGFR β -negative blood vessels was observed in either MC38 or CT26 tumors, whatever the treatment (Figure 4B). Thus, increased blood vessel density in MC38 tumors after GARP:TGF- β 1/PD-1 blockade can be attributed to an increased density of those vessels that are stabilized by pericytes, a parameter often taken as an indicator of blood vessel normalization in tumors (17).

GARP:TGF- β 1 complexes are known to be expressed on endothelial cells (2, 8, 18). We therefore verified whether treatment with anti-GARP:TGF- β 1 mAbs modified the densities and proportions of blood vessels containing GARP⁺ BECs in frozen MC38 and CT26 tumors (Figure 4C), as GARP can be detected by mIF in frozen tissues only (8). In untreated tumors, $45.3\% \pm 6.2\%$ (median $\pm$ IQR) of blood vessels contained GARP⁺ BECs in MC38, versus $30.7\% \pm 7.7\%$ in CT26 tumors. Combined GARP:TGF- β 1/PD-1 blockade increased GARP⁺ blood vessel density about 2-fold in MC38, but not in CT26 tumors (Figure 4D). Densities of GARP-negative blood vessels did not change in MC38 or CT26 tumors, whatever the treatment. In a follow up experiment, we co-stained frozen tumor sections for GARP and PDGFR β . The two markers were frequently co-expressed in intratumoral blood vessels: whilst $63.8\% \pm 5.8\%$ (median $\pm$ IQR) of GARP⁺ blood vessels were also PDGFR β ⁺ in MC38 tumors, only $13.6\% \pm 4.2\%$ of GARP⁺ vessels expressed PDGFR β (Figure S5). We conclude that combined

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GARP:TGF- β 1/PD-1 blockade increased the density of GARP⁺ blood vessels covered by pericytes in MC38, but not in CT26 tumors.

Active TGF- β 1 Inhibits the Proliferation of Murine GARP⁺ Endothelial Cells *In Vitro*

TGF- β 1 exerts a cytostatic effect on many cell types. By blocking TGF- β 1 activation on the surface of GARP⁺ endothelial cells, anti-GARP:TGF- β 1 mAbs could increase endothelial cell proliferation and the formation of new GARP⁺PDGFR β ⁺ functional blood vessels in MC38 tumors. *In vitro* proliferation of MS1, an endothelial cell line which is derived from a pancreatic sarcoma and expressing surface GARP:TGF- β 1 complexes (**Figure 5A**), was

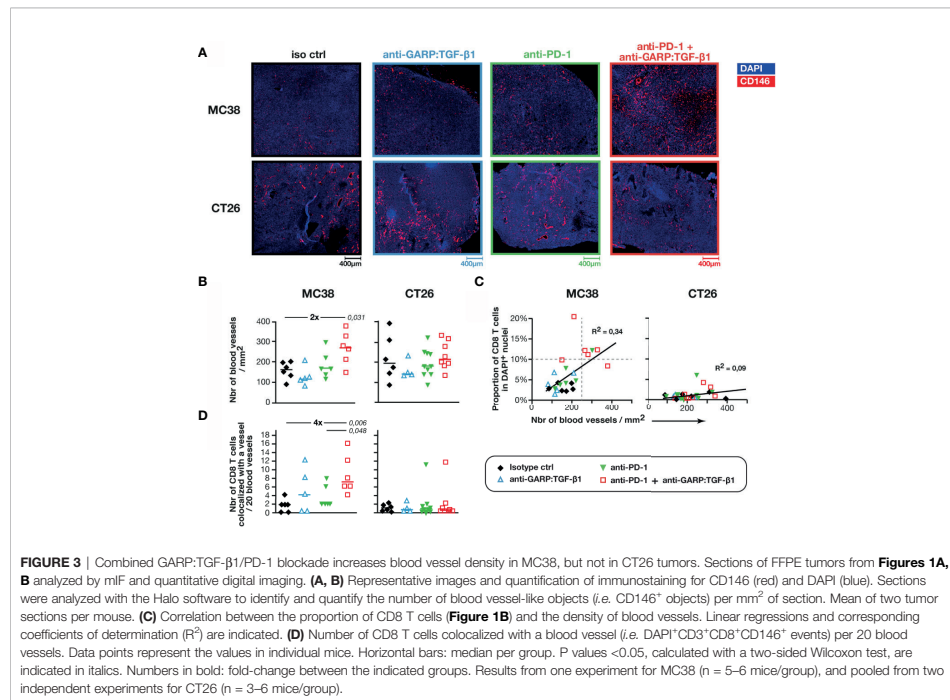
inhibited by recombinant active TGF- β 1 (rTGF- β 1) in a dose-dependent manner (**Figure 5B**). Noteworthy, incubation with neutralizing anti-active TGF- β 1,2,3 (clone 1D11) or blocking anti-GARP:TGF- β 1 (clone 58A2) mAbs did not increase MS1 proliferation (**Figure S6**), indicating that they are not under the influence of autocrine TGF- β 1 activity. This result indicates that in the absence of a stimulus or another cell type, MS1 endothelial cells are unable to activate the latent TGF- β 1 presented by GARP on their surface. We also tested the effect of rTGF- β 1 on another murine endothelial cell line, C166, which originates from yolk sac. We observed no inhibition of proliferation (**Figures 5A, B**).

The inhibition of MS1 proliferation by rTGF- β 1 *in vitro* suggests that anti-GARP:TGF- β 1 mAbs could favor the

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proliferation of GARP⁺ endothelial cells *in vivo*, and consequently increase blood vessel densities in MC38 tumors.

Combined GARP:TGF- β 1/PD-1 Blockade Increases Expression of E-Selectin by BECs in MC38, but Not in CT26 Tumors

TGF- β 1 regulates expression of adhesion molecules in hematopoietic and endothelial cells (19–21). We measured the expression of genes encoding various adhesion molecules in MC38 and CT26 tumor samples from control and mAb-treated mice. No difference was observed for expression of *Vcam1* and *Icam1* relative to *Actb* in any treatment group as compared with controls (**Figure S7**). In contrast, expression of *Lfa1* and *Sell*, encoding adhesion molecules expressed on leukocytes, and that of *Sele*, encoding E-selectin expressed on BECs, were increased after combined GARP:TGF- β 1/PD-1 blockade in MC38, but not in CT26 tumors (**Figure 6A** and **Figure S7**). We measured *Lfa1/Cd3e*, *Sell/Cd3e* and *Sele/Cd146* mRNA ratios, to normalize adhesion molecule expression to the abundance of CD3⁺ T cells or CD146⁺ BECs (**Figure 6A** and **Figure S7**). No significant difference in *Lfa1/Cd3e* or *Sell/Cd3e* ratios was observed in MC38 samples from control or mAb-treated mice. Thus, increased expression of these genes relative to

Actb after GARP:TGF- β 1/PD-1 blockade in MC38 tumors is probably mostly due to increased T cell infiltration. This was different for expression of *Sele*: ratios of *Sele/Actb* and *Sele/Cd146* were significantly increased after combined GARP:TGF- β 1/PD-1 blockade in MC38, but not in CT26 tumors. This suggests that increased *Sele* expression in MC38 tumors results not only from increased BEC densities but also from increased *Sele* mRNA levels per BEC. We verified whether exposure to rTGF- β 1 regulates *Lfa1* or *Sele* expression by murine activated T cells or endothelial cells *in vitro*, respectively. rTGF- β 1 marginally increased (1.3- to 1.5-fold) *Lfa1* expression by murine splenocytes stimulated or not with anti-CD3/CD28 beads (**Figure 6B**). In contrast, rTGF- β 1 reduced *Sele* expression by more than 2-fold in MS1 endothelial cells (**Figure 6C**), confirming previous observations on HUVECs and murine lung, heart and liver endothelial cells (22, 23).

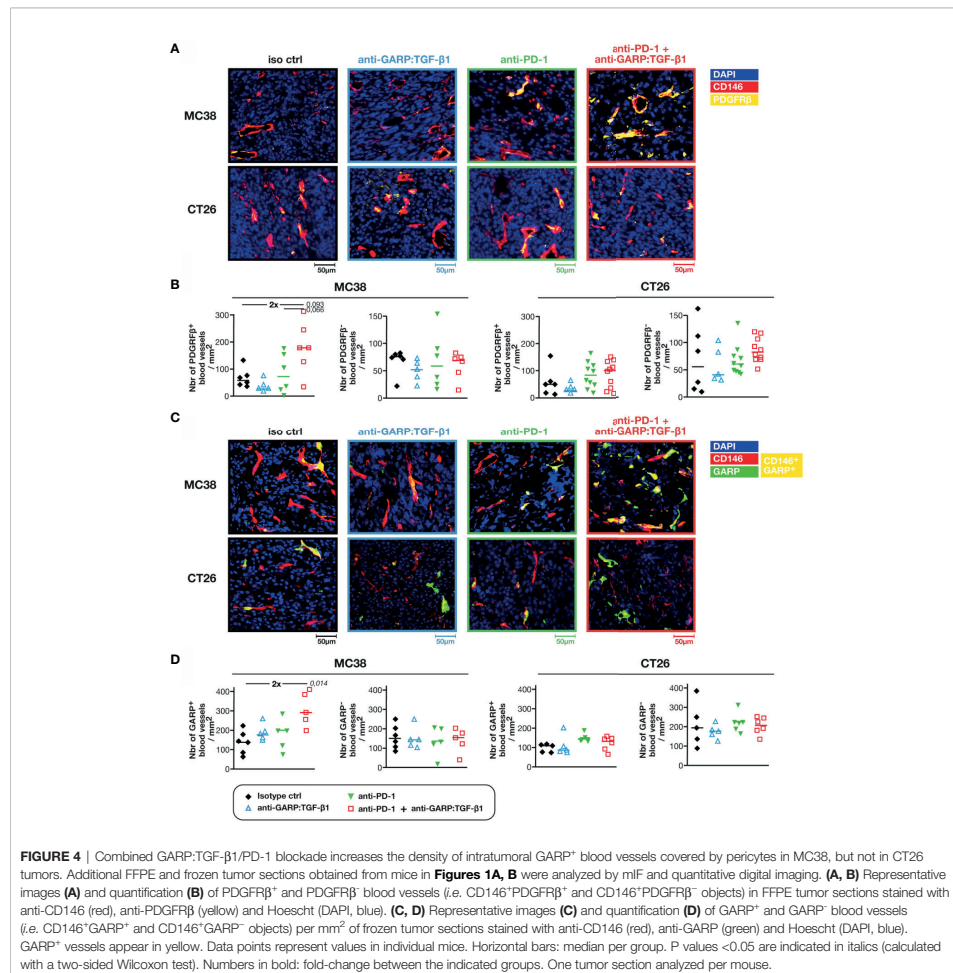
DISCUSSION

Altogether, our results suggest that combined GARP:TGF- β 1/PD-1 blockade exerts anti-tumor activity in MC38 tumors by increasing the density of intratumoral GARP⁺ blood vessels

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covered by PDGFR β ⁺ pericytes, the expression of E-selectin by BECs, and the extravasation and infiltration of T cells, including activated anti-tumor CD8 T cells.

