HUMAN IMMUNOLOGY

Monoclonal antibodies against GARP/TGF-β1 complexes inhibit the immunosuppressive activity of human regulatory T cells in vivo

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Regulatory T cells (T_{regs}) are essential to prevent autoimmunity, but excessive T_{reg} function contributes to cancer progression by inhibiting antitumor immune responses. T_{regs} exert contact-dependent inhibition of immune cells through the production of active transforming growth factor–β1 (TGF-β1). On the T_{reg} cell surface, TGF-β1 is in an inactive form bound to membrane protein GARP and then activated by an unknown mechanism. We demonstrate that GARP is involved in this activation mechanism. Two anti-GARP monoclonal antibodies were generated that block the production of active TGF-β1 by human T_{regs}. These antibodies recognize a conformational epitope that requires amino acids $GARP_{137–139}$ within GARP/TGF-β1 complexes. A variety of antibodies recognizing other GARP epitopes did not block active TGF-β1 production by T_{regs}. In a model of xenogeneic graft-versus-host disease in NSG mice, the blocking antibodies inhibited the immunosuppressive activity of human T_{regs}. These antibodies may serve as therapeutic tools to boost immune responses to infection or cancer via a mechanism of action distinct from that of currently available immunomodulatory antibodies. Used alone or in combination with tumor vaccines or antibodies targeting the CTLA4 or PD1/PD-L1 pathways, blocking anti-GARP antibodies may improve the efficiency of cancer immunotherapy.

INTRODUCTION

Regulatory T cells (T_{regs}) are a subset of $CD4^+$ T cells that inhibit T cell responses. They prevent autoimmunity by suppressing self-reactive T cells (*1*, *2*). Their differentiation and function depend on transcription factor FOXP3, whose stable expression is a hallmark of fully differentiated T_{regs} in both mice and humans. This stable expression depends on an epigenetic modification in the *FOXP3* gene consisting in the demethylation of an intronic region called *FOXP3i1*, *TSDR*, or *CNS2* (*3*–*5*). *FOXP3i1* demethylation can serve to identify T_{regs} and is currently the only specific marker of these cells in humans (*6*–*8*).

Pharmacological targeting of T_{regs} could be beneficial in various pathologies. Strategies to increase their numbers are tested to treat autoimmune diseases or allogeneic graft rejections (*9*). T_{reg} inhibition, on the other hand, may be advantageous in cancer or chronic infections, where immune responses are suppressed by T_{regs}. Depleting human T_{regs} remains a challenge because of the absence of a specific surface marker. Targeting the high-affinity interleukin-2 (IL-2) receptor with anti-CD25 antibodies or toxin-conjugated IL-2 failed to meet expectations, because many non-T_{reg} immune cells also express this receptor (*10*, *11*). The alternative to depletion is inhibition of T_{reg} function. Several mechanisms of immune suppression by T_{regs} have been described in mouse models (*12*). They have been less studied in humans, and which one, if any, could be safely targeted in patients is not known. Using clones of human T_{regs} characterized by a demethylated *FOXP3i1*, we have previously shown that T_{regs}, but not other T lymphocytes, upon T cell receptor stimulation, produce the active form of transforming growth factor–β1 (TGF-β1), a potently immunosuppressive cytokine (*4*, *13*, *14*).

Treatment with anti–TGF-β1 antibodies could decrease T_{reg} function but will inhibit the TGF-β1 produced by other cell types than T_{regs}, bringing forth the risk of severe side effects. Anti–TGF-β1 antibodies could stimulate the growth of preneoplasic lesions, because TGF-β1 exerts a potent cytostatic effect on premalignant cells (*15*). Accordingly, the development of squamous cell carcinomas was observed in a phase 1 trial evaluating an anti–TGF-β antibody in melanoma patients (*16*, *17*).

An interesting alternative would be to inhibit the production of active TGF-β1 by T_{regs} but not by other cell types. This might be possible because the mechanisms of active TGF-β1 production, a tightly regulated multistep process, are cell type–specific (*18*). The homodimeric precursor pro–TGF-β1 is cleaved by furin to produce latent TGF-β1, in which the C-terminal fragment, or mature TGF-β1, remains noncovalently bound to the N-terminal fragment known as LAP (latency associated peptide). This latent complex is inactive because LAP prevents mature TGF-β1 from binding to its receptor. Further processing, referred to as "TGF-β1 activation," is required to release mature TGF-β1 from LAP. Virtually all immune cells secrete soluble latent TGF-β1 (*4*, *19*, *20*), but very few activate the cytokine. Some epithelial and dendritic cells activate TGF-β1 through binding of integrins $\alpha_V\beta_6$ and $\alpha_V\beta_8$, respectively, to an RGD motif in LAP (*18*). To date, how T_{regs} activate TGF-β1 is not known. Active TGF-β1 is not detected in supernatants but exerts its paracrine actions when T_{regs} contact target cells, indicating that activation occurs close to the T_{reg} surface (*4*). T_{regs} carry latent TGF-β1 bound to GARP, a transmembrane protein expressed by T_{regs} but not by other immune cells (*19*, *21*–*25*). Forced expression of GARP in non-T_{reg} cells is sufficient to present latent TGF-β1 on the surface but not to activate it (*19*).

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We hypothesized that GARP, although not sufficient, is required for the activation of TGF-β1 by human T_{regs}, and that this activation could be inhibited by anti-GARP antibodies. We derived 31 monoclonal antibodies (mAbs) that recognize human GARP (hGARP), and showed that two of them blocked TGF-β1 activation by human T_{regs}. The blocking anti-hGARP mAbs inhibited the immunosuppression exerted by human T_{regs} in vitro and in vivo.

RESULTS

Thirty-one new anti-hGARP mAbs

We used two approaches to derive mAbs against hGARP (Fig. 1A). First, lymphocytes from mice immunized with murine cells expressing hGARP were used to generate hybridomas producing mouse anti-human GARP (MHG-1 to MHG-14) mAbs. Second, phage complementary DNA (cDNA) libraries encoding immunoglobulin light and heavy chain variable regions were prepared from lymphocytes of llamas immunized with murine cells expressing *hGARP* and *hTGFB1* or with plasmids encoding these molecules (*26*). *Escherichia coli* were transfected with independent V_H/V_κ or V_H/V_λ libraries to produce phages displaying Fab fragments on their surface. Phage clones were selected by several rounds of binding to immobilized recombinant hGARP/hTGF-β1 complexes, elution, and infection of *E. coli*. Fab-coding regions from selected clones were sequenced to construct full-length mAbs by subcloning into a human immunoglobulin G1 (IgG1) backbone, generating llama-derived anti-human GARP mAbs (LHG-1 to LHG-17). MHG-1 to MHG-14 and LHG-1 to LHG-17 bound to a human T helper (T_H) clone transduced with *hGARP* but not to the untransduced T_H clone, which does not express hGARP (*4*, *19*) (Fig. 1B).

Inhibition of active TGF-β1 production by two anti-hGARP mAbs

The autocrine activity of TGF-β1 produced by stimulated human T_{regs} induces SMAD2 phosphorylation (*4*). We stimulated a T_{reg} clone, that is, a pure population of cells bearing a demethylated *FOXP3i1* allele (*4*), in the presence or absence of anti-hGARP mAbs and assessed SMAD2 phosphorylation by Western blot. Phosphorylated SMAD2 (pSMAD2) was detected in stimulated T_{regs} but not in nonstimulated T_{regs} or in T_{regs} stimulated in the presence of a neutralizing anti–TGF-β antibody (Fig. 2). pSMAD2 was reduced by 80 to 90% in the presence of MHG-8 or LHG-10. The 29 other MHG and LHG mAbs, as well as 4 commercially available anti-hGARP mAbs, did not block active TGF-β1 production by T_{regs} (Fig. 2). Similar results were obtained with another population of human T_{regs}, namely, polyclonal blood $CD4^+CD25^{hi}$-$CD127^{lo}$ cells that were shortly amplified in vitro and contained 55 to 97% cells with a demethylated *FOXP3i1* allele (table S1). MHG-8 and LHG-10, and none of the other anti-GARP mAbs tested, prevented SMAD2 phosphorylation in activated polyclonal T_{regs} (fig. S1). This inhibitory activity of MHG-8 and LHG-10 indicated that GARP is involved in the production of active TGF-β1 by human T_{regs}.

