Selective inhibition of TGF-β1 produced by GARP-expressing Tregs overcomes resistance to PD-1/PD-L1 blockade in cancer

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TGF-β1, β2 and β3 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-β1 production by Tregs with antibodies against GARP:TGF-β1 complexes induces regressions of mouse tumors otherwise resistant to anti-PD-1 immunotherapy. Effects of combined GARP:TGF-β1/PD-1 blockade are immune-mediated, do not require FcγR-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-β1 in one third of human melanoma metastases. Our results suggest that anti-GARP:TGF-β1 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.
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 immunity1-3, and Tregs appear deleterious to anti-tumor immunity in most patients and cancer types4-12. 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 potently immunosuppressive TGF-β1 cytokine. Like most other immune cells, Tregs produce TGF-β1 in a latent, inactive form, in which the mature TGF-β1 dimer is non-covalently associated to the latency associated peptide (LAP). LAP forms a ring around mature TGF-β1, masking the interaction sites with the TGF-β1 receptor chains4,14,15. Only a few cell types are able to activate the cytokine by releasing mature TGF-β1 from LAP, through cell-type-specific mechanisms16. Upon T-cell receptor (TCR) stimulation, Tregs present latent TGF-β1 on their surface via disulfide linkage of LAP to a transmembrane protein called GARP15,17,18. Integrin αVβ8 interacts with GARP:(latent)TGF-β1 complexes, leading to release of active TGF-β1 close to the surface of stimulated Tregs19,20. Treg-derived active TGF-β1 exerts paracrine, short-distance immunosuppressive effects on immune cells, including T cells16. We derived anti-GARP:TGF-β1 mAbs that block the TGF-β1 activation by TCR-stimulated human Tregs, through a molecular mechanism elucidated via X-ray crystallography15,21. These mAbs do not bind complexes of GARP and latent TGF-β2 or β3, which are produced by non-T-cell types and once activated, signal via the same receptor as TGF-β115. Blocking anti-GARP:TGF-β1 mAbs inhibited the immunosuppression by human Tregs of a xenogenic graft-versus-host disease induced by transfer of human PBMCs into immunodeficient NSG mice21.

Here, we derive an anti-mouse GARP:TGF-β1 mAb that blocks release of active TGF-β1 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-β1 mAbs in the clinics.

Results

Anti-GARP:TGF-β1 mAb blocks TGF-β1 activation by mouse Tregs. Previously described blocking antibodies against human GARP:TGF-β1 complexes do not recognize mouse GARP:TGF-β1. To derive mAbs that block TGF-β1 activation from GARP: TGF-β1 complexes on murine Tregs, we immunized llamas with plasmids encoding mouse GARP and TGF-β1, and constructed V\(_H\)/V\(_k\) and V\(_H\)/V\(_\lambda\) cDNA libraries to select Fab clones binding mouse GARP:TGF-β1 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-β1 complexes but not free GARP or free latent TGF-β1 (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-β1 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 (FdC) mIgG2a subclass antibody. The FcD mIgG2a contains two amino-acid substitutions in the Fc region (D265A/N295A) that preclude binding to all mouse FcYRs22,23 (Supplementary Fig. 1). Binding and blocking activities of clone 58A2 closely resembled those of blocking anti-human GARP:TGF-β1 mAbs24,25. Clone 58A2 was thus further selected to test whether treatment with blocking anti-GARP:TGF-β1 mAbs could improve anti-tumor immune responses and favor tumor rejections in mice.

Anti-GARP:TGF-β1 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-β1 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-β1 or block TGF-β1 activation from GARP:TGF-β1 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 FcYRs, 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 FcγR-dependent agonistic activity on PD-1 expressed by CD8+ T cells25.

As shown in Fig. 2b, combination with anti-GARP:TGF-β1 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-β1 did not require its binding to FcYRs: tumor rejection was also more frequent (CR: 4/10) when anti-GARP:TGF-β1 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-β1 (WT or FcD), but not with anti-TGF-β (Fig. 2c). This indicates that in CT26-bearing mice, blocking the activity of TGF-β1 emanating from GARP:TGF-β1-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-β1 significantly increased the anti-tumor activity of anti-PD-1 against established CT26 tumors in seven independent
Fig. 1 An anti-GARP:TGF-β1 mAb that blocks TGF-β1 activation by mouse Tregs in vitro. a 293T cells were transfected with constructs encoding HA-tagged mouse GARP, mouse TGF-β1 (i.e. LAP + mature TGF-β1), or both, to induce surface expression of free GARP, free latent TGF-β1, or GARP:TGF-β1 complexes, respectively (Cuenode 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-β1 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-β1 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-β1 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.