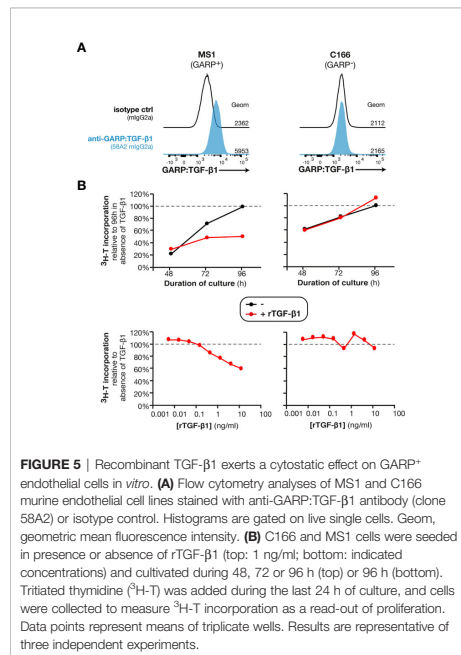
Because rTGF- β 1 inhibited the proliferation of GARP⁺ endothelial cells *in vitro*, we propose that anti-GARP:TGF- β 1 mAbs act by inhibiting TGF- β 1-mediated endothelial cell cytostasis in MC38 tumors, thereby favoring BEC proliferation and densification of the intratumoral blood vasculature. Anti-PD-1 mAbs, alone or in combination with anti-CTLA-4, were previously shown to increase pericyte coverage of blood vessels

in mouse tumors, but these treatments did not increase, and even reduced intratumoral blood vessel densities (24–27). Combining anti-PD-1 with anti-GARP:TGF- β 1 could therefore increase pericyte coverage of blood vessels as a result of PD-1 blockade, while also increasing BEC proliferation and blood vessel density as a result of GARP:TGF- β 1 blockade. This would explain the densification of pericyte-covered blood vessels observed in MC38 tumors upon combined GARP:TGF- β 1/PD-1 blockade. Increased density of pericyte-covered blood vessels is an indicator of tumor vasculature normalization, which could

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improve tumor perfusion (17, 28). Other approaches targeting the tumor vasculature to overcome resistance to PD-1/PD-L1 blockade were tested previously, *e.g.* with anti-VEGFR2 mAbs, which enhanced vascular normalization but did not increase blood vessel density in mouse models of breast and pancreatic cancer (29). Anti-GARP:TGF- β 1 could represent a more efficient and safer alternative, densifying in addition to normalizing the blood vasculature by targeting GARP-expressing cells only.

The cellular source of the active TGF- β 1 exerting cytostatic effects on BECs in MC38 tumors is not yet clearly identified. We observed that approximately half of tumor blood vessels in MC38 tumors contained endothelial cells expressing GARP:TGF- β 1 on their surface. It would thus be tempting to speculate that anti-GARP:TGF- β 1 improves the anti-tumor efficacy of anti-PD-1 by blocking TGF- β 1 activation on the surface of GARP⁺ endothelial cells. However, we observed that *in vitro*, endothelial cells were not able to activate latent TGF- β 1 presented by GARP on their surface. This suggests that an unidentified stimulus, or another cell type, may be required to allow TGF- β 1 activation on the surface of endothelial cells *in vivo*. In T cells, the mere presence of surface GARP:TGF- β 1 is not sufficient to induce TGF- β 1 activation. TGF- β 1 activation by Tregs requires TCR stimulation and interaction of GARP:TGF- β 1 complexes with integrin α V β 8 (3). The situation might be similar for endothelial cells, which

would require an additional stimulus and/or the intervention of an α V β 8-expressing cell type to activate latent TGF- β 1 presented by GARP on their surface *in vivo*. Tregs themselves, entering the tumor *via* blood vessels, represent interesting candidates. Indeed, we previously showed that the anti-tumor activity of combined GARP:TGF- β 1/PD-1 blockade was lost in Treg-specific *Garp* knock-out mice bearing MC38 tumors, suggesting that targeting GARP-expressing Tregs is indispensable for anti-GARP:TGF- β 1 to exert anti-tumor activity (8). It could thus very well be that the cellular source of the active TGF- β 1 exerting cytostasis on BECs in MC38 tumors is the immunosuppressive Tregs. Considering that tumor-infiltrating T cells, which include Tregs, are more abundant in MC38 than in CT26 tumors, this could explain at least in part why GARP:TGF- β 1 blockade increases the density of the tumor blood vasculature in MC38, but not in CT26 tumors.

Whichever the cellular source of TGF- β 1 signals blocked by anti-GARP:TGF- β 1, increased tumor perfusion will increase T cell influx in the MC38 tumor vasculature. We observed that expression of *Sele*, a gene encoding E-selectin and repressed by TGF- β 1, was increased in MC38 tumors upon combined GARP:TGF- β 1/PD-1 blockade. E-selectin on endothelial cells allows for the adhesion of activated leukocytes and facilitates their extravasation by slowing down their rolling on blood vessel walls. Therefore, anti-GARP:TGF- β 1 mAbs, by blocking TGF- β 1 activity and increasing *Sele* expression, could facilitate T cell extravasation and infiltration within MC38 tumors.

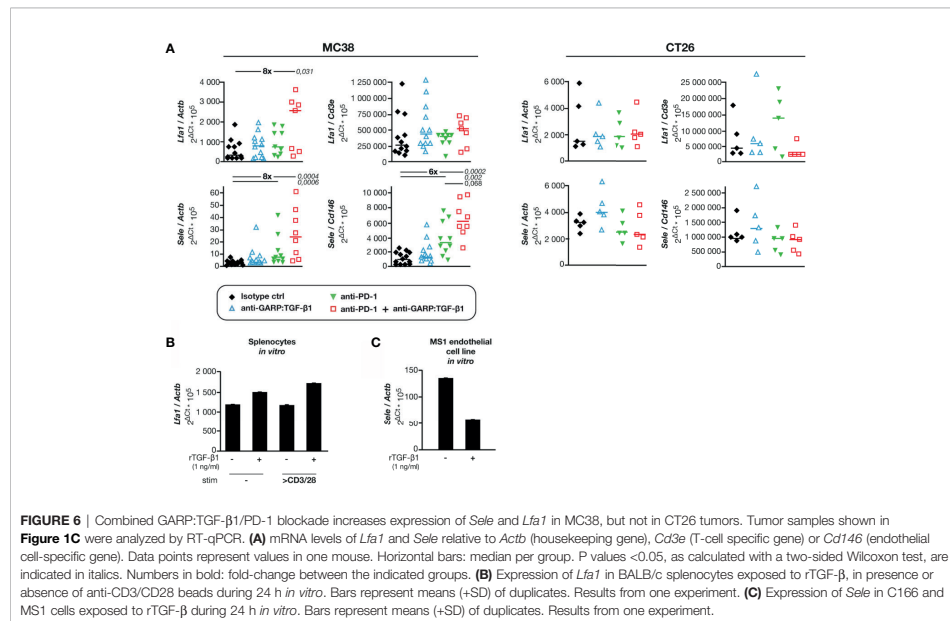
It is noteworthy that activated intratumoral T cells can themselves enhance vascular functionality by secreting TNF α , known to upregulate E-selectin on blood endothelial cells (23), and IFN γ , which induces expression of chemokines involved in pericyte recruitment (25, 30, 31). We observed a significant increase in *Ifng* mRNA levels and proportions of IFN γ -producing CD4 T cells in MC38 tumors after combined GARP:TGF- β 1/PD-1 blockade. Thus, increased tumor blood vessel density and functionality induced by combined GARP:TGF- β 1/PD-1 blockade could be further reinforced as a consequence of increased infiltration by activated T cells, in a positive feedback loop.

The mode of action of combined GARP:TGF- β 1/PD-1 blockade in MC38 tumors contrasts with that previously described in CT26 tumors, in which it increased effector functions of already present anti-tumor CD8 T cells, without increasing T cell infiltration or blood vessel density. It is unclear why combined GARP:TGF- β 1/PD-1 blockade did not densify the blood vasculature and increase T cell infiltration in CT26 tumors, and conversely, why it did not increase effector functions of tumor-infiltrating CD8 T cells in MC38 tumors. Whether different modes of action result from using different colon carcinoma cell lines (MC38 vs CT26) or different syngeneic mouse strains (C57BL/6 vs BALB/c, respectively) could be assessed by comparing the two tumor models in F1 mice. The proportion of blood vessels containing GARP⁺ BECs in CT26 tumors was lower as compared with MC38 tumors, perhaps contributing to the lack of effect of anti-GARP:TGF- β 1 on the blood vasculature in these tumors. Nevertheless, combined GARP:TGF- β 1/PD-1 blockade increased immune-mediated tumor rejections to a similar extent in CT26 and MC38 tumors (8),

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indicating that both modes of action can significantly contribute to the anti-tumor activity of the combination.