Epitopes recognized by anti-hGARP mAbs

Our results suggested that MHG-8 and LHG-10 recognized GARP epitopes distinct from those bound by the noninhibitory anti-hGARP mAbs. We first examined whether the anti-hGARP antibodies recognized free GARP or GARP/TGF-β1 complexes on the surface of cells (Fig. 3A). We used 293T cells, which contain no GARP and very low

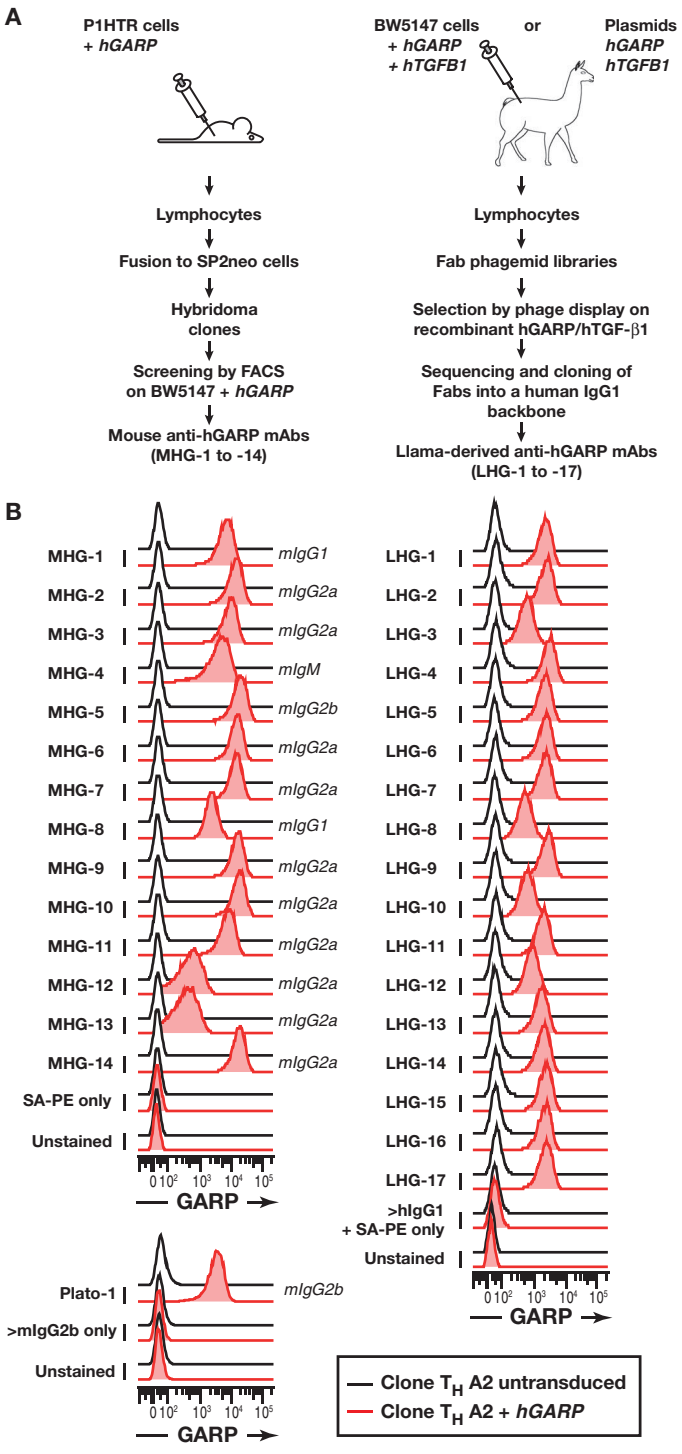

Fig. 1. Generation of 31 anti-GARP mAbs. (**A**) Strategies used to derive anti-hGARP mAbs. (**B**) Flow cytometry analyses of clone T_H A2 (human $CD4^+$ T_H cells that do not express hGARP) or T_H A2 cells transduced with *hGARP*, after labeling with biotinylated MHG-1 to MHG-14 followed by streptavidin coupled to phycoerythrin (SA-PE), or with LHG-1 to LHG-17 followed by biotinylated anti-hIgG1 and SA-PE, or with a commercially available mouse anti-hGARP mAb (clone Plato-1) followed by anti-mIgG2b antibodies coupled to AF647.

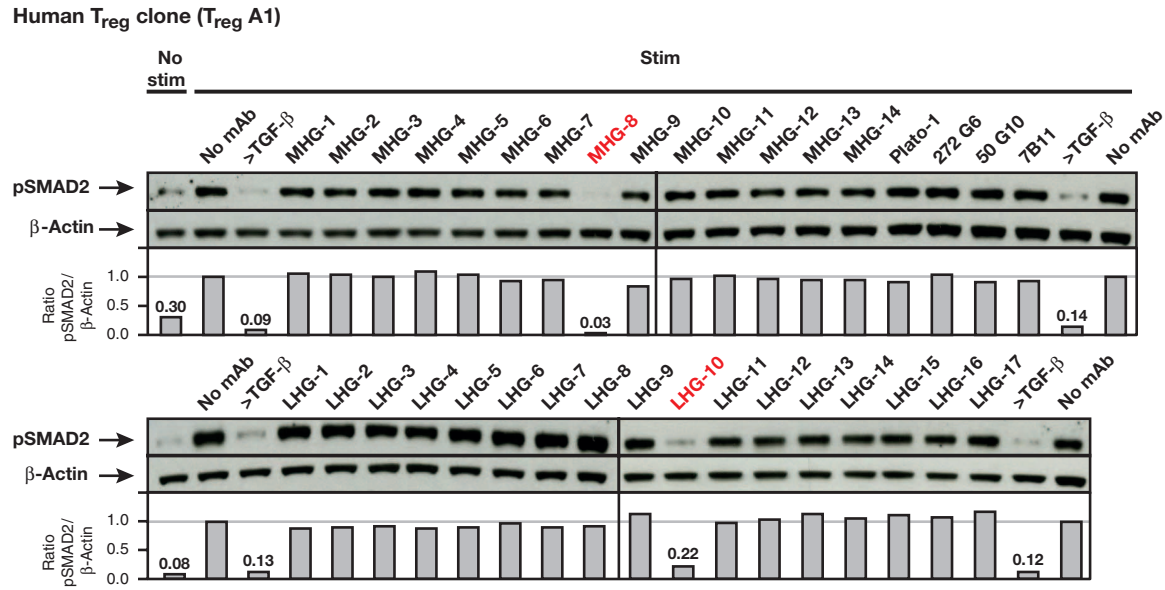

Fig. 2. MHG-8 and LHG-10 inhibit production of active TGF-β1 by a human T_{reg} clone. Clone T_{reg} A1 was stimulated with immobilized anti-CD3 and soluble anti-CD28 in the presence or absence of the indicated mAbs. Plato-1, 272G6, 50G10, and 7B11 are commercially available anti-hGARP mAbs. Cell lysates were analyzed by Western blot with antibodies against pSMAD2 as a readout for active TGF-β1 production or β-actin as loading control. Bar graphs show quantification of enhanced chemiluminescence (ECL) signals represented as ratios of pSMAD2 to β-actin, normalized to the ratio in cells stimulated in the absence of anti-hGARP mAb (No mAb) measured on the same gel.

amounts of TGF-β1. Accordingly, no surface latent TGF-β1 is detected with an anti-LAP antibody (Fig. 3B). Transfection of a hemagglutinin (HA)–tagged *GARP* alone leads to the staining by anti-HA but not by anti-LAP antibodies, indicating the presence of free GARP (Fig. 3B). Transfection of *TGFB1* leads to low surface LAP expression in the absence of GARP. Cotransfection of *GARP* and *TGFB1* leads to higher surface LAP, corresponding to the presentation of GARP/TGF-β1 complexes (Fig. 3B).

A first group (n = 8) of anti-hGARP mAbs bound free GARP only: they recognized cells transfected with *GARP* but not with *GARP* and *TGFB1*. Three such antibodies are shown in Fig. 3B, and all are listed in Table 1. The recognition of free GARP was confirmed by immunoprecipitation: the antibodies immunoprecipitated GARP but did not coimmunoprecipitate pro–TGF-β1 or latent TGF-β1 (fig. S2). A second group (n = 17) bound free GARP and GARP/TGF-β1 complexes: they recognized 293T cells transfected with *GARP* alone or with *TGFB1* (Fig. 3B). They coimmunoprecipitated pro–TGF-β1 and latent TGF-β1 with GARP (fig. S2). A third group (n = 6) bound GARP/TGF-β1 complexes but neither free GARP nor free TGF-β1: they recognized cells transfected with *GARP* and *TGFB1* but not with either *GARP* alone or *TGFB1* alone (Fig. 3B). They coimmunoprecipitated pro–TGF-β1 and latent TGF-β1 with GARP (fig. S2). Both inhibitory antibodies MHG-8 and LHG-10 belonged to this group, together with four other mAbs that were not inhibitory (Table 1).