Anti-GARP:TGF-β1 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-β1 WT were injected 47 days after the last mAb administration with live CT26 cells in the right flank, and live RENCA or EMT6 syngeneic tumors 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-β1 combined with anti-PD-1 induces protective T-cell-mediated immunity against CT26-specific tumor antigens.

Anti-GARP:TGF-β1 does not modify numbers of TILs. We examined whether treatment with anti-GARP:TGF-β1, 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-β1 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 ~9 × 10^4 leukocytes (CD45+ cells) per mm^3 (Fig. 5b). Tumor-infiltrating leukocytes (TILs) comprised 16 ± 2% (mean ± sem) and 17 ± 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 mm^3 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
antigen, using an AH1/H-2Ld tetramer. AH1 is an immunodominant peptide presented by H-2Ld 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 cells. Anti-AH1 CD8+ T cells represented 31 ± 2% of the total CD8+ T cells in infiltrating tumors on day 13 (Supplementary Fig. 5a). Again, proportions and numbers of anti-AH1 CD8+ T cells per mm3 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
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 ± 3% of the CD4+ TILs were Tregs (FOXP3+), and 54 ± 2% of the tumor-infiltrating Tregs 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 is not T regs.
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Fig. 4 Combined blockade of GARP:TGF-β1 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-β1 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 mm². 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 mm^3 of tumor were not decreased in mice that had received an anti-GARP:TGF-β1 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-β1 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-β1 antibodies, either in a WT or an FcD format, did not deplete intra-tumoral Tregs in this model.

GARP:TGF-β1/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 TNFα and IFNγ and direct perforin/granzyme-dependent killing of tumor cells. Signaling induced in CD8^+ T cells by binding of TGF-β1 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 Prf1, Gzmβ, Tnf, and Ifng mRNAs were observed in tumors from mice treated with a combination of anti-PD-1 and anti-GARP:TGF-β1 WT or FcD 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 IFNγ, TNFα, IL-2, and inflammation were observed in mice treated with the anti-GARP:TGF-β1 (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-β1 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 IFNγ and TNFα, and surface CD107α 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 IFNγ, TNFα, or CD107α, 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-β1 WT or FcD mAbs, but not with either mAb alone (Fig. 5f and Supplementary...
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
Fig. 5 Combined blockade of GARP:TGF-β1 and PD-1 increases effector functions of anti-tumor CD8+ T cells. BALB/c mice (n = 5/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. 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. d 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: IFNγ = 179; TNFa = 184; inflammatory = 164; and IL-2 = 175. e 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 effectors functions (shown in f) with tumor weight in the corresponding mouse at day 13. z = 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 ≥5% of anti-AH1 CD8+ T cells with multiple effector functions and received the combination of anti-PD-1 and anti-GARP:TGF-β1 WT or FcD mAbs (Fig. 5g).

Altogether, these data indicate that treatment with a combination of anti-GARP:TGF-β1 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 IFNγ 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-β1 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-β1 WT and anti-PD-1 WT mAbs. Depletion of CD8+ T cells abrogated the anti-tumor efficacy of the combination treatment (Fig. 6a). Second, we administered a neutralizing anti-IFNγ mAb into tumor-bearing mice on the same days as treatments with anti-GARP:TGF-β1 and anti-PD-1. Neutralization of IFNγ also abrogated the anti-tumor efficacy of the combination treatment (Fig. 6b). Together, these results suggest that increased IFNγ production by CD8+ T cells contributes to the anti-tumor activity of anti-GARP:TGF-β1 combined with anti-PD-1.