Taken together, our results suggest that combined GARP:TGF- β 1/PD-1 blockade can exert anti-tumor activity *via* multiple mechanisms, not only by increasing effector functions of anti-tumor T cells already present within tumors, but also by increasing tumor blood vessel density and infiltration by new anti-tumor T cells. Anti-GARP:TGF- β 1 mAbs could thus be tested in the clinics to overcome resistance to PD-1/PD-L1 blockade in patients with a broad range of cancer types, including not only tumors already infiltrated by T cells, but also poorly vascularized tumors with low immune cell infiltration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité d'Ethique pour l'Expérimentation Animale Secteur des Sciences de la Santé Université catholique de Louvain.

AUTHOR CONTRIBUTIONS

SL conceived the study. CB, PM, GS, AN, PC, NB, and SL analyzed the data. CB, PM, GS, NV-B, FB, and LR performed experiments. SL and CB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.704050/full#supplementary-material>

Supplementary Figure 1 | Combined GARP-TGF- β 1/PD-1 blockade and anti-PD-1 alone reduce growth of MC38 and CT26 tumors. Volume and weight of tumors collected on day 13 for analyses shown in **Figures 1–4** and **Figure 6**. Data points represent values in individual mice. Horizontal bars: median per group. P values < 0.05, as calculated with a two-sided Wilcoxon test, are indicated by numbers in italics. Results from one experiment for MC38 (n=5–6 mice/group), and pooled from two independent experiments for CT26 (n=3–6 mice/group in each experiment).

Supplementary Figure 2 | CD8 T cells infiltrating MC38 tumors include activated anti-tumor T cells, but combined GARP-TGF- β 1/PD-1 blockade does not increase their proportions or functions. MC38 tumors from mice treated as indicated in **Figure 1** were collected on day 13 to perform flow cytometry and RT-qPCR. **(A)** Proportions (%) of the indicated cell types among tumor infiltrating leukocytes (CD45⁺) or CD8 T cells (CD45⁺ CD8⁺). Results from one experiment (n=6–10 mice/group). **(B)** Proportions (%) of cells producing IFN γ , TNF α , and/or expressing surface CD107a among the indicated tumor-infiltrating cell subsets, obtained on unstimulated bulk processed tumors. Results from one experiment (n=6–10 mice/group). **(C)** Expression of *Ifng*, *Tnfa*, *Prf1* and *Gzmb* relative to *Actb* in MC38 tumors. Results from 2 independent experiments (n=4–7 mice/group in each experiment). Data points represent values in individual mice. Horizontal bars: median per group. P values < 0.05, as calculated with a two-sided Wilcoxon test are indicated in italics. Numbers in bold: fold-change between the indicated groups.

Supplementary Figure 3 | Combined GARP-TGF- β 1/PD-1 blockade does not increase the proliferation of tumor infiltrating CD8 T cells in MC38 and CT26. MC38 tumors from mice treated as indicated in **Figure 1** were collected on day 13. CT26 tumors from mice treated as indicated in **Figure 1** (with the exception that antibodies were injected on days 6, 10 and 14) were collected on day 14. Tumors were dissociated and analyzed by flow cytometry. Results from one experiment for MC38 (n=5 mice/group) and one experiment for CT26 (n=4/group). **(A)** Percentage of Ki-67⁺ cells in live single CD8 T cells (CD45⁺CD8⁺ for MC38, and CD3⁺CD8⁺ for CT26). **(B)** Geometric

mean fluorescence intensity (geomean) for Ki-67 staining in live single Ki-67⁺ CD8 T cells. Data points represent values in one mouse. Horizontal bars: median per group.

Supplementary Figure 4 | Combined GARP-TGF- β 1/PD-1 blockade does not modify mean area of blood vessels in MC38 and CT26 tumors. mIF and quantitative digital imaging of sections shown in **Figure 4**. Mean area of CD146⁺ objects in tumor sections (n=134 to 26 939 CD146⁺ objects per section). Data points represent the mean values of two tumor sections analyzed in individual mice. Horizontal bars: median per group.

Supplementary Figure 5 | A majority of GARP⁺ blood vessels also express PDGFR β in MC38 tumors. Frozen MC38 tumor sections from mice shown in **Figures 1A, B** were used for mIF and quantitative imaging. **(A)** Representative image of a MC38 control tumor stained with anti-CD146 antibody (red), anti-PDGFR β (yellow) and anti-GARP (green). Left: CD146 and PDGFR β signals. Right: GARP and PDGFR β signals on the same area. White arrow: CD146⁺PDGFR β ⁺GARP⁺ vessel (top images) or CD146⁺PDGFR β ⁺GARP⁺ vessel (bottom images). **(B)** Proportion (%) of PDGFR β ⁺ vessels in GARP⁺ or GARP⁺ blood vessels. Data points represent values for individual mice. Horizontal bars: median per group. P value < 0.05, as calculated with a two-sided Wilcoxon test, are indicated in italics. Number in bold: fold-change between the indicated groups. One tumor section analyzed per mouse.

Supplementary Figure 6 | C166 and MS1 endothelial cells do not activate TGF- β 1 in vitro in absence of stimulus or another cell type. C166 and MS1 cells were used to perform a proliferation assay as described in **Figure 5B**. Incorporation of ³H-T was measured after 96 hours of culture in the presence of blocking anti-GARP-TGF- β 1 (clone 58A2) or neutralizing anti-TGF- β 1, β 2, β 3 (clone 1D11) mAbs. Bars indicate means (+ SD) for triplicate wells. Data representative of 3 independent experiments.

Supplementary Figure 7 | Combined GARP-TGF- β 1/PD-1 blockade increases expression of *Sei* but not of *Icam1* and *Vcam1* in MC38 tumors. RT-qPCR analyses relative to **Figure 6A**. Graphs show the expression level of *Icam1*, *Vcam1* and *Sei* normalized by *Actb*, *Cd3e* or *Cd146*. Data points represent values measured in individual mice. Horizontal bars: median per group. Number in italics indicate P values < 0.05 as calculated with a two-sided Wilcoxon test, and numbers in bold indicate fold-changes between groups for selected comparisons.

Supplementary Table 1 | Primers and probes sequences.

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Conflict of Interest: Patents pertaining to results obtained with the 58A2 antibody clone (anti-mouse GARP-TGF- β 1) have been filed under the Patent Cooperation Treaty (International application Number PCT/IB2019/053753), with SL, GS, PC as inventors and UCLouvain and argenx as applicants. SL received research support from argenx and owns stock options in argenx.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RESULTS

Supplementary figures

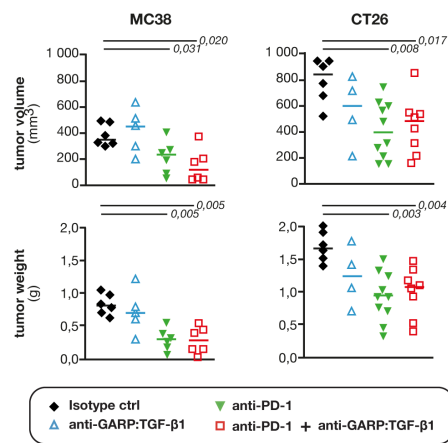


Figure S1

RESULTS

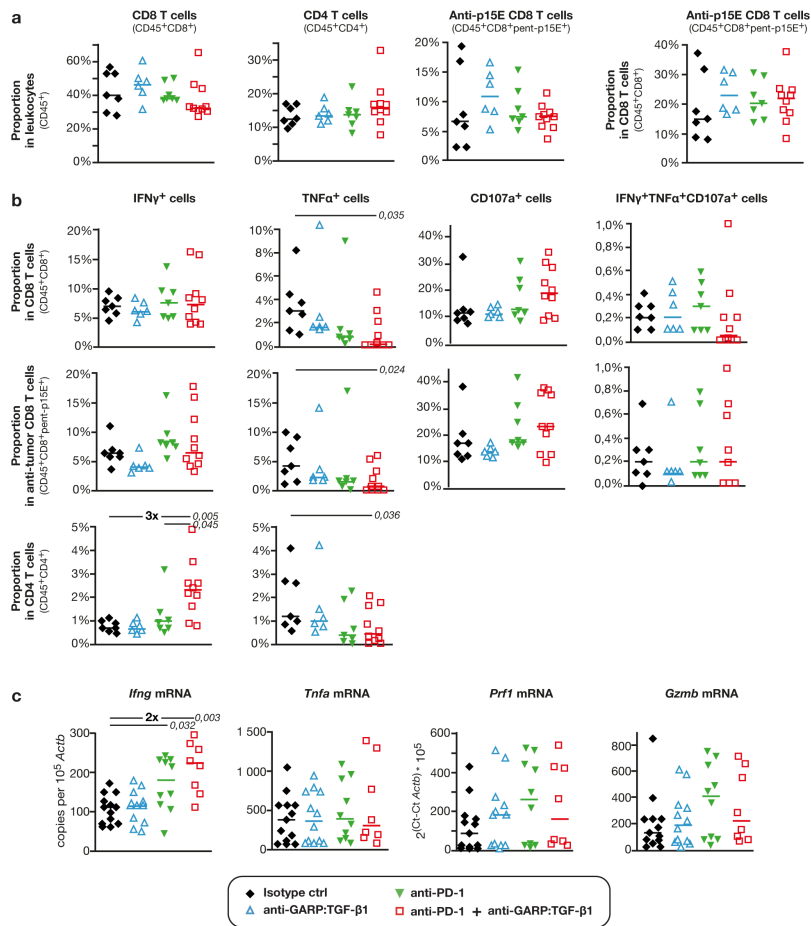


Figure S2

RESULTS

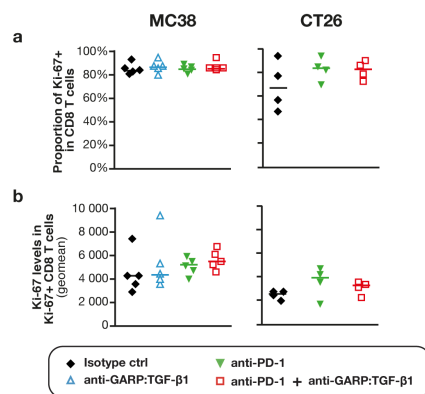


Figure S3

RESULTS

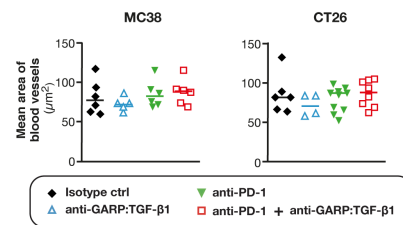


Figure S4

RESULTS

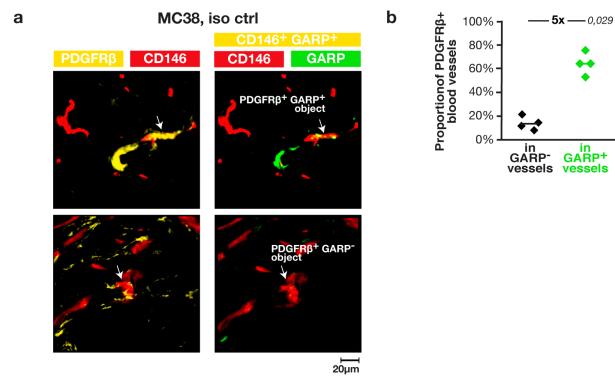


Figure S5

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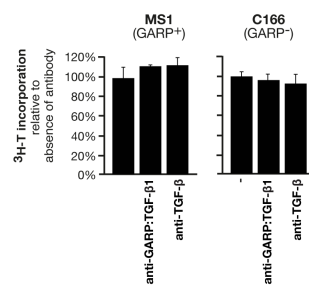