We next defined the GARP regions that were recognized by our antibodies. Because the latter did not recognize mouse GARP (mGarp), we transfected 293T cells with HA-tagged *mGarp/hGARP* chimeras, with or without *TGFB1* depending on the binding requirements determined above. Labeling with anti-HA antibodies indicated that all chimeras were expressed at similar levels. Labeling with the panel of anti-hGARP antibodies indicated that 5 mAbs required a region comprising amino acids 20 to 101 ($hGARP_{20–101}$), 15 mAbs required $hGARP_{101–141}$, 6 required $hGARP_{141–207}$, 1 required $hGARP_{265–332}$, and 4 required $hGARP_{332–628}$ (Fig. 3C and Table 1). Both inhibitory antibodies MHG-8 and LHG-10 required $hGARP_{101–141}$, a requirement shared with the four other antibodies that only recognized GARP/TGF-β complexes (Table 1).

The sequences of mouse and $hGARP_{101–141}$ differ at 14 positions, comprising three clusters of three contiguous amino acids. We constructed three mutated versions of HA-tagged hGARP, in which three contiguous residues were replaced by those found in mGarp, and transfected them into 293T, with or without *hTGFB1* as above. Binding patterns to mutants indicated that five antibodies, including MHG-8 and LHG-10, required $hGARP_{137–139}$ (Fig. 3D and Table 1). Of these, MHG-8 and LHG-10 were the only antibodies that could not recognize free GARP (Table 1). We concluded that the inhibition of T_{reg} TGF-β1 production by anti-hGARP mAbs MHG-8 and LHG-10 is associated with the recognition of an epitope that requires amino acids 137 to 139 in hGARP complexed to TGF-β1.

Inhibition of human T_{reg} function by MHG-8 and LHG-10 in vitro

We previously showed that the production of active TGF-β1 was involved in the immunosuppressive activity of human T_{regs} toward other $CD4^+$ T cells (*4*). We therefore tested whether MHG-8 and LHG-10 could inhibit human T_{reg} function in in vitro suppression assays. We used clone T_{reg} A1 as a source of T_{regs} and freshly isolated blood $CD4^+CD25^-CD127^{hi}$ cells (T_H cells) as targets for suppression. Cells were stimulated at a 1 T_{reg}/1 T_H ratio in the presence or absence of anti-hGARP mAbs. The T_{reg} clone inhibited the proliferation of T_H cells by 66%. This effect was decreased to nonsignificant levels in the presence of MHG-8 or LHG-10 but not in the presence of the six other anti-hGARP antibodies tested (Fig. 4A). We also measured the suppression by clone T_{reg} A1 on another T_H target, clone T_H A2,

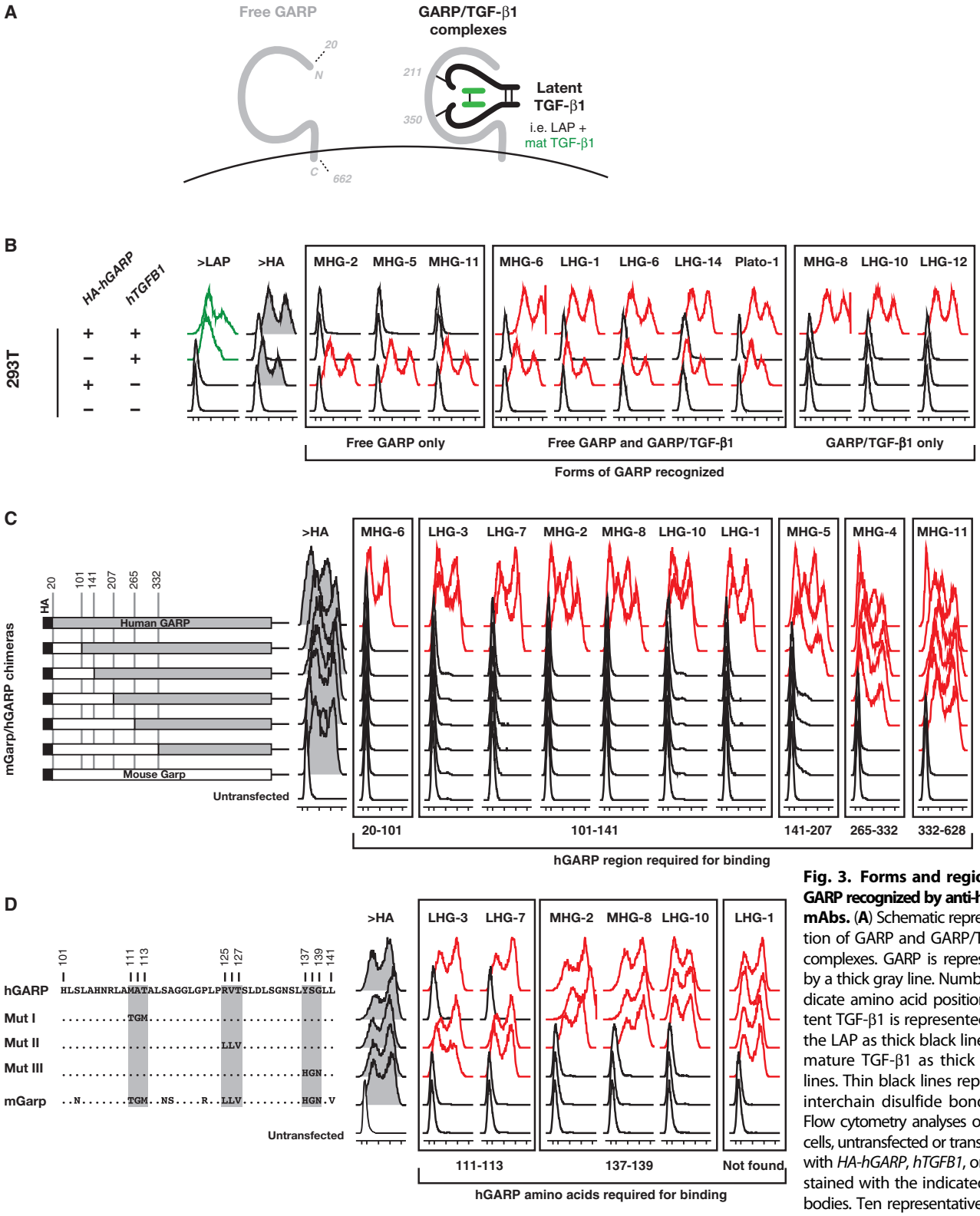

Fig. 3. Forms and regions of GARP recognized by anti-hGARP mAbs. (**A**) Schematic representation of GARP and GARP/TGF-β1 complexes. GARP is represented by a thick gray line. Numbers indicate amino acid positions. Latent TGF-β1 is represented with the LAP as thick black lines and mature TGF-β1 as thick green lines. Thin black lines represent interchain disulfide bonds. (**B**) Flow cytometry analyses of 293T cells, untransfected or transfected with *HA-hGARP*, *hTGFB1*, or both, stained with the indicated antibodies. Ten representative MHG and LHG antibodies are shown, but all were tested in this assay. (**C**) Flow cytometry analyses of 293T cells transfected with plasmids encoding the HA-tagged mGarp/hGARP chimeras shown on the left. *hTGFB1* was cotransfected with *mGarp/hGARP* chimeras for the analyses of mAbs that bind hGARP/hTGF-β1 complexes only (LHG-3, MHG-8, and LHG-10). (**D**) As in (C), except that 293T cells were transfected with plasmids encoding mutated forms of full-length HA-tagged hGARP. In each mutant, three amino acids of hGARP were replaced by the three amino acids found in mGarp, as shown on the left.

Table 1. Classification of anti-hGARP mAbs based on binding requirements.

