

# Monoclonal antibodies against GARP/TGF- $\beta$ 1 complexes inhibit the immunosuppressive activity of human regulatory T cells in vivo

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Regulatory T cells ( $T_{\text{regs}}$ ) are essential to prevent autoimmunity, but excessive  $T_{\text{reg}}$  function contributes to cancer progression by inhibiting antitumor immune responses.  $T_{\text{regs}}$  exert contact-dependent inhibition of immune cells through the production of active transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). On the  $T_{\text{reg}}$  cell surface, TGF- $\beta$ 1 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- $\beta$ 1 by human  $T_{\text{regs}}$ . These antibodies recognize a conformational epitope that requires amino acids GARP<sub>137–139</sub> within GARP/TGF- $\beta$ 1 complexes. A variety of antibodies recognizing other GARP epitopes did not block active TGF- $\beta$ 1 production by  $T_{\text{regs}}$ . In a model of xenogeneic graft-versus-host disease in NSG mice, the blocking antibodies inhibited the immunosuppressive activity of human  $T_{\text{regs}}$ . 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 ( $T_{\text{regs}}$ ) are a subset of CD4<sup>+</sup> 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  $T_{\text{regs}}$  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  $T_{\text{regs}}$  and is currently the only specific marker of these cells in humans (6–8).

Pharmacological targeting of  $T_{\text{regs}}$  could be beneficial in various pathologies. Strategies to increase their numbers are tested to treat autoimmune diseases or allogeneic graft rejections (9).  $T_{\text{reg}}$  inhibition, on the other hand, may be advantageous in cancer or chronic infections, where immune responses are suppressed by  $T_{\text{regs}}$ . Depleting human  $T_{\text{regs}}$  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- $T_{\text{reg}}$  immune cells also express this receptor (10, 11). The alternative to depletion is inhibition of  $T_{\text{reg}}$  function. Several mechanisms of immune suppression by  $T_{\text{regs}}$  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  $T_{\text{regs}}$  characterized by a demethylated FOXP3i1, we have pre-

viously shown that  $T_{\text{regs}}$ , but not other T lymphocytes, upon T cell receptor stimulation, produce the active form of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), a potentially immunosuppressive cytokine (4, 13, 14).

Treatment with anti-TGF- $\beta$ 1 antibodies could decrease  $T_{\text{reg}}$  function but will inhibit the TGF- $\beta$ 1 produced by other cell types than  $T_{\text{regs}}$ , bringing forth the risk of severe side effects. Anti-TGF- $\beta$ 1 antibodies could stimulate the growth of preneoplastic lesions, because TGF- $\beta$ 1 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- $\beta$  antibody in melanoma patients (16, 17).

An interesting alternative would be to inhibit the production of active TGF- $\beta$ 1 by  $T_{\text{regs}}$  but not by other cell types. This might be possible because the mechanisms of active TGF- $\beta$ 1 production, a tightly regulated multistep process, are cell type-specific (18). The homodimeric precursor pro-TGF- $\beta$ 1 is cleaved by furin to produce latent TGF- $\beta$ 1, in which the C-terminal fragment, or mature TGF- $\beta$ 1, remains non-covalently bound to the N-terminal fragment known as LAP (latency associated peptide). This latent complex is inactive because LAP prevents mature TGF- $\beta$ 1 from binding to its receptor. Further processing, referred to as “TGF- $\beta$ 1 activation,” is required to release mature TGF- $\beta$ 1 from LAP. Virtually all immune cells secrete soluble latent TGF- $\beta$ 1 (4, 19, 20), but very few activate the cytokine. Some epithelial and dendritic cells activate TGF- $\beta$ 1 through binding of integrins  $\alpha_V\beta_6$  and  $\alpha_V\beta_8$ , respectively, to an RGD motif in LAP (18). To date, how  $T_{\text{regs}}$  activate TGF- $\beta$ 1 is not known. Active TGF- $\beta$ 1 is not detected in supernatants but exerts its paracrine actions when  $T_{\text{regs}}$  contact target cells, indicating that activation occurs close to the  $T_{\text{reg}}$  surface (4).  $T_{\text{regs}}$  carry latent TGF- $\beta$ 1 bound to GARP, a transmembrane protein expressed by  $T_{\text{regs}}$  but not by other immune cells (19, 21–25). Forced expression of GARP in non- $T_{\text{reg}}$  cells is sufficient to present latent TGF- $\beta$ 1 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- $\beta$ 1 by human T<sub>regs</sub>, 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- $\beta$ 1 activation by human T<sub>regs</sub>. The blocking anti-hGARP mAbs inhibited the immunosuppression exerted by human T<sub>regs</sub> in vitro and in vivo.