Figure S6

RESULTS

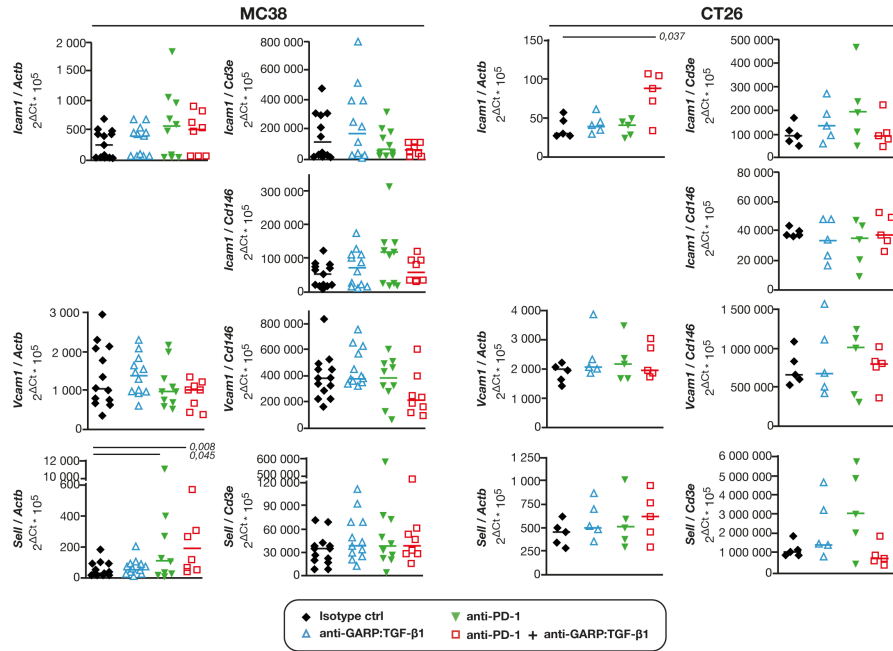


Figure S7

RESULTS

Supplementary Table 1. Primers sequences	
Sequence (5' - 3')	Definition
TCAAGTGGCATAGATGTGGAAGAA	Forward primer in <i>mlfng</i> mRNA
TGGCTCTGCAGGATTTTCATG	Reverse primer in <i>mlfng</i> mRNA
TCACCATCCTTTTCCAGTTCCTCCAG	Taqman probe in <i>mlfng</i> mRNA
AAGAGAGCAAGGACAACACTC	Forward primer in <i>mGzmb</i> mRNA
CATGTCCCCCGATGATCTC	Reverse primer in <i>mGzmb</i> mRNA
ACAAGGTCAGCAGTAGCAGGAGGA	Taqman probe in <i>mGzmb</i> mRNA
CAGTAGAGTGTGCGCATGTACAG	Forward primer in <i>mPrf1</i> mRNA
GATGAGCCTGTGGTAAGCAT	Reverse primer in <i>mPrf1</i> mRNA
TCGCCTGGTACAAAAACCTCCACTC	Taqman probe in <i>mPrf1</i> mRNA
CATCTTCTCAAAATTCGAGTGACAA	Forward primer in <i>mTnfa</i> mRNA
GGGTTGTACCTTGTCTACTCCCA	Reverse primer in <i>mTnfa</i> mRNA
CACGTCGTAGCAAACCAAGTGGA	Taqman probe in <i>mTnfa</i> mRNA
ATAGGAAGGCCAAGGCCAAG	Forward primer in <i>mCd3e</i> mRNA
TTGCGGATGGGCTCATAGTC	Reverse primer in <i>mCd3e</i> mRNA
ACCCGAGGAACCGGTGCTGGT	Taqman probe in <i>mCd3e</i> mRNA
GTGGTTGATGTCTTCTCTACAA	Forward primer in <i>mCd8b</i> mRNA
TCCGCACACAGTAAAGTAGAC	Reverse primer in <i>mCd8b</i> mRNA
AATGCCAGCAGAAGCAGGATGCAGACTA	Taqman probe in <i>mCd8b</i> mRNA
CGTGATAGCTGTGCTCTGAA	Forward primer in <i>mCd4</i> mRNA
GTTCTCTCCATGTCCAACCTAA	Reverse primer in <i>mCd4</i> mRNA
ACTGAGAGTGTCATGCCGAACCAG	Taqman probe in <i>mCd4</i> mRNA
GGTCCTTGCCTACTTGCTG	Forward primer in <i>mlcam1</i> mRNA
CTGTGCTTTGAGAACTGTGG	Reverse primer in <i>mlcam1</i> mRNA
CCGCTACCATCACCGTGATTCGTT	Taqman probe in <i>mlcam1</i> mRNA
CCATCCTTTCTTGAGATTCTTGC	Forward primer in <i>mSell</i> mRNA
CTTCATTCTGTAGCCGTCAT	Reverse primer in <i>mSell</i> mRNA
TTAACCGCCTTGCCAGCCAAATG	Taqman probe in <i>mSell</i> mRNA
GTCCTTCCGACAGTTTCTCTC	Forward primer in <i>mLfa1</i> mRNA
GGAGTCATGGAGTGTGGTATC	Reverse primer in <i>mLfa1</i> mRNA
TCCCAATGTAGCCAGACTCACACC	Taqman probe in <i>mLfa1</i> mRNA
AGTTCAATTTCTGCTGTCTTCA	Forward primer in <i>mSele</i> mRNA
ATGTGCCTTCTTACAACGTCT	Reverse primer in <i>mSele</i> mRNA
CCACGATGCATTTGTGTTCTGATTGTT	Taqman probe in <i>mSele</i> mRNA

DISCUSSION

DISCUSSION

1 How does combined GARP:TGF- β 1/PD-1 blockade induce tumor rejections?

My thesis work consisted in studying the effect of anti-GARP:TGF- β 1 mAbs in murine tumor models. By performing longitudinal analysis of tumor growth for up to 50 days, we showed that anti-GARP:TGF- β 1 mAbs induce rejection of tumors inoculated by subcutaneous (s.c.) injection of two colorectal cancer cell lines, namely CT26 and MC38, in mice otherwise resistant to anti-PD-1 therapy [73].

In this first article, we showed that tumor rejections induced by combined GARP:TGF- β 1/PD-1 blockade were immune-mediated, because mice that had rejected their CT26 primary tumor were protected against a rechallenge with CT26 cells, but not with other syngeneic cell lines such as RENCA or EMT6 [73]. Also, injection of antibodies depleting CD8 T cells or neutralizing IFN γ abrogated the anti-tumor effects of the combined therapy in CT26-bearing mice. Combination of anti-PD-1 and anti-GARP:TGF- β 1 mAbs did not increase CT26 tumor infiltration of T cells, but rather enhanced the effector functions of tumor-specific CTLs already present in the tumor bed [73]. This latter observation strikingly contrasted with observations made by others who had combined PD-(L)1 blockade with TGF- β blockade. Three publications reported that combined TGF- β /PD-(L)1 blockade increased CD8 T cell density within EMT6, CCK-168 or MBT-2 tumors, respectively [62, 65, 117]. Differences with observations made in CT26 with GARP:TGF- β 1 blockade might result from the fact that our blocking approach is more specific, blocking only TGF- β 1 emanating from GARP-expressing cells, or from the fact that we examined a different tumor type, and hence a different tumor microenvironment. In our first article, we had also showed that in MC38 tumor-bearing mice, anti-tumor effects of GARP:TGF- β 1/PD-1 blockade required the presence of GARP:TGF- β 1 complexes on Tregs, but not on platelets [73]. This indicates that anti-GARP:TGF- β 1 mAbs target Tregs to exert anti-tumor activity in MC38. However, we had not explored further the mode of action of combined GARP:TGF- β 1/PD-1 blockade in

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MC38 with regards to tumor infiltration of T cells and contrasting observations with combined TGF- β /PD-(L)1 blockade.