Form of hGARP recognized			hGARP amino acid required	
Free GARP only	**Free GARP and GARP/TGF-β1**	**GARP/ TGF-β1 only**	**Specific amino acid**	**Region**
	MHG-6			**20–101**
	MHG-7			
	MHG-9			
	LHG-5			
	LHG-11			
	LHG-7	LHG-3	**111–113**	**101–141**
	LHG-9			
			125–127	
MHG-2 MHG-3	LHG-17	**MHG-8 LHG-10**	**137–139**	
	LHG-1	LHG-8	**Others**	
	LHG-2	LHG-12		
	LHG-4	LHG-13		
	LHG-15			
MHG-1	LHG-6			**141–207**
MHG-5	LHG-16			
	MHG-10			
	MHG-14			
				207–265
	(Plato-1)			**264–332**
MHG-11	LHG-14			**332–628**
MHG-12				
MHG-13				

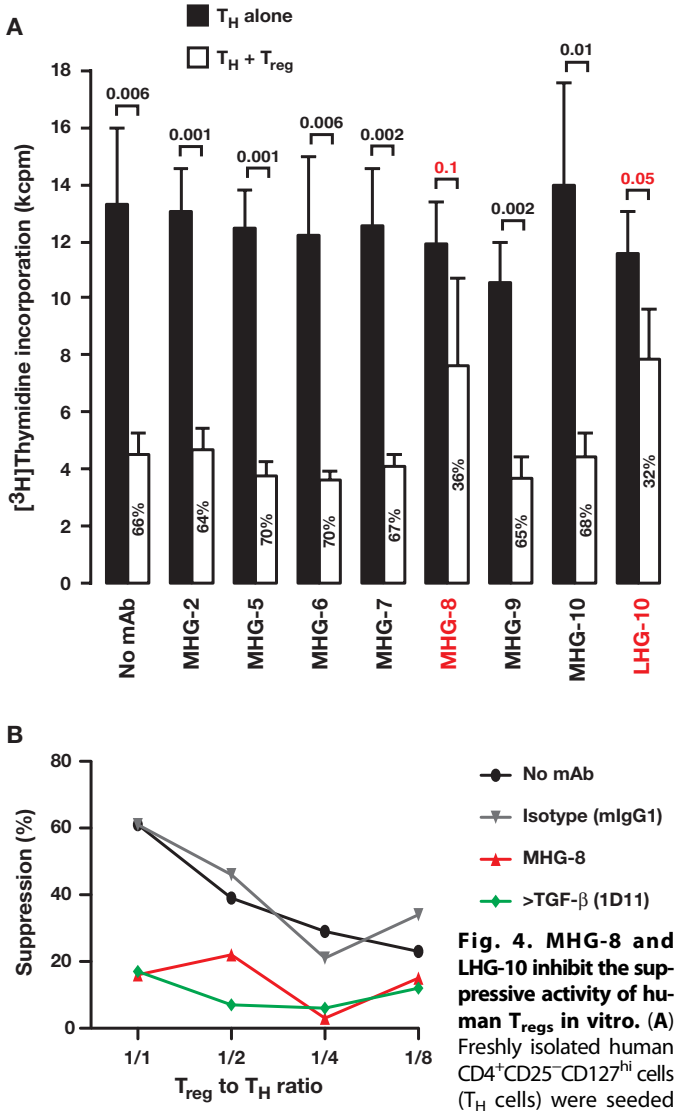

Fig. 4. MHG-8 and LHG-10 inhibit the suppressive activity of human T_{regs} in vitro. (**A**) Freshly isolated human $CD4^+CD25^-CD127^{hi}$ cells (T_H cells) were seeded alone or with clone T_{reg} A1 at a 1:1 T_{reg}/T_H ratio. Cells were stimulated with coated anti-CD3 and soluble anti-CD28 in the presence or absence of the indicated anti-hGARP mAbs. [^{3}H]Thymidine was added during the last 16 hours of the 4-day culture, and incorporation was measured in a scintillation counter as a readout for proliferation. Bar histograms and error bars indicate mean kilocounts per minute (kcpm) and SD, respectively (n = 3 biological replicates). Clone T_{reg} A1 did not proliferate in the absence of T_H cells (T_{reg} alone: 0.5 ± 0.04 kcpm). Suppression of T_H proliferation in the presence of T_{regs} is indicated inside white bars and is calculated as follows: % suppression = 1 − [kcpm (T_H alone)/kcpm (T_H + T_{reg})]. Proliferation in the presence of T_{regs} was compared to proliferation in the absence of T_{regs} with a Student's t test. P values are indicated above brackets. (**B**) Clone T_H A2 cells were seeded with clone T_{reg} A1 at the indicated T_{reg}/T_H ratios in the presence or absence of MHG-8, of an anti–TGF-β mAb, or of an isotype control. Stimulation, measure of proliferation, and calculation of suppression were performed as in (A).

at various T_{reg}/T_H ratios (Fig. 4B). Antibody MHG-8 decreased suppression by T_{reg} A1 at all ratios tested, in a manner similar to that of an anti–TGF-β mAb. These results confirm the activity of TGF-β in the immunosuppression by human T_{regs} and indicate that anti-GARP antibodies can partially block this activity in vitro.

Inhibition of human T_{reg} function by MHG-8 and LHG-10 in vivo

We next evaluated whether an inhibitory anti-hGARP mAb could inhibit T_{reg} function in vivo. Because our antibodies do not bind mGarp, we used a model of xenogeneic graft-versus-host disease (GVHD) induced by the transfer of human peripheral blood mononuclear cells (PBMCs) into immunocompromised *NOD/Scid/Il2rg*$^{-/-}$ (NSG) mice. NSG mice have defective cytokine signaling and lack functional T, B, and natural killer cells, allowing very efficient engraftment of human T cells. Thirty to 40 days after PBMC transfer, the mice develop GVHD because of the activity of human T cells against murine tissues (*27*). In this model, the cotransfer of human T_{regs} attenuates GVHD (*28*).

We transferred human PBMCs into NSG mice with or without autologous T_{regs}, namely, blood $CD4^+CD25^{hi}CD127^{lo}$ cells shortly amplified in vitro as described above (Fig. 5A). One day before the graft and weekly thereafter, mice were injected with antibodies or phosphate-buffered saline (PBS). Objective signs of GVHD were monitored biweekly to establish a disease score. We tested MHG-8 in five independent