## RESULTS

### Thirty-one new anti-hGARP mAbs

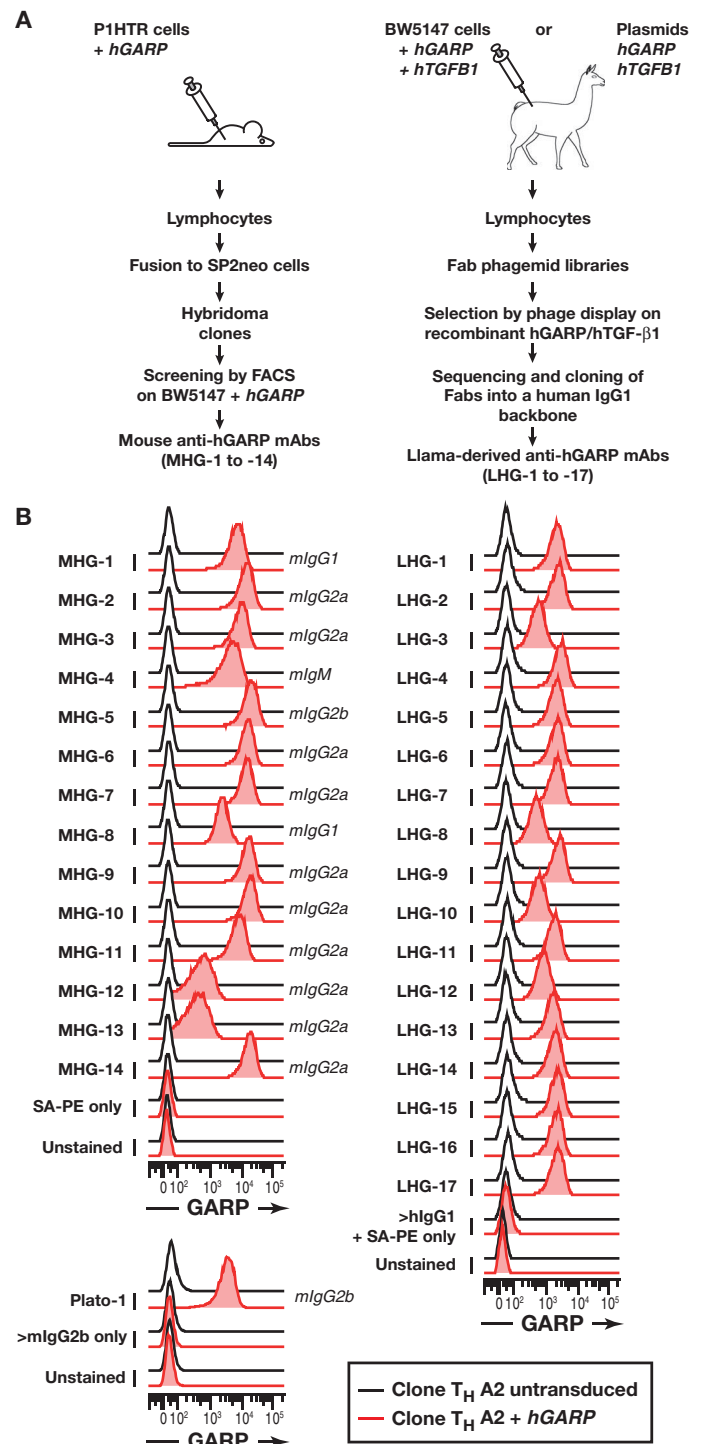
We used two approaches to derive mAbs against hGARP (Fig. 1A). First, lymphocytes from mice immunized with murine cells expressing hGARP were used to generate hybridomas producing mouse anti-human GARP (MHG-1 to MHG-14) mAbs. Second, phage complementary DNA (cDNA) libraries encoding immunoglobulin light and heavy chain variable regions were prepared from lymphocytes of llamas immunized with murine cells expressing hGARP and hTGF $\beta$ 1 or with plasmids encoding these molecules (26). *Escherichia coli* were transfected with independent V<sub>H</sub>/V<sub>K</sub> or V<sub>H</sub>/V<sub>L</sub> libraries to produce phages displaying Fab fragments on their surface. Phage clones were selected by several rounds of binding to immobilized recombinant hGARP/hTGF- $\beta$ 1 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<sub>H</sub>) clone transduced with hGARP but not to the untransduced T<sub>H</sub> clone, which does not express hGARP (4, 19) (Fig. 1B).