In our second paper [183], we directly compared MC38 and CT26 tumors to examine T cell infiltration, but also changes in the tumor vasculature after treatment with anti-PD-1 and/or anti-GARP:TGF- β 1 mAbs. We observed that, in contrast to what happens in CT26 tumors, combined GARP:TGF- β 1/PD-1 blockade in MC38 increased the density of tumor infiltrating T cells, which include tumor-specific (anti-p15E) CD8 T cells. T cells were located close to blood vessels in both tumor types. The proportion of CD8 T cells clustering close to blood endothelial cells (BECs) in MC38 was increased by combined GARP:TGF- β 1/ PD-1 blockade (69%) as compared to untreated tumors (48%). This strongly suggests that T cells enter the MC38 tumor bed *via* intratumoral blood vessels. However, we found no evidence of increased penetration of T cells farther within the tumor bed, either from blood vessels, or from the periphery towards the center of the tumor. Increased density of tumor-infiltrating CD8 T cells in MC38 was correlated with increased density of blood vessels containing GARP⁺ endothelial cells and covered by pericytes. Pericyte coverage is a hallmark of vessel normalization and is associated with increased vessel perfusion and immune cell infiltration [163]. We hypothesize that GARP:TGF- β 1 blockade increases proliferation of pericyte-covered, GARP-expressing BECs, because we observed that recombinant TGF- β 1 exerts a cytostatic effect on the GARP⁺ endothelial cell line MS1 *in vitro*. Increased entry of T cells in the tumor bed may thus result from increased density of functional vessels. In addition, we propose that it may also result from increased expression of leukocyte-endothelial adhesion molecule E-selectin, a selectin specifically expressed on endothelial cells. In MC38 however, GARP:TGF- β 1/PD-1 blockade did not increase effector functions of anti-tumor CD8 T cells already present within tumors, as it does in CT26. Interestingly, it did appear to increase production of IFN γ by CD4 T cells. We have no indication that the CD4 T cells producing IFN γ are directed against tumor-specific antigens. But IFN γ produced by CD4 T cells may contribute to vascular normalization [162, 184]. Figure 12 summarizes the changes observed in the immune and vascular

DISCUSSION

compartments of CT26 and MC38 tumors after combined GARP:TGF- β 1/PD-1 blockade.

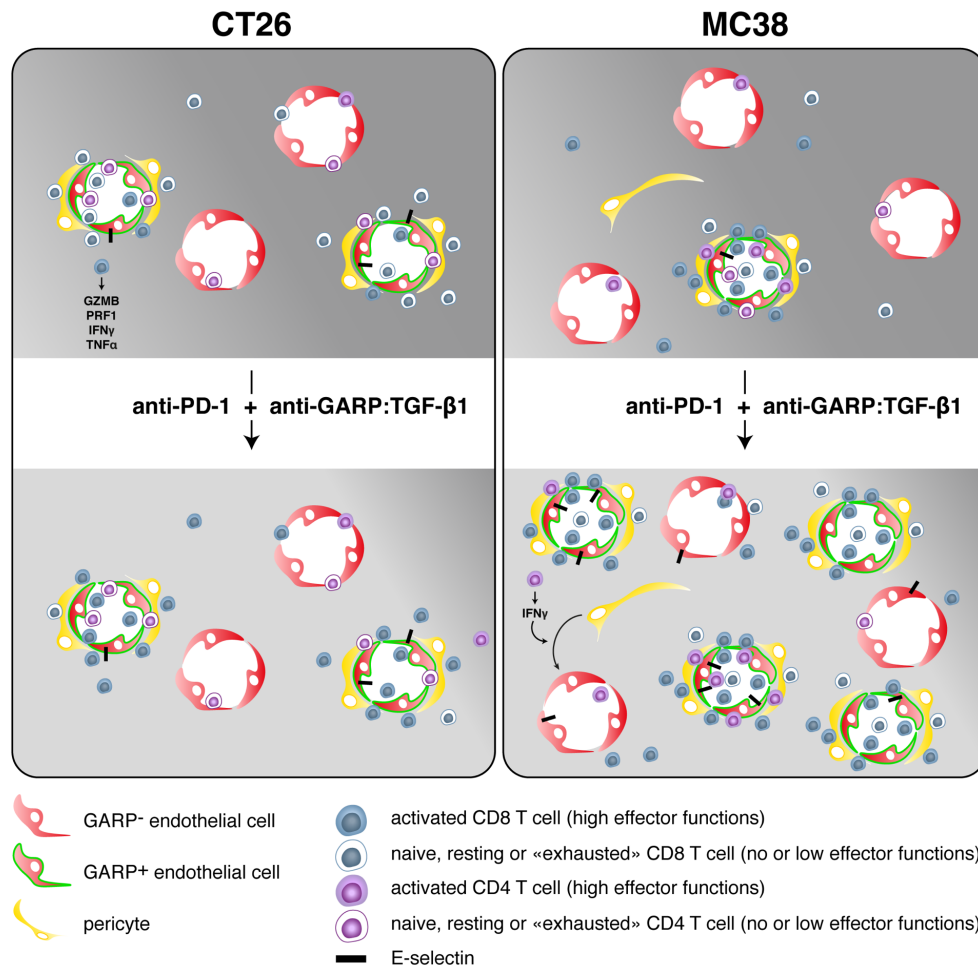


FIGURE 12. Graphical summary of modes of action for combined blockades of GARP:TGF- β 1 and PD-1 in CT26 vs MC38 tumors.

Overview of immune and vascular status in CT26 (left) and MC38 (right) tumor-bearing mice, untreated (upper panel) or treated with anti-PD-1 and anti-GARP:TGF- β 1 mAbs (lower panel). In CT26 tumors, combined therapy increases effector functions of tumor-specific CD8 T cells (blue) without increasing their density. In MC38 tumors, infiltration of CD8 T cells and CD4 T cells (purple) is increased by combined treatment, most probably as a result of increased density of blood vessels

DISCUSSION

comprising GARP⁺ (green) endothelial cells (red) surrounded by pericytes (yellow), and increased expression of E-selectin (black) by endothelial cells. IFN γ production by CD4 T cells is also increased and might contribute to vascular normalization by inducing recruitment of mural cells to blood vessels.

2 Do anti-GARP:TGF- β 1 mAbs target TGF- β 1 activation by GARP-expressing blood endothelial cells ?

To answer that question, we would first need to determine if BECs can activate TGF- β 1 in a GARP-dependent manner. Results previously generated in the laboratory show that HUVECs do not activate TGF- β 1 *in vitro*, at least in the absence of a required yet unidentified stimulus, or other cell type. This was evidenced by examining pSMAD2 levels by western blot. pSMAD2 levels remain unchanged after incubation with anti-active TGF- β 1,2,3 or anti-GARP:TGF- β 1 mAbs. Several studies reported that purified isolated endothelial cells can activate TGF- β 1 after exposure to pro-inflammatory cytokines or hypoxia [95, 96]. Pro-inflammatory signals such as IL-1 β or TNF α are known to activate endothelial cells, which then undergo physical changes to mediate diapedesis [185]. Using cytotaxis as a read-out for TGF- β activity, Phan *et al* observed that treatment of rat pulmonary artery endothelial cells with IL-1 β and TNF α increased production of active TGF- β in the media, while IL-1 β or TNF α alone had only modest effects [95]. Hypoxia was shown by others to increase ALK1 (*i.e.* a TGF- β type 1 receptor chain) expression and signaling in human aortic endothelial cells [96]. VEGF was shown to increase levels of integrin α V β 3, an RGD-binding integrin described to activate TGF- β 1 *in vitro*, on human dermal microvascular endothelial cells [186]. Amphiregulin (AREG), which signals *via* the EGF (Epidermal Growth Factor) receptor, was shown to induce integrin α V-dependent activation of TGF- β 1 by pericytes, which restores blood-barrier function after tissue injury [187]. To try to confirm that appropriately stimulated endothelial cells activate TGF- β 1, as suggested by others, and determine if they do so in a

DISCUSSION

GARP-dependent manner, I exposed human and murine endothelial cell lines expressing GARP:TGF- β 1 complexes (HUVEC and MS1) or not (EA.hy926 and C166) to various stimuli. Production of active TGF- β by the treated cells was measured in co-culture with reporter TMLEC, a cell line stably transfected with a construct containing the luciferase gene under the control of a TGF- β -responsive promoter (SMAD-binding elements). None of the stimuli previously reported to induce activation of TGF- β 1 by endothelial cells in the literature (*i.e.* IL-1 β + TNF α , VEGF), nor other stimuli (*i.e.* AREG, EGF, IL-6), increased TGF- β 1 production by GARP⁺ or GARP⁻ endothelial cell lines in our hands (Figure 13). In addition, baseline reporter signals were not reduced by an anti-TGF- β antibody (clone 1D11) or by the anti-GARP:TGF- β 1 mAb. Altogether, we concluded that endothelial cells do not activate TGF- β 1 in resting conditions or when stimulated with various molecules, at least *in vitro*.

DISCUSSION

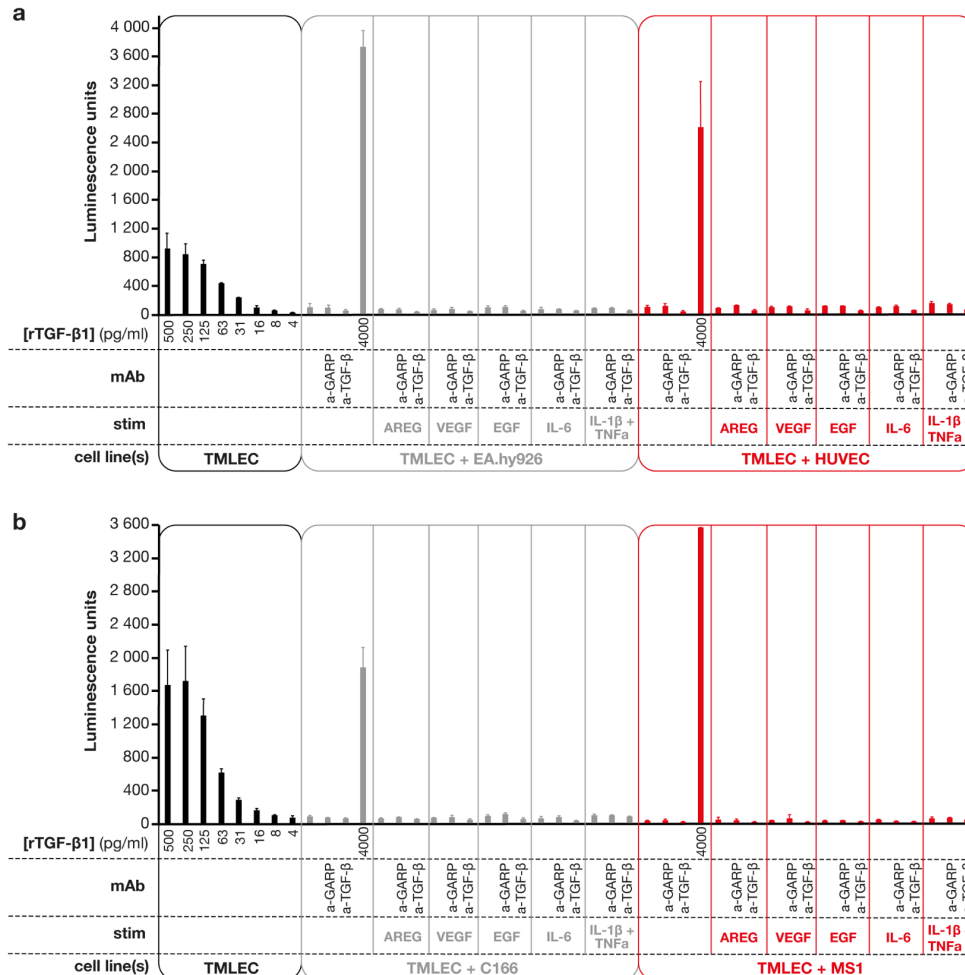


FIGURE 13. Human and murine endothelial cell lines do not activate TGF-β1 *in vitro*.