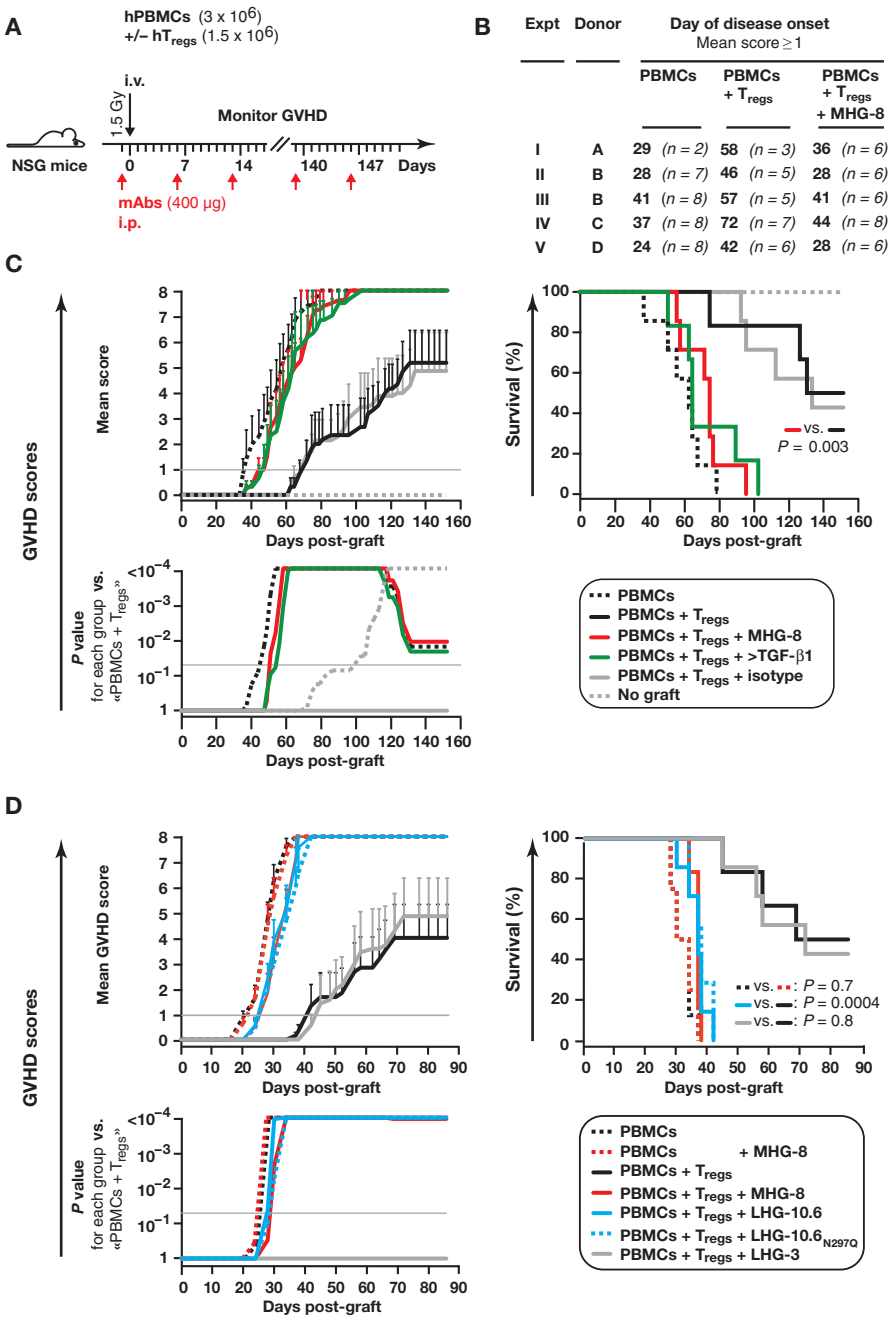

Fig. 5. Anti-hGARP mAbs that block TGF-β production inhibit suppression by human T_{regs} in vivo. (**A**) Experimental setup. On day 0, PBMCs and autologous T_{regs} were injected intravenously in preconditioned NSG mice (1.5 Gy on day −1). The mice received weekly intraperitoneal injections of mAbs or PBS, as indicated. Clinical GVHD was monitored biweekly to establish a score based on weight loss, reduced mobility, anemia or icterus, and hair loss. (**B**) Results of five independent experiments (I to V) performed with cells from four donors (table S1). The day of disease onset is when the mean disease score becomes ≥1. *n*, number of mice per experimental group. (**C** and **D**) Detailed results from experiments IV (C) and V (D) showing progression of disease score and survival. For disease scores, top graphs show means per group + SEM; bottom graphs show *P* values of comparisons between each group and the group receiving PBMCs + T_{regs} calculated using two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test. Statistical significance of differences in survival was calculated using a log-rank (Mantel-Cox) test.

experiments with PBMCs from four donors. A summary of the data is shown in Fig. 5B and detailed results in Fig. 5 (C and D) and fig. S3. In mice that received no antibodies, cotransfer of T_{regs} delayed GVHD onset from 24 to 41 days to 42 to 72 days, confirming the protective activity of T_{regs}. The administration of MHG-8 abrogated this protection: GVHD occurred 28 to 44 days after transfer, as early as in mice receiving PBMCs only. Abrogation of the protective effect of T_{regs} was also observed with a neutralizing anti–TGF-β1 antibody but not with an isotype control (Fig. 5C). As expected, immunohistochemical analyses of lung and liver of mice with a GVHD score of 6 showed massive infiltrations by human T cells, a hallmark of xenogeneic GVHD (fig. S4).

Protection by T_{regs} and its abrogation by MHG-8 were also seen on survival curves (Fig. 5C). Whereas 50% of mice grafted with PBMCs and T_{regs} showed long-term survival (>152 days), all mice either grafted with PBMCs only or grafted with PBMCs and T_{regs} and treated with MHG-8 or anti–TGF-β1 antibodies had died by day 103.

We verified that MHG-8 did not aggravate GVHD in mice grafted with PBMCs alone; thus, its effect depended on the co-injection of T_{regs} (Fig. 5D). We also examined whether abrogation of T_{reg} protection by MHG-8 depended on its ability to block TGF-β production. We thus compared MHG-8 to LHG-10.6, which also blocks TGF-β production by T_{regs}, and to LHG-3, which does not. Antibody LHG-10.6 is a variant of LHG-10 with increased affinity for GARP/TGF-β1 complexes that was selected by phage display from Fabs in which the heavy chain of LHG-10 was combined to the $V_κ$ library. Like MHG-8, LHG-10.6 aggravated GVHD, whereas nonblocking antibody LHG-3 had no effect (Fig. 5D). This suggested that MHG-8 and LHG-10.6 abrogate T_{reg} protection by blocking T_{reg} production of TGF-β1 and not by inducing T_{reg} depletion. To further exclude the latter possibility, we also tested a mutated version of LHG-10.6, named LHG-10.6$_{N297Q}$. The N297Q mutation results in loss of Fc glycosylation, thus loss of Fc receptor and C1q binding, and consequently loss of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) functions (*29*, *30*). LHG-10.6$_{N297Q}$ aggravated GVHD in mice grafted with PBMCs and T_{regs} as potently as LHG-10.6, confirming that anti-GARP antibodies do not act by depleting T_{regs} (Fig. 5D).

We measured human cytokines in the serum of mice 20 days after cell transfer (Fig. 6A). Human IL-2 and interferon-γ (IFNγ) were detected at high levels in mice grafted with PBMCs only, indicating a strong xenogeneic activation of human T cells. They were significantly reduced by the cotransfer

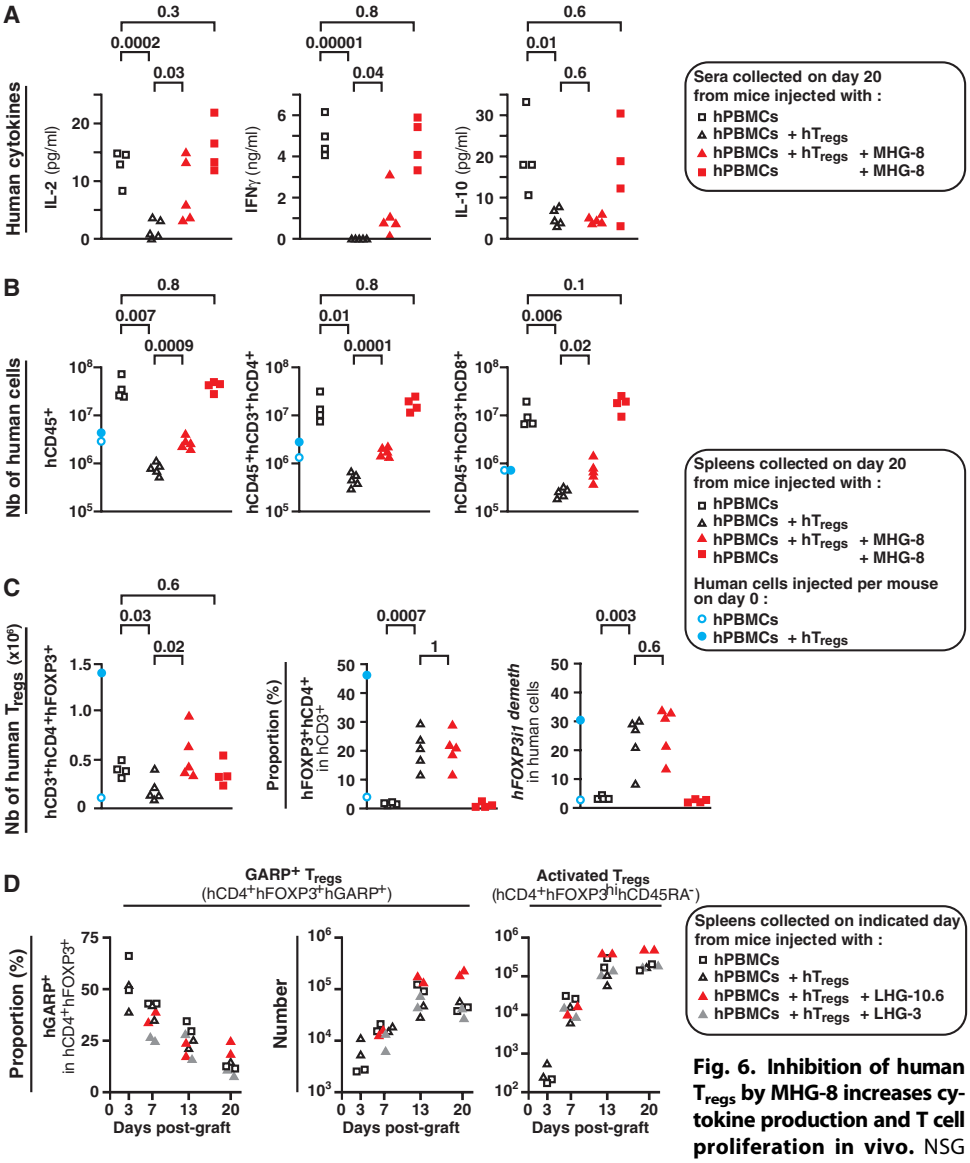

Fig. 6. Inhibition of human T_{regs} by MHG-8 increases cytokine production and T cell proliferation in vivo. NSG mice injected as in Fig. 5A were sacrificed 20 days after cell transfer. (**A**) Serum levels of human cytokines were measured in a multiplex bead assay. (**B** to **D**) Numbers and proportions of the indicated human cells in the spleens were evaluated by flow cytometry. Squares and triangles represent individual mice ($n = 4$ to 5 mice per group). Circles represent numbers or proportions of the corresponding human cells injected per mouse on day 0. Statistical significance between groups was determined with a Student's *t* test. *P* values are indicated above brackets. Data from one experiment representative of two.