Anti-GARP:TGF-β1 acts by blocking TGF-β1 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-β1 exerts anti-tumor activity by blocking TGF-β1 activation by Tregs but not by other GARP-expressing cell types. However, TGF-β1 activation by GARP+ platelets was also suggested to suppress anti-tumor immunity33. We thus derived two C57BL/6 mouse strains carrying a T-reg 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-β1 (Fig. 7a). As shown in Fig. 7b, the proportions of complete responses to anti-GARP:TGF-β1 + 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-β1 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-β1 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, contributing to the anti-tumor activity of GARP+FOXP3+ cells 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) 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 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 IFNγ responsive genes, indicating that the more T cells and activated IFNγ-producing T cells, the more Tregs and GARP+ Tregs in the melanoma samples (Supplementary Data 1).
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, IFNγ, TNFα, 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-1β-, TNFα-, or TGF-β1-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-β1 mAbs could serve as an immunotherapeutic approach to inhibit TGF-β1-dependent immunosuppression by intra-tumoral Tregs in patients with cancer.

In mice bearing CT26 or MC38 tumors, blocking anti-GARP:TGF-β1 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-β1 mAbs significantly increased the frequency of tumor rejection relative to anti-PD-1 alone. This does not necessarily imply that anti-GARP:TGF-β1 mAbs will have no
anti-tumor activity as monotherapy in patients with cancer, but 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-β1 emanating from GARP-expressing Tregs is required for anti-GARP:TGF-β1 to exert anti-tumor activity. They suggest also that blocking the activity of TGF-β1 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 IFNγ 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-β1, anti-PD-1, or a combination of the two. This suggests that in MC38 or CT26 models at least, blocking the activity of TGF-β1 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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**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-β1 and isotype control mAbs (250 μg/mouse) were injected i.p. on day 3, 6, 8, 10, 13, 16, and 20 (gray and black arrows). Anti-PD-1 mAbs (25 μg/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). Same as b, for mice carrying a Treg-specific deletion of GARP (left) and their littermate controls (right). Results pooled from two independent experiments. **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).
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 alone. Variation in the proposed modes of action of anti-GARP:TGF-β1 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-β1 only blocks TGF-β1 emanating from GARP-expressing cells such as Tregs, whereas anti-TGF-β neutralizes activity of all three TGF-β1, β2, and β3 isoforms, regardless of their cellular source. Our data suggest that blocking only the TGF-β1 produced on Treg surfaces with anti-GARP:TGF-β1 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.
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-β1 activity with anti-GARP:TGF-β1 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.

FcγR-dependent functions of immunostimulatory mAbs affect their anti-tumor activity in different ways. Anti-CTLA-4 and anti-PD-L1 mAbs must bind activating FcγRs to exert potent anti-tumor activity through ADCC or ADPC-mediated depletion of Tregs, or myeloid and tumor cells, respectively. The inability of FcγRs to mediate depletion of Tregs, or myeloid and tumor cells, respectively, because this prevents cross-linking and agonistic activity or depletion of PD-1-expressing anti-tumor CD8+ T cells. Figure 1A shows that mAbs do not require FcγR-dependent functions to exert anti-tumor activity in mice, supporting a mode of action by which they block TGF-β1 activation and downstream signaling without depleting GARP-expressing Tregs. This suggests that in cancer patients, blocking anti-GARP:TGF-β1 mAbs that are unable to bind FcγRs 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-β1 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, GarpYSG/YSG, C57BL/6, Treg-specific, and platelet-specific-Garp KO mice were bred at the SPF animal facility of the KULeuven. Cell type specific-Garp KO and WT littermates were obtained by crossing Lrc32−/−/129 with B6.129 (Cg)-Foxp3tm4(YFP/icre)Ayr/J. 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: ideogram with FOXP3+ and CD3G+ cells and CD3G. Indicated P-values for Pearson correlation were calculated with a two-sided t-test. 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+ 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 curves are plotted as green curves, with the Enrichment Score (ES) corresponding to the maximum value. Top: gene set of the hallmark IFN-γ signature (MsigDB). Middle and bottom panels: gene sets induced by TGF-β1 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-β1 in at least one of the five cell types; bottom panel: genes induced by TGF-β1 in CD4+ T cells; See also Supplementary Data 2). FES (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.