Human GARP⁻ (EA.hy926) and GARP⁺ (HUVEC) endothelial cell lines (**a**), and murine GARP⁻ (C166) and GARP⁺ (MS1) endothelial cell lines (**b**) were co-cultured with reporter TMLEC, stimulated with amphiregulin (AREG), VEGF, EGF, IL-6 or IL-1β and TNFα and treated with mAb directed against GARP:TGF-β1 (clone MHG-8 for human cell lines; clone 58A2 for murine cell lines) or against active TGF-β1, 2 and 3 (clone 1D11), or treated with recombinant TGF-β1 (rTGF-β1). TMLEC alone were treated with different doses of rTGF-β1. After 24 hours, luciferase activity (read-out of TGF-β1 activation) is assessed by addition of Bright-Glo (Promega) and luminescent signal is quantified using GloMax MicroPlate Reader (Promega). Each

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condition was tested in duplicate wells. Bars represent mean of duplicates. Error bars represent standard deviation.

This does not necessarily exclude that GARP-expressing endothelial cells could activate TGF- β 1 *in vivo*. Of note, GARP⁺ endothelial cell lines used in our assays either come from embryonic tissue (HUVEC, C166, EA.hy926) or were immortalized (MS1, EA.hy926), which may question their relevance and their representative value as compared to non-transformed endothelial cells *in vivo*. In that case, isolation of primary endothelial cells from organs would be an interesting alternative to the use of established cell lines. I tried to isolate endothelial cells from mouse lungs, livers and tumors. After tissue dissociation, I managed to obtain GARP⁺ endothelial cells by flow cytometry, but in insufficient numbers and only to poor purity. I could not further enrich these cells by differential centrifugation, use of a density gradient or sorting with magnetic beads. FACS (Fluorescent-Associated Cell Sorting) was not tested and could be an interesting alternative, although the number of endothelial cells obtained after processing remains a significant problem. Mouse liver is a particularly interesting organ to process, because Carambia *et al* have shown that isolated liver sinusoidal endothelial cells express GARP:TGF- β 1 and induce differentiation of naïve T cells into Foxp3⁺ Tregs in a TGF- β -dependent manner in co-culture assays [188]. The fact that we did not observe TGF- β 1 activity after endothelial cell stimulation *in vitro* could also suggest that the presence of a cellular partner is required for TGF- β 1 activation by these cells. Several studies have shown that co-culture of endothelial cells with mural cells (*i.e.* pericytes or SMCs) is required for this process [98-100]. So far, we have not tested this by co-culture of endothelial and mural cell lines, because the latter are quite difficult to obtain.

In the first article of my thesis [73], we showed that GARP expression on Tregs is required for the anti-tumor efficacy of combined GARP:TGF- β 1/PD-1 blockade in MC38 tumors. Indeed, anti-tumor effects of the combination are abolished in C57BL/6 mice harboring a Treg-specific deletion of *Garp* (*Foxp3^{Cre}* x *Garp^{fl/fl}*). The fact that GARP-expressing Tregs are targeted by the antibody does not exclude the

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possibility that GARP-expressing blood endothelial cells could also be targeted in addition to Tregs, and that this could constitute a complementary mechanism to mediate the anti-tumor effects of anti-GARP:TGF- β 1. To test this hypothesis, our goal was to use C57BL/6 mice that harbor an endothelial-specific deletion of *Garp* (*Tie2^{Cre}* x *Garp^{fl/fl}*). Unfortunately, preliminary experiments performed on this mouse strain revealed that GARP expression was not lost in all endothelial cells (incomplete deletion in endothelial cells), and more importantly yet, that the deletion was not specific of endothelial cells, as rather large proportions of Tregs, B cells and platelets had also lost *Garp* expression in these mice (Figure 14). Thus, the *Tie2^{Cre}* x *Garp^{fl/fl}* mouse strain is not suitable to perform conclusive tumor experiments *in vivo*. We will need to use another promoter to drive expression of the Cre recombinase if we want to address this question.

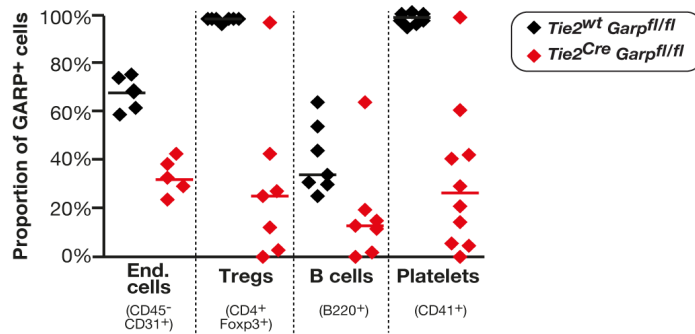


FIGURE 14. Deletion of GARP is not complete and specific of endothelial cells in *Tie2^{Cre} Garp^{fl/fl}* mice.

Proportion of GARP⁺ cells, from left to right, in endothelial cells (End. cells; CD45⁻CD31⁺ cells) isolated from liver, TCR-stimulated Tregs (CD4⁺Foxp3⁺ cells), BCR-stimulated B cells (B220⁺ cells) isolated from spleen and platelets (CD41⁺ cells) isolated from blood of *Tie2^{wt} Garp^{fl/fl}* (black) or *Tie2^{Cre} Garp^{fl/fl}* (red) mice, as analyzed by flow cytometry. Data points represent the values in individual mice, and horizontal bars represent the median (n=5-7 mice/genotype).

DISCUSSION

Another approach on-going in the laboratory aims at determining which cell types in the tumor microenvironment produce active TGF- β 1 by identifying the spatial relationships between pSMAD2⁺ cells and GARP-expressing Tregs and BECs in sections of frozen human tumors by multiplex immunofluorescence (mIF). This method could be applied to sections of murine tumors from mice treated or not with the anti-GARP:TGF- β 1 mAb. As we know that the antibody targets GARP⁺ Tregs in MC38 tumors, we would expect a decrease in the density of pSMAD2⁺ cells at proximity of GARP⁺FOXP3⁺ cells following the treatment. To determine if GARP⁺ endothelial cells produce active TGF- β 1 and are also directly targeted by the anti-GARP:TGF- β 1 mAb, similar analyses could be performed to measure pSMAD2⁺ cells at proximity of GARP⁺CD146⁺ cells.

Altogether, despite our efforts, we have not yet been able to determine whether GARP:TGF- β 1 complexes on endothelial cells serve as a source of active TGF- β 1 in the tumor microenvironment, and whether this cellular source needs to be targeted, in addition to Tregs, to observe anti-tumor effects upon treatment with anti-GARP:TGF- β 1 mAbs.

3 How does combined GARP:TGF- β 1/PD-1 blockade increase the density of pericyte-covered GARP⁺ blood vessels in MC38, and why not in CT26 tumors?

At the present time, we still cannot propose a clear molecular mechanism that would explain how combined blockades of PD-1 and GARP:TGF- β 1 increase the density of blood vessels containing GARP⁺ endothelial cells. In our second article [183], we show that tumor-infiltrating T cells, which include Tregs, are preferentially located very close to blood vessels. We could speculate that Tregs are even closer to GARP⁺ than GARP⁻ blood vessels, because GARP⁺ vessels are also more frequently

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stabilized by pericytes, and pericyte coverage increases perfusion and thus potentially T cell infiltration. We could not test this hypothesis in our mIF analyses, because we have not performed co-staining of Foxp3 and CD146. Nevertheless, by decreasing TGF- β 1 activation by Tregs, including Tregs located close to GARP⁺ BECs, anti-GARP:TGF- β 1 mAbs could decrease the paracrine activity of Treg-derived TGF- β 1 on endothelial cells. This could lead to increased proliferation of GARP⁺ BECs. In our hypothesis, GARP:TGF- β 1 complexes on endothelial cells would represent a marker of stabilized blood vessels, but not necessarily a source of active TGF- β 1. Indeed, this remains in line with results of experiments testing anti-GARP:TGF- β 1 mAbs in *Foxp3^{Cre} x Garp^{fl/fl}* mice bearing MC38 tumors, which indicates that Tregs need to express GARP to observe anti-tumor effects with anti-GARP:TGF- β 1 mAbs. Notwithstanding this, our observation that GARP:TGF- β 1/PD-1 blockade increased the intra-tumor expression of *Sele*, an endothelial cell-specific mRNA known to be repressed by TGF- β 1, may indicate indeed that TGF- β 1 signaling is reduced in endothelial cells in response to GARP:TGF- β 1/PD-1 blockade in MC38 tumors. Increased *Sele* expression upon GARP:TGF- β 1/PD-1 blockade was not observed in CT26 tumors.