of T_{regs}, confirming suppressive activity. MHG-8 decreased the suppression by T_{regs} but had no effect in mice transferred with PBMCs alone. Finally, we observed that IL-10 levels were not increased but instead reduced in the presence of T_{regs}, suggesting that T_{regs} do not suppress through production of IL-10 in this model (Fig. 6A). Noteworthy, the effects of MHG-8 on suppression of cytokine production were measured at an early time point before disease onset to preclude confounding effects from severe inflammation that develops in some groups at later time points. This probably explains why the effects of MHG-8 are less pronounced on cytokine production than on disease progression.

In spleens collected 20 days after transfer, human hematopoietic cells ($hCD45^+$) comprised mostly T lymphocytes ($CD4^+$ and $CD8^+$), which had considerably proliferated in mice grafted with PBMCs alone. This proliferation was inhibited by the cotransfer of T_{regs}, an effect that was decreased by MHG-8 (Fig. 6B). Notably, we observed that numbers and proportions of T_{regs} ($hCD3^+hCD4^+hFOXP3^+$ cells or cells with a demethylated *FOXP3i1* allele) were not reduced in mice treated with MHG-8. On the contrary, T_{reg} numbers were significantly increased in mice transferred with T_{regs} and treated with MHG-8 as compared to untreated mice (Fig. 6C). This suggests that the MHG-8–mediated blockade of autocrine TGF-β1 activity favors T_{reg} proliferation while concomitantly inhibiting T_{reg} function. It also supports our hypothesis that inhibitory anti-GARP mAbs do not act by depleting T_{regs}.

However, it could still be that inhibitory mAbs deplete a minor subpopulation of T_{regs} without affecting total T_{reg} numbers. For example, this could occur if only a small proportion of T_{regs} expressed GARP as a result of activation in this model. In vitro, GARP was shown to be expressed only on activated T_{regs} (*19*, *23*). First, we thus measured the proportions and numbers of $GARP^+$ T_{regs} at several time points after the transfer of human PBMCs ± T_{regs} in NSG mice (Fig. 6D and fig. S5). Three days after transfer, about 50% of $CD4^+FOXP3^+$ cells expressed GARP, indicating that $GARP^+$ cells do not represent a minor subpopulation of T_{regs}. Proportions of $GARP^+$ cells decreased progressively to 10 to 20% of $CD4^+FOXP3^+$ by day 20, and no difference was observed between untreated mice and mice treated with the anti-GARP mAb LHG-10.6, which is inhibitory, or LHG-3, which is not. The numbers of $GARP^+$ T_{regs} were not reduced in mice treated with either anti-GARP mAb by comparison to untreated mice. They were even increased at day 20 in mice treated with LHG-10.6 compared to all other conditions. Second, we used another identification procedure for activated human T_{regs}, as proposed by Miyara *et al.* (*31*), who defined these cells as a subset of $FOXP3^+$ T_{reg} cells characterized by high FOXP3 levels and no CD45RA expression ($CD4^+FOXP3^{hi}$ $CD45RA^-$ cells). A subpopulation of human $FOXP3^{hi}$ cells appeared in mice 7 days after the transfer of human PBMCs ± T_{regs}. $FOXP3^{hi}$ cells expressed the highest levels of GARP and most were $CD45RA^-$ (fig. S5). Therefore, in vivo, GARP expression was maximal in the activated human $CD4^+FOXP3^{hi}CD45RA^-$ T_{regs}. The numbers of $CD4^+FOXP3^{hi}$ $CD45RA^-$ cells were not decreased in mice treated with anti-GARP

mAbs by comparison to untreated mice (Fig. 6D), confirming that inhibitory anti-GARP mAbs did not act in this model by depleting GARP-expressing cells. Together, these results indicate that the inhibitory anti-GARP mAbs are capable of inhibiting the immunosuppressive activity of human T_{regs} in vivo without inducing T_{reg} depletion.

DISCUSSION

GARP is expressed on stimulated T_{regs} but not on other types of T lymphocytes. It has been shown to play a role in TGF-β1 processing by T_{regs} by forming disulfide bonds with pro–TGF-β1, favoring its cleavage into latent TGF-β1 and inducing the secretion and surface presentation of GARP/latent TGF-β1 complexes (*19*, *23*, *32*, *33*). Several results suggested that GARP mediated the activation of latent TGF-β1 by T_{regs} (*4*, *32*, *34*), but direct evidence was lacking. Mouse *Garp*$^{-/-}$ T_{regs}, in contrast to wild-type T_{regs}, could not induce the differentiation of cocultured naïve CD4$^+$ T lymphocytes into T_H17 or iT_{regs}, two processes known to depend upon active TGF-β1 (*34*). In transfected 293T cells, $\alpha_V\beta_6$ and $\alpha_V\beta_8$ integrins were shown to activate latent TGF-β1 bound to GARP (*32*). On the other hand, lentiviral-mediated expression of GARP was not sufficient to induce the production of active TGF-β1 by human T_H cells (*4*). Our results with anti-hGARP mAbs are the first demonstrating that GARP is required for the production of active TGF-β1 by stimulated human T_{regs}. Note that at least one other T_{reg}-specific molecule must cooperate with GARP to activate TGF-β1, because GARP alone is not sufficient. Integrin $\alpha_V\beta_8$ appears to be the GARP partner for T_{reg} TGF-β activation in mouse T_{regs} (*35*). If this is true in human T_{regs} also, blocking anti-hGARP mAbs could function by disrupting the interaction between GARP/latent TGF-β1 complexes and integrins.

GARP is present on other cells than T_{regs}, for example, megakaryocytes, platelets, and some endothelial or fibroblast cell lines (*36*–*39*), but does not appear to activate latent TGF-β1 in these cells (fig. S6). Therefore, in contrast to anti–TGF-β antibodies, anti-hGARP antibodies like MHG-8 and LHG-10 might be T_{reg}-specific TGF-β inhibitors. This should greatly reduce the adverse events resulting from the inhibition of tumor suppression by TGF-β1.

The role of active TGF-β1 production in T_{reg} suppression is still controversial. In murine models, *Tgfb1*$^{-/-}$ or *Garp*$^{-/-}$ T_{regs} show no defect in their suppressive activity in vitro (*34*, *40*). But in vivo, in a model of colitis induced by the transfer of naïve T cells, the cotransfer of *Tgfb1*-deficient T_{regs} failed to inhibit disease development (*41*). Moreover, naïve T cells that resist TGF-β1 signaling because of the expression of a dominant-negative TGF-βRII or to a T cell–specific deletion of TGF-βRII cannot be suppressed by wild-type T_{regs} (*42*–*44*). Together, these results support a role for TGF-β1 in suppression by murine T_{regs} in vivo. It must be noted that in contrast to results mentioned above (*41*), others reported that *Tgfb1*-deficient T_{regs} do inhibit colitis induced by the transfer of wild-type naïve T cells (*42*). In this latter study, however, suppression by *Tgfb1*-deficient T_{regs} was not as efficient as that by wild-type T_{regs} and was abrogated by anti–TGF-β1 antibodies. We suggest that *Tgfb1*-deficient T_{regs} inhibited autoimmune colitis by activating latent TGF-β1 produced by a non-T_{reg} cell type. In support of this hypothesis, exogenously added latent TGF-β1 was shown to bind GARP present on human T_{regs} treated with *siTGFB1* (*23*). Mice with a targeted deletion of *Garp* in T cells do not show severe spontaneous autoimmunity (*34*). This indicates that Garp is not required for the development and homeostasis of the murine immune system, but it does not exclude that GARP is important for T_{reg} function in specific pathologies in mice or humans. Little is known regarding the T_{reg} suppressive mechanisms in humans. We show here that the in vivo administration of anti-hGARP mAbs blocking TGF-β1 production by human T_{regs} abrogates their ability to suppress xenogeneic GVHD. We also provide evidence that their mode of action is based on the inhibition of T_{reg} function but not on T_{reg} depletion. Accordingly, we observed an increase instead of a reduction in T_{reg} numbers upon treatment with MHG-8. Increased T_{reg} numbers may result from the blockade of autocrine TGF-β1 cytostatic activity in T_{regs}. In line with this, increased T_{reg} proliferation or numbers were observed in mice with a T cell–specific deletion of the *TGFB1*, *TGFBRI*, or *TGFBRII* gene (*41*, *44*, *45*).