Binding of mAbs to Fc receptors. Recombinant biotinylated extracellular domains of immunoglobulin Fc receptors (FcRs) were purchased from Sino-Biological. Interactions of mAbs with FcγRs were measured using the Biacore T200 (GE Healthcare). Briefer, the biotin CAPture kit was used to immobilize biotinylated FcγRs to the sensor chip. Prior to measurements, one conditioning cycle consisting of three serial regeneration steps (1 min, flow rate 10 µL/min) was performed. In each measurement cycle, Biotin CAPture reagent was applied to the reference and measurement channel (5 min, flow rate 2 µL/min). Biotinylated FcγRs at a concentration of 1 µg/mL were injected (2 min, flow rate 10 µL/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 FcγR1, 12.8–8000 nM for FcγRII/III, and 0.8–8000 nM for FcγRI. Analysis interaction was performed in the single-cycle kinetic mode (2 min at 30 µL/min) followed by 10 min of dissociation. Flow cells were regenerated (2 min, 20 µL/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 × 107 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 × 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 folowed: V = π × D × d/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 IgG1 (clone S9A2, mAb-1A8, or mouse 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
injected in their left flank. Average tumor size per group is calculated for all tumors, including the corresponding mouse was euthanized. The last tumor size measured prior to

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ruption with the Tissue Lyser (Quiagen), total RNA was extracted using fragments were collected and stored at (Thermo

under either standard conditions (95 °C for 3 fi

Master Mix (Eurogentec), 300 nM of each primer, and 100 nM of Takyon probe (Sigma, 5 µg/ml), anti-CD107a coupled to BV421 and the H2-Ld/AH1 tetramer (negative control) for 90 min, washed twice with TBS-T, incubated with secondary antibody (clone MHG-6, in-house), in Dako Antibody Diluent or no primary antibody (T cells).)

Multiplexed immunofluorescence on tumor mouse tissues. Two adjacent 7-µm-thick cryosections freshly cut from frozen tonsil or tumor tissue were used for automated selection of FOXP3 nuclei surrounded by GARP stain, respectively. Adequate selection of FOXP3 by automated counting of FOXP3

Multiplexed immunofluorescence on mammalian cells. Melanoma cell lines LB2259-MEL.A and LB2667-MEL were established from the resected metastasis of two melanoma patients, and grown in DMEM medium (Invitrogen Life Technologies) supplemented with 10% FCS and amino acid mix; 100 U/mL penicillin and 100 µg/mL streptomycin. Primary keratinocytes were derived from the detached epidermis layer of a healthy skin sample and were cultured in selective Ca2+ free 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 + HMGS2 (ThermoFischer Scientific) and Endothelial Cell Growth Medium (Promocell, respectively). The cultured cells were treated with either IFNy (100 U/mL), IL-1β (10 ng/ml), TGF-β (5 ng/ml), TNFa (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 Geneprobe 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 levels for each cell line were then treated or not with TGF-β1 were obtained from the 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-β1 signature in all melanoma cell lines or in the CD4+ T cells). Genes of the experimental signatures are listed in Supplementary Data 2.

Multiplexed immunofluorescence on mammalian cells. Melanoma cell lines LB2259-MEL.A and LB2667-MEL were established from the resected metastasis of two melanoma patients, and grown in DMEM medium (Invitrogen Life Technologies) supplemented with 10% FCS and amino acid mix; 100 U/mL penicillin and 100 µg/mL streptomycin. Primary keratinocytes were derived from the detached epidermis layer of a healthy skin sample and were cultured in selective Ca2+ free 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 expanded in Medium 254 + HMGS2 (ThermoFischer Scientific) and Endothelial Cell Growth Medium (Promocell, respectively). The cultured cells were treated with either IFNy (100 U/mL), IL-1β (10 ng/ml), TGF-β (5 ng/ml), TNFa (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 Geneprobe 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 levels for each cell line were then treated or not with TGF-β1 were obtained from the 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-β1 signature in all melanoma cell lines or in the CD4+ T cells). Genes of the experimental signatures are listed in Supplementary Data 2.
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 transformed volumes. This approach is recommended for repeated measures over time on individual mice. 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. S 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_TNF_ALPHA_SIGNALING.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 GSE154588. 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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Competing interests

G.D.B., L.M., I.V.D.W., B.v.d.W., H.d.H., M.S., and M.G. are full-time employees of argenx and own stock options in argenx. The remaining authors declare no competing interests.

Additional information

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