Based on the above, it is tempting to speculate that differences in blood vessel proliferation in MC38 and CT26 tumors could result from differences in Treg densities in the two tumor models. More precisely, it could be that anti-GARP:TGF- β 1 mAbs do not increase blood vessel density in CT26 because Tregs close to blood vessels are less abundant in these tumors. mIF analyses of FFPE (Formalin-Fixed Paraffin-Embedded) non-treated control tumors showed that the density of CD4 T cells is 2,5 fold higher in MC38 ($3,7\% \pm 1,7\%$ of DAPI⁺ nuclei; median $\pm$ interquartile range or IQR) compared with CT26 tumors ($1,5\% \pm 0,2\%$). Flow cytometry confirmed that this difference was also true for Tregs: proportions of CD4⁺ FOXP3⁺ cells in live single cells is 1,7 fold higher in MC38 tumors ($1,5\% \pm 0,1\%$) as compared with CT26 tumors ($0,9\% \pm 0,3\%$). Further experiments need to be performed to support our hypothesis, notably to determine if *i)* density of T cells, including Tregs, is indeed higher close to GARP⁺ vessels as compared to GARP⁻ vessels in MC38 tumors; and

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ii) GARP⁺ BECs are under the influence of paracrine TGF- β 1 produced by GARP⁺ expressing Tregs.

Performing thymidine incorporation assays on murine endothelial cell lines, I observed that not all endothelial cells respond to TGF- β 1-mediated cytostasis *in vitro*. Whilst MS1 cell proliferation was decreased in a dose-dependent manner by treatment with recombinant TGF- β 1, this was not the case for C166 cells. *In vivo*, similar situations could occur, with some endothelial cells susceptible to cytostasis by TGF- β 1, but others not. This is especially relevant as TGF- β 1 has been shown to exert dual opposing effects on angiogenesis, by both promoting or blocking endothelial proliferation depending on the signaling pathway engaged [130]. Another explanation to the difference observed between MC38 and CT26 after GARP:TGF- β 1/PD-1 blockade in terms of blood vessel density could thus be that BECs are less sensitive to TGF- β 1 cytostasis in CT26 than in MC38 tumors.

Finally, the fact that anti-GARP:TGF- β 1 mAbs do not modify the density of GARP⁺ blood vessels in CT26 could also be explained by the lower proportions of blood vessels containing GARP⁺ BECs in these tumors by comparison to MC38. Quantification of vascular structures by digital imaging of mIF stainings showed that in untreated MC38 tumors, 45,3% $\pm$ 6,2% (median $\pm$ IQR) of blood vessels were GARP⁺, as compared to 30,7% $\pm$ 7,7% in CT26 tumors. This difference could impact the effects of anti-GARP:TGF- β 1 mAbs, particularly if they were to directly target GARP⁺ endothelial cells, a hypothesis that we still have not excluded completely.

4 How does anti-GARP:TGF- β 1 modulate T cell activity in CT26 and MC38 tumors and how does that mediate anti-tumor efficacy?

In CT26 tumors, combined GARP:TGF- β 1/PD-1 blockade increases the effector functions of tumor-specific (anti-AH1) CD8 T cells. Two preliminary observations

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made in our lab indicate that this occurs *via* a mechanism that may not involve direct blocking of TGF- β 1 signaling in anti-tumor CD8 T cells.

First, we observed by flow cytometry that the ratio of total (nuclear + cytoplasmic) to cytosolic SMAD2, which is indicative of TGF- β 1 signaling, was not decreased in CT26 tumor-infiltrating anti-AH1 CD8 T cells following treatment of mice with anti-GARP:TGF- β 1 mAb (Figure 15a, left panel). In contrast, the total-to-cytoplasmic SMAD2 ratio was decreased in tumor-infiltrating Tregs after GARP:TGF- β 1/PD-1 blockade, although only a few samples were analyzed and the difference with untreated tumors did not reach statistical significance (Figure 15a, right panel). These data suggest that blocking GARP:TGF- β 1 complexes reduce autocrine TGF- β 1 signaling in Tregs, but not paracrine signaling by Treg-derived TGF- β in CD8 T cells. Of note, we used total-to-cytoplasmic SMAD2 ratios to evaluate TGF- β 1 signaling by flow cytometry, because detection of pSMAD2 does not work in our hands with this method. Interestingly, we observed increased total-to-cytoplasmic SMAD2 ratios in CT26-infiltrating GARP⁺ Tregs after *ex vivo* treatment with rTGF- β 1, but not in anti-AH1 CD8 T cells obtained from mice injected intraperitoneally (*i.p.*) with rTGF- β 1 (Figure 15b-c). This may suggest that tumor-infiltrating anti-AH1 CD8 T cells are less sensitive to TGF- β 1 than Tregs. It is unlikely that such difference would be due to a lower efficiency of rTGF- β 1 *in vivo* treatment as compared to *ex vivo* treatment, because *i.p.* injection of rTGF- β 1 increased by 2-fold the total-to-cytoplasmic SMAD2 ratio in CD4 T cells, which includes Tregs.

Second, Grégoire de Streel in our lab observed that anti-AH1 CD8 T cells infiltrating CT26 tumors do not express CD103, a tissue-resident marker that is typically induced by TGF- β 1 signaling [174]. This also suggests that anti-tumor CD8 T cells may not be under the influence of TGF- β 1 signaling within CT26 tumors.

If combined GARP:TGF- β 1/PD-1 blockade does not result in reduced TGF- β 1 signaling in CD8 T cells, increase in CD8 T cell effector functions could be the consequence of the reduction of an immunosuppressive activity by Tregs that does not imply TGF- β 1 production. TGF- β 1 is known to be required to sustain Treg

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immunosuppressive mechanisms [189]. We thus hypothesize that decreased TGF- β 1 signaling in Tregs themselves would reduce the putative TGF- β 1-independent immunosuppressive activity that they exert on anti-tumor CD8 T cells. Another possibility is that blocking TGF- β 1 activation by GARP-expressing Tregs decreases TGF- β 1 signaling in antigen-presenting cells such as DCs, which would in turn increase priming and activation of naïve T cells, and thus indirectly increase effector functions of anti-tumor CD8 T cells.

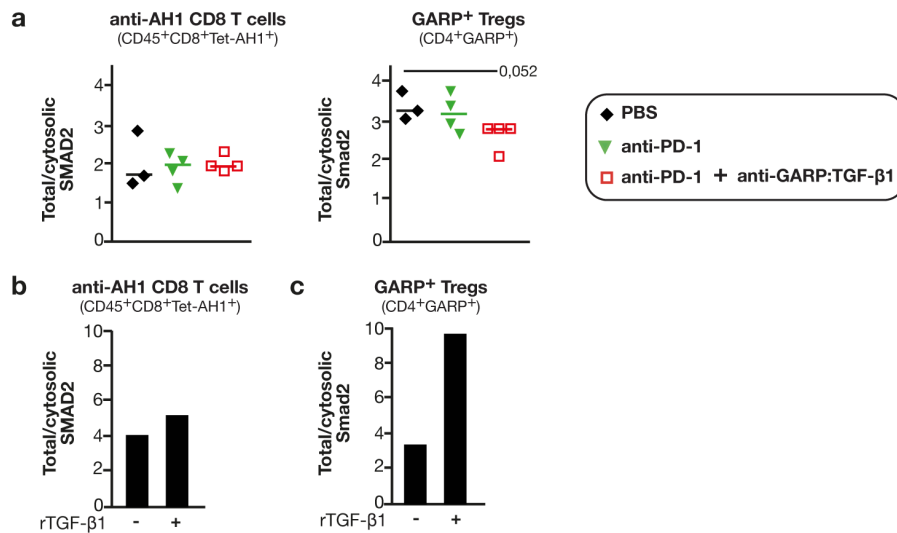


FIGURE 15. TGF- β 1 signaling is decreased in GARP+ Tregs but not in tumor-specific CD8 T cells in CT26 tumors following combined GARP:TGF- β 1/PD-1 blockade.

(a) Mice were injected s.c. with CT26 cells and treated with PBS, anti-PD-1 (clone RMP1-14 mIgG2a FcSilent) or a combination of anti-PD-1 and anti-GARP:TGF- β 1 (clone 58A2 mIgG2a DANA) 2 to 3 times. Tumors were collected and dissociated on day 15 (left panel) or 10 (right panel).

(b) Mice were injected s.c. with CT26 cells on day 0 and intraperitoneally (*i.p.*) with recombinant TGF- β 1 (rTGF- β 1, 500ng/mouse) 20 hours prior to tumor collection. Tumors were collected and dissociated on day 15.

(c) Mice were injected s.c. with CT26 cells. Tumors were collected and dissociated on day 10, and treated *ex vivo* with rTGF- β 1 (10ng/ml) for 30 minutes.

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SMAD2 levels were assessed by flow cytometry after nuclear permeabilization (total SMAD2) or cytosolic permeabilization (cytosolic SMAD2) on the same samples. Total-to-cytosolic ratio of SMAD2 in tumor-infiltrating anti-AH1 CD8 T cells (CD45⁺CD8⁺Tet-AH1⁺) and GARP-expressing Tregs (CD45⁺CD4⁺GARP⁺) is shown. Dots represent individual values (n=3-5 mice/group), and horizontal bars represent median **(a)**. Bar graphs show median of 2 **(b)** to 3 mice **(c)** analyzed.

In MC38 tumors, combined GARP:TGF- β 1/PD-1 blockade does not increase the activity of anti-tumor CD8 T cells, but it does increase the proportion of IFN γ -producing CD4 T cells. Tian and colleagues have shown that immune checkpoint blockade therapies promote blood vessel normalization in tumors *via* activation of Th1 CD4 helper T cells [162]. Another study underlines the involvement of CD8 T cells, but not CD4 T cells, in this process [155]. Notwithstanding their differences, the two publications identify IFN γ as the molecular mediator of the immune-mediated induction of vascular normalization in tumors. Thus, we propose that the combined GARP:TGF- β 1/PD-1 blockade increases the density of normalized vessels by creating a positive feedback loop that induces: *i*) the proliferation of pericyte-covered GARP⁺ endothelial cells as a result of blocking TGF- β 1 signals in BECs; and *ii*) the IFN γ -mediated normalization of blood vessels due to the action of anti-PD-1. Requirement for a synergistic action of both processes might explain why treatment with anti-PD-1 or anti-GARP:TGF- β 1 mAb alone fail to increase the density of blood vessels on their own.