Although we provide strong evidence that the production of active TGF-β1 by human T_{regs} can be inhibited with anti-GARP mAbs in a model of xenogeneic GVHD, an important limitation to our study is that T_{regs} exert a desirable immune-suppressive activity in the GVHD model, and that reduction of T_{reg} function by anti-GARP mAbs aggravates the experimental disease. Because our anti-GARP mAbs do not cross-react on mGarp and because we lack inhibitory anti-mGarp mAbs, we have not been able to test whether such mAbs could inhibit T_{reg} function in murine models of cancer or chronic infection, where T_{regs} exert adverse immune suppression.

Inhibition of T_{reg} function with anti-GARP mAbs could prove beneficial in human diseases such as cancer or chronic infections, for which immune-stimulatory therapeutic approaches are being pursued. In cancer, striking results were recently obtained with immunostimulatory antibodies that modulate endogenous mechanisms of T cell regulation (*46*). Clinical efficacy was demonstrated with mAbs against CTLA4, PD1, or PD-L1. The activity of anti-CTLA4 mAbs in mice appears to depend in part on the ADCC-mediated depletion of tumor-infiltrating T_{regs} (*47*, *48*). The clinical efficacy of anti-CTLA4 and anti-PD1 is associated with severe autoimmune side effects resulting from the broad T cell stimulation that they induce (*49*–*51*). To date, no therapeutic strategy was shown to specifically reduce T_{reg} function. Anti-GARP antibodies, alone or combined with antigenic stimulation, may be a less toxic alternative or a complement to the existing immunostimulatory antibodies to fight cancer and infectious diseases.

MATERIALS AND METHODS

Study design

Our study was designed to determine whether TGF-β1 production by human T_{regs} can be inhibited in vitro and in vivo with anti-GARP mAbs. We derived 31 new anti-GARP mAbs and identified 2 that inhibit TGF-β1 production in vitro. To examine their inhibitory activity in vivo, we used a model of xenogeneic GVHD induced by the transfer of human PBMCs in NSG mice. GVHD can be partially prevented in this model by cotransfer of autologous T_{regs}, providing a means to test the ability of anti-GARP mAbs to inhibit human T_{reg} function. Sample sizes of in vivo experiments were defined on the basis of the number of available human autologous T_{regs}. T_{regs} are rare in human blood, no specific surface marker is available that allows their isolation to very high purity, and therefore the in vitro amplification that is required to obtain sufficient T_{reg} numbers must be as short as possible.

This considerably limits the number of T_{regs} available for experiments. Mice were assigned randomly to experimental groups. Studies were not blinded. Numbers of replicates are indicated on the figures or in the corresponding legends for all experiments.

Mice

Mice (DBA/2, Balb/c, and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/*SzJ* or NSG from Jackson Laboratory) were bred at the animal facility of the Université catholique de Louvain, Belgium. Handling of mice and experimental procedures were conducted in accordance with national and institutional guidelines for animal care.

Cells and transfections

P1.HTR cells, a highly transfectable variant of the P815 mastocytoma derived from DBA/2 mice (*52*), were electroporated with a plasmid encoding the full-length hGARP and selected in puromycin (1.6 μg/ml) under limiting dilution conditions. Two clones expressing high surface hGARP (P1.HTR + *hGARP*) were isolated and used to immunize H-2^{d} mice. A stable clone of murine BW5147.C2 cells expressing high levels of hGARP (BW5147 + *hGARP*) was derived as described (*33*). This clone was electroporated with a plasmid encoding full-length human TGF-β1 and selected in neomycin (3 mg/ml) under limiting dilution conditions. A subclone expressing high levels of surface hGARP/hTGF-β1 complexes (BW5147 + *hGARP* + *hTGFB1*) was isolated and used to immunize llamas.

Human T_{reg} and T_H clones were derived and cultured as previously described (*4*). Total human PBMCs were purified from the blood of hemochromatosis donors by centrifugation on a Lymphoprep gradient. Human polyclonal T_{regs} were obtained by sorting $CD4^+CD25^+CD127^{lo}$ cells by fluorescence-activated cell sorting (FACS) from total PBMCs, followed by in vitro stimulation with anti-CD3/CD28–coated beads in the presence of IL-2 during 12 to 13 days, as described (*33*). 293T cells were transiently transfected with hGARP- and hTGF-β1–encoding plasmids using the TransIT-LT1 transfection reagent (Mirus Bio).

Generation of MHG mAbs

DBA/2 or Balb/c mice were immunized with live P1.HTR + *hGARP* cells, following a previously described injection scheme (*53*). Lymphocytes from mice with high titers of anti-hGARP antibodies, as determined by FACS, were fused to SP2/neo cells in the presence of polyethylene glycol. Hybridomas were selected in HAT medium and cloned under limiting dilution conditions. Supernatants of hybridoma clones were screened by FACS for the presence of antibodies binding to BW5147 + *hGARP* cells. Fourteen positive clones were selected, further subcloned to ensure clonality, and amplified for large-scale production and purification of 14 new anti-hGARP mAbs (MHG-1 to MHG-14).

Generation of LHG mAbs

Immunizations of llamas, harvesting of peripheral blood lymphocytes (PBLs), RNA preparation, and amplification of antibody fragments were performed as described (*26*). Briefly, four llamas were injected six times at weekly intervals with 1×10^7 BW5147 + *hGARP* + *hTGFB1* cells and Freund's incomplete adjuvant in two regions of the neck muscles located a few centimeters apart. Another four llamas were injected four times biweekly with a mix of plasmids containing *hGARP* cDNA and *hTGFB1* cDNA, respectively. Blood samples (10 ml) were collected to monitor IgG1 responses against hGARP/TGF-β1 complexes by enzyme-linked immunosorbent assay (ELISA) using immobilized recombinant GARP/TGF-β1 complexes [produced in human embryonic kidney (HEK) 293 cells cotransfected with *hTGFB1* and *hGARP* truncated from the transmembrane-coding region] for capture, followed by a mouse anti-llama IgG1 antibody [clone 27E10 (*54*)] and a horseradish peroxidase (HRP)–conjugated donkey anti-mouse antibody (Jackson) for detection. Three to 4 days after the last immunization, 400 ml of blood was collected from responding llamas, PBLs were isolated on a Ficoll-Paque gradient, and total RNA was extracted as described (*55*). On average, 450 μg of RNA was obtained and used for random cDNA synthesis followed by polymerase chain reaction (PCR) amplification of the immunoglobulin heavy and light chain variable regions (V_H, V_λ, and Vκ). Two independent phagemid libraries, coding for V_H/V_λ and V_H/V_κ Fabs, respectively, were constructed as previously described (*26*) to obtain a diversity of 1×10^8 to 7×10^8 Fabs in each library. Phages expressing Fabs were produced and selected according to standard protocols. Briefly, two to three rounds of phage selections were performed by binding on immobilized recombinant GARP/TGF-β1, washing, and elution with trypsin. In some instances, counter selections with soluble hGARP ($hGARP_{1–628}$ fused to a TEV-3× Strep-tag produced in 293E cells) and soluble latent TGF-β1 were used to enrich for Fabs binding hGARP/TGF-β1 complexes only. Individual colonies were isolated and periplasmic fractions containing soluble Fabs were produced by isopropyl1-β-D-thiogalactoside induction. Fabs in periplasmic fractions were then screened by ELISA for binding to immobilized hGARP/TGF-β1. V_H and V_L regions of Fab clones binding to hGARP/TGF-β1 complexes were sequenced. Fab clones were divided into 17 families on the basis of similarities in the sequences coding for the V_H CDR3 region. V_H and V_L sequences from one representative clone of each family were subcloned in a full human IgG1 backbone, and the resulting plasmids were transfected into HEK-293E cells to produce and purify 17 new anti-hGARP mAbs (LHG-1 to LHG-17).