5 What to expect from the use of anti-GARP:TGF- β 1 mAbs in the clinics?

A phase I clinical study was launched in 2019 to test the safety and efficacy of anti-GARP:TGF- β 1 in combination with anti-PD-1 in cancer patients (ClinicalTrials.gov: NCT03821935). Cancer types included in the study comprise locally advanced or metastatic triple-negative breast cancer, pancreatic adenocarcinoma, urothelial

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cancer, hepatocellular carcinoma and head and neck squamous cell carcinoma. Indications were selected based on data suggesting high prevalence of TGF- β 1 signaling and/or T cell activity in these cancer types.

5.1 Anti-tumor efficacy and tumor infiltration of T cells

We observed that combined GARP:TGF- β 1/PD-1 blockade increases the effector functions of tumor-specific CD8 T cells already present in CT26 tumors. We thus initially proposed that anti-GARP:TGF- β 1 mAbs should ideally be tested in the clinics in patients with so-called “hot” or “inflamed” tumors, *i.e.* tumors that are abundantly infiltrated by immune cells which are unable control tumor growth. However, our follow-up work with the MC38 tumor model revealed a potential additional mode of action of combined GARP:TGF- β 1/PD-1 blockade, which enhances the density of stabilized blood vessels and T cell infiltration of these tumors. A similar mode of action could thus perhaps contribute to the ability of anti-GARP:TGF- β 1 mAbs to overcome resistance to PD-1/PD-L1 blockade in patients suffering from a broader range of cancer types than we had initially proposed. Based on our results in MC38, we suggest that anti-GARP:TGF- β 1 mAbs could be tested not only in patients with inflamed tumors, but also with tumors that are poorly vascularized and poorly infiltrated with immune cells (*i.e.* so called “cold” tumors, comprising “immune-excluded” and “immune desert” tumors) [190].

Of note however, our suggestion that anti-GARP:TGF- β 1 may overcome resistance to anti-PD-1 by increasing tumor vascularization and T cell infiltration derives from observations in mice bearing tumors that cannot be qualified as “cold”. MC38 tumors are infiltrated by $\pm$ 3 times more T cells than CT26 tumors. Subcutaneously transplanted MC38 tumors were qualified by others as a model of immune-excluded tumors, where T cells are confined in stromal regions (*i.e.* fibrotic regions surrounding the tumor bed) [62]. We did not observe exactly the same distribution of T cells in MC38: in our hands, T cells in MC38 tumors were not confined to the tumor periphery, but they were indeed enriched close to the tumor margin by comparison to the tumor center. Thus, we cannot qualify MC38 tumors as cold tumors, because

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they are neither immune desert nor completely immune-excluded. To further support our hypothesis that anti-GARP:TGF- β 1 mAb could help increase TIL density in “cold” tumors, we should assess changes occurring after combined GARP:TGF- β 1/PD-1 blockade in the microenvironment of murine tumors that are poorly infiltrated with T cells, such as subcutaneously implanted B16F10 tumors [191]. We did test monotherapies and combinations of anti-PD-1 (clone RMP1-14 WT) and anti-GARP:TGF- β 1 mAbs in B16F10, but none of the treatment induced tumor rejections. However, we had observed in CT26 that the anti-PD-1 antibody in a wild-type format was less potent than the anti-PD-1 antibody in a Fc-silent version [73]. This may indicate that anti-GARP:TGF- β 1 would need to be combined with the anti-PD-1 FcSilent mAbs to exert synergistic anti-tumor efficacy in B16F10 tumors. But we have not tested this combination to date.

5.1 Potential toxicities

The use of anti-GARP:TGF- β 1 mAbs in cancer patients may raise concerns regarding potential toxicities, given the pleiotropy of TGF- β 1 actions. More specifically, TGF- β 1 and Tregs are crucial for the maintenance of immune tolerance in the periphery, as evidenced by the phenotype of *Tgfb*^{-/-} mice, and mice harboring loss-of-function mutations in *Foxp3*, that both die from autoimmune-like disorders [82, 94].

The precise contribution of GARP in maintaining peripheral tolerance is unclear. It is noteworthy that *Garp* KO mice are not viable: they die shortly after birth due to the presence of a cleft palate and a consequent inability of pups to thrive [192]. Thus, the role of GARP has been mainly studied in conditional, cell type-specific *Garp* knock-outs.

Deletion of *Garp* specifically in Tregs (*Foxp3*^{Cre} $\times$ *Garp*^{fl/fl} mice) does not affect their ability to suppress effector T cell responses in steady state conditions, and these mice do not display overt auto-immunity or inflammation [193]. However, in the same study, the authors show that *Foxp3*^{Cre} $\times$ *Garp*^{fl/fl} mice spontaneously develop a mild lupus-like syndrome upon aging. We examined mice from a similar strain (*Foxp3*^{Cre}

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x Garp^{fl/fl} mice housed in our SPF facility), but we did not observe these spontaneous lupus-like manifestations upon aging (Zhang *et al*, in preparation). In contrast, we observed moderate lupus-like symptoms *i*) upon aging in mice with a B cell-specific *Garp* deletion (*Mb1^{Cre} x Garp^{fl/fl}* mice), and *ii*) after persistent overexpression of IFN α induced by plasmid electroporation in the tibialis muscle of *Foxp3^{Cre} x Garp^{fl/fl}* mice. The mild lupus-like phenotypes included moderate leukopenia and IgG depositions in the kidneys (Zhang *et al*, in preparation). We concluded from our observations that the absence of GARP on Tregs increases susceptibility to moderate lupus-like auto-immunity in conditions of chronic inflammation, and that the absence of GARP on B cells increases susceptibility to the development of spontaneous lupus-like autoimmunity upon aging. We do not know whether differences between our observations and those by the group of Zihai Li in Treg-specific GARP KO mice are due to different microbiota in our mouse strains or to other factors.

TGF- β 1 signals regulate many processes involved in mucosal immunity, such as CD4 T cell differentiation towards Th17 or pTregs and B cell switch to IgA production. Thus, we and others also examined the roles of GARP-expressing Tregs in the immune homeostasis of the gut. Again, contradictory results were obtained. On the one hand, Salem *et al* have shown that Tregs lacking *Garp* expression could not control colitis, whether it was induced by DSS (Dextran Sulfate Sodium) or by transfer of naïve CD4 T cells [193]. On the other hand, we have seen that *Garp* deletion in Tregs did not modify the ability of Tregs to control colitis induced by naïve CD4 T cells transfer in immunodeficient mice (Xuhao Zhang, personal communications). Treatment with anti-GARP:TGF- β 1 mAbs did not aggravate intestinal inflammation induced by oral gavage of *Citrobacter rodentium* in highly susceptible IL-22 receptor KO mice. It also did not induce systemic dissemination of the bacteria, nor alteration of the innate and adaptive immune responses to the infection (Gaignage, Zhang *et al*, submitted).

Thus, results from our laboratory are reassuring with regard to the risk of severe inflammation or auto-immune manifestations that could be provoked by treatment

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with anti-GARP:TGF- β 1 in cancer patients. Of course, this will need to be confirmed in the currently ongoing clinical trial.

To date, no study has reported roles for GARP on endothelial cells. We have not yet been able to determine whether endothelial cells activate TGF- β 1 *via* GARP *in vivo*. Importantly though, Vermeersch and colleagues did show that deletion of *Garp* in endothelial cells (*Tie2^{Cre} x Garp^{fl/fl}* mice) did not affect thrombus formation or thrombo-inflammation [74]. However, *Garp* deletion in endothelial cells is neither complete nor strictly restricted to endothelial cells in these mice (as discussed above). This might explain why a putative, non-redundant function for GARP on endothelial cells has not been identified yet, and suggests that a more adapted *Cre* transgenic mouse strain should be used to analyze the role of *Garp* on these cells, and predict possible toxicities of GARP:TGF- β 1 blockade in cancer patients.

Although we have shown that GARP-expressing platelets are not required for the anti-tumor effects of GARP:TGF- β 1/PD-1 blockade in MC38 tumors [73], the anti-GARP:TGF- β 1 antibody may still exert platelet-related effects outside of the tumor. Our group and others have shown that platelets activate TGF- β 1 in a GARP-dependent manner during blood coagulation *in vitro*, but the relevance of this observation *in vivo* still remains to be elucidated [79, 194]. Mice harboring a deletion of *Garp* specifically in platelets and megakaryocytes (*Pf4^{Cre} x Garp^{fl/fl}* mice) do not develop auto-immune-like disorders and have normal hemostasis. Absence of GARP on platelets thus does not appear to affect platelet aggregation, nor thrombosis [74].

We could not detect GARP-expressing B cells in human or murine tumors using mIF analyses or flow cytometry. In preliminary experiments, treatment of tumor-bearing *Mb1^{Cre} x Garp^{fl/fl}* mice with anti-PD-1 and/or anti-GARP:TGF- β 1 mAbs indicate that GARP expression on B cells is not required to observe anti-tumor effects upon combined GARP:TGF- β 1/PD-1 blockade (unpublished data). We have previously shown that BCR-stimulated human B cells activate TGF- β 1 *via* GARP *in vitro*, and that this favors isotype switching towards IgA production [72]. In mouse however,

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production of antigen-specific IgM, IgG and IgA following immunization with OVA (ovalbumin) or CTB (B subunits of cholera toxin) was not altered in mice carrying a deletion of *Garp* in B cells, nor in wild-type mice treated with anti-GARP:TGF- β 1 mAbs (Gaignage, Zhang *et al*, submitted).

Altogether, our observations are encouraging with regard to the safety of anti-GARP:TGF- β 1 mAbs in the clinics. No major alteration of immune homeostasis was observed following treatment of wild-type mice with this antibody, or in conditional *Garp* KO mouse strains.

Of course, the phase I clinical trial that was recently initiated to test anti-GARP:TGF- β 1 in combination with anti-PD-1 will allow for a more thorough evaluation of the safety profile of anti-GARP:TGF- β 1 mAbs in cancer patients. Hopefully, it may also provide encouraging preliminary indications of putative anti-tumor efficacy for this novel immunotherapeutic approach of metastatic cancer.

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