Analysis of *FOXP3i1* methylation

Proportions of cells with a demethylated *FOXP3i1* in human PBMCs, in human polyclonal T_{reg} populations, or in splenocytes from NSG mice grafted with human cells were measured by methyl-specific quantitative PCR as described (*7*) using the following primers (sense, antisense, and TaqMan probe, 5′-3′ with underlined nucleotides corresponding to LNA-modified bases): total *FOXP3i1* alleles: AAACCTACTACAAAACAAAACAAC/GGAGGAAGAGAAGAGGGTA/CCTATAAAATAAAATATCTACCCTC; demethylated *FOXP3i1* alleles: TCTACCCTCTTCTCTTCCTCCA/GATTTTTTTGTTATTGATGTTATGGT/AAACCCAACACATCCAACCA.

Flow cytometry

Intact or permeabilized cells were labeled according to standard protocols using combinations of the following primary and/or secondary reagents as indicated in the figures. Primary antibodies: biotinylated MHG-1 to MHG-14; LHG-1 to LHG-17; anti-hGARP clone Plato-1 (Enzo Life Sciences); anti–hCD45-PerCP (peridinin chlorophyll protein), anti–hCD3-FITC (fluorescein isothiocyanate) or anti–hCD3-APC (allophycocyanin), anti–hCD4-FITC or anti–hCD4-APC, and anti–hCD45RA-PE-Cy7 (BioLegend); anti–hCD8α-APC-H7, anti–CD25-PE-Cy7, and anti–hCD127-PE (BD Biosciences); anti–hFOXP3-PE or anti–hFOXP3-APC (eBioscience); anti–hLAP-APC (R&D Systems); and anti-HA (Eurogentec). Secondary antibodies or reagents: anti–hIgG1-biotine (Jackson ImmunoResearch); anti–mIgG1-AF647,

anti–mIgG2b-AF647, and LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies); and SA-PE (BD Biosciences). Labeled cells were analyzed on an LSRFortessa cytometer or sorted on a FACSAria III (both from BD Biosciences), and results were computed with the FlowJo software (Tree Star). Gating strategies, isotype controls, and representative stains are shown for all flow experiments in fig. S7.

Assay to measure active TGF-β1 production by human T_{reg} cells

A human T_{reg} clone (1×10^6 cells/ml) was stimulated in serum-free medium with coated anti-CD3 (Orthoclone OKT3; Janssen-Cilag, 1 μg/ml) and soluble anti-CD28 (BD Biosciences; 1 μg/ml) in the presence or absence of an anti-hGARP mAb (10 μg/ml) (clones tested: MHG-1 to MHG-14; LHG-1 to LHG-17; Plato-1 from Enzo Life Sciences; 272G6 and 50G10 from Synaptic Systems; and 7B11 from BioLegend) or of an anti–TGF-β antibody (clone 1D11, R&D Systems). Cells were lysed after 24 hours and submitted to SDS–polyacrylamide gel electrophoresis under reducing conditions. Gels were blotted on nitrocellulose membranes with the iBlot system (Life Technologies). After blocking, membranes were incubated with primary antibodies directed against pSMAD2 (Cell Signaling Technology) or β-actin (Sigma), then with secondary HRP-coupled antibodies, and revealed with an ECL substrate (Thermo Fisher Scientific). The presence of pSMAD2 indicates production of active TGF-β1 by the stimulated T_{reg} clone. ECL signals were quantified by measuring the density of the 55-kD pSMAD2 and 40-kD β-actin bands on autoradiographs using the ImageJ software. Source data for Western blot analyses are shown in fig. S8.

In vitro suppression assays

T_H cells (2×10^4) were seeded alone or with the indicated numbers of T_{regs} and stimulated with coated anti-CD3 (Orthoclone OKT3, Janssen-Cilag, 1 μg/ml) and soluble anti-CD28 (BD Biosciences, 1 μg/ml) in the presence or absence of an anti-hGARP mAb (MHG or LHG), an anti–TGF-β antibody (clone 1D11, R&D Systems), or an isotype control (mIgG1 clone 11711, R&D Systems) (10 μg/ml). [Methyl-^{3}H]thymidine (0.5 mCi per well) was added during the last 16 hours of the 4-day culture.

Xenogeneic GVHD in NSG mice

NSG mice were irradiated (1.5 Gy) 1 day before tail vein injections of human PBMCs (3×10^6 per mouse) alone or mixed with autologous polyclonal T_{regs} (1.5×10^6 per mouse). One day before graft and weekly thereafter, mice received intraperitoneal injections of PBS or 400 μg of MHG-8 (mIgG1), an anti–TGF-β1 antibody [mIgG1 clone 13A1/A26 (*56*)], or an isotype control [mIgG1 anti-TNP clone B8401H5.M (*57*)]. Mice were monitored biweekly for the development of GVHD. A global disease score was established by adding up scores attributed in the presence of the following symptoms: weight loss (1 if ≥10%; 2 if ≥20%); anemia or icterus (1 if white or yellow ears; 2 if white or yellow ears and tail); humped posture (1); reduced activity (1 if limited activity; 2 if no activity); and hair loss (1). Mice were euthanized when reaching a global score ≥6. Death corresponds to a maximum score of 8.

Cytokine concentrations in sera

Concentrations of human IL-2, IL-10, and IFNγ in mouse serum were determined using a Bio-Plex Pro Human Cytokine 17-plex Assay according to the manufacturer's recommendations (Bio-Rad Laboratories). Limits of detection in this assay were as follows: 0.12 pg/ml for IL-2, 1.56 pg/ml for IFNγ, and 2.48 pg/ml for IL-10.

Histology and immunohistochemistry

Lungs and liver from euthanized mice were collected, fixed in 10% formalin, and routinely processed for paraffin embedding. Four-micrometer sections were stained with hematoxylin and eosin for histologic examination. Additional sections were deparaffinized in xylene and rehydrated through a graded ethanol series, and antigens were retrieved with citrate buffer combined with heating in a pressure cooker for CD8 and combined with heating in a microwave for CD4. Slides were then blocked with a peroxidase blocking solution (Dako) for 10 min and incubated with the primary antibody anti-CD4 (Abcam) or anti-CD8 (Abcam) for 60 min and detected with the Envision kit (Dako) for 30 min. Colorimetric detection was completed with diaminobenzidine (Dako) for 5 min. Slides were then counterstained with hematoxylin. Xenogeneic GVHD was assessed in lung and liver sections by two pathologists.

Statistical analyses

Statistical analyses were performed with Prism 6.0 (GraphPad Software Inc.) or Excel 2011 (Microsoft). Statistical significance of differences observed in data presented in Figs. 4 and 6 was determined with a two-sided Student's *t* test. Curves of disease score in Fig. 5 were compared using two-way ANOVA followed by a Bonferroni post hoc test. Survival curves in Fig. 5 were generated using the Kaplan-Meier method and compared using a log-rank test (Mantel-Cox). All *n* (numbers of biological replicates) and *P* values are indicated on the figures or in the corresponding legends (α level for all statistical analyses: 0.05).

SUPPLEMENTARY MATERIALS

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Fig. S1. MHG-8 and LHG-10 inhibit production of active TGF-β1 by human polyclonal T_{regs}.
Fig. S2. Some anti-hGARP mAbs bind free GARP only.
Fig. S3. Detailed results of experiments I, II, and III summarized in Fig. 5B.
Fig. S4. Analyses of tissues from NSG mice grafted with human PBMCs.
Fig. S5. FACS analysis of splenocytes collected from NSG mice at various time points after transfer of human PBMCs ± T_{regs}.
Fig. S6. Human fibroblasts express GARP but do not produce active TGF-β1.
Fig. S7. Gating strategies, isotype controls, and representative stains of all flow experiments.
Fig. S8. Source data for figures containing Western blot analyses.
Table S1. Purity of polyclonal human T_{regs}.

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Editor's Summary

Immunotherapy according to GARP

Regulatory T cells (T_{regs}) play a critical role in preventing autoimmunity but can be co-opted by cancer cells to block immune surveillance of tumors. Cuende *et al.* report that a membrane protein, GARP, which binds transforming growth factor –β1 (TGF-β1) on the cell surface of T_{regs}, is involved in T_{reg}-mediated inhibition of immune responses. What's more, the authors develop anti-GARP monoclonal antibodies that block TGF- β1 production by T_{regs} and inhibit the activity of these cells in a xenogeneic mouse model of graft-versus-host disease. Thus, blocking GARP, either alone or in combination with other checkpoint inhibitors, could add to our arsenal for cancer immunotherapy.

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