### Inhibition of active TGF- $\beta$ 1 production by two anti-hGARP mAbs

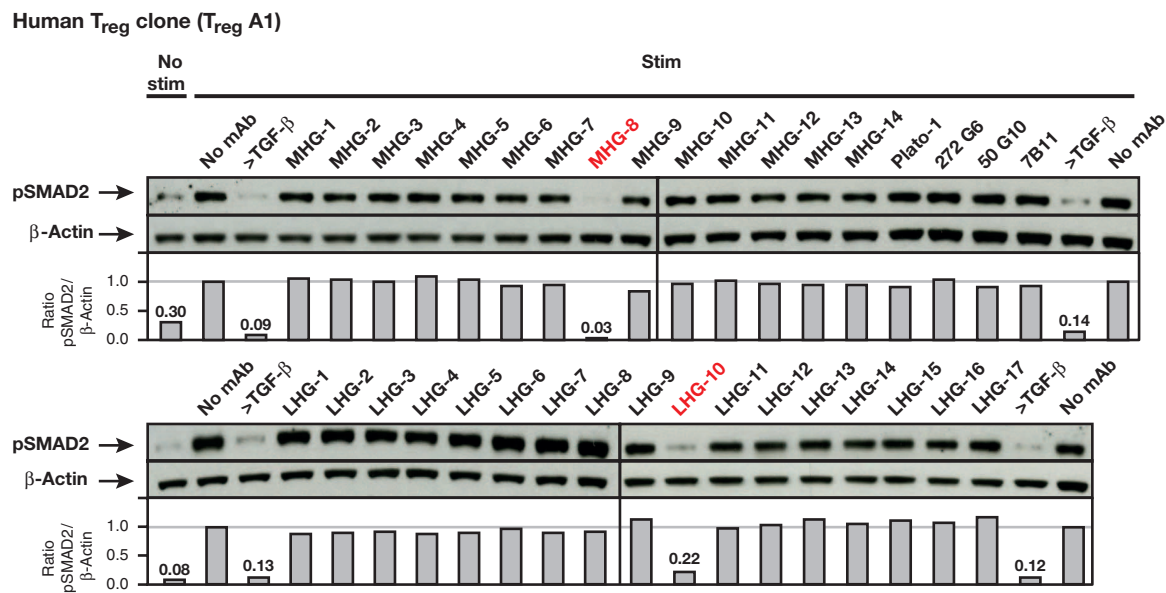
The autocrine activity of TGF- $\beta$ 1 produced by stimulated human T<sub>regs</sub> induces SMAD2 phosphorylation (4). We stimulated a T<sub>reg</sub> clone, that is, a pure population of cells bearing a demethylated *FOXP3i1* allele (4), in the presence or absence of anti-hGARP mAbs and assessed SMAD2 phosphorylation by Western blot. Phosphorylated SMAD2 (pSMAD2) was detected in stimulated T<sub>regs</sub> but not in nonstimulated T<sub>regs</sub> or in T<sub>regs</sub> stimulated in the presence of a neutralizing anti-TGF- $\beta$  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- $\beta$ 1 production by T<sub>regs</sub> (Fig. 2). Similar results were obtained with another population of human T<sub>regs</sub>, namely, polyclonal blood CD4<sup>+</sup>CD25<sup>hi</sup>-CD127<sup>lo</sup> 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<sub>regs</sub> (fig. S1). This inhibitory activity of MHG-8 and LHG-10 indicated that GARP is involved in the production of active TGF- $\beta$ 1 by human T<sub>regs</sub>.

### 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- $\beta$ 1 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<sub>H</sub> A2 (human CD4<sup>+</sup> T<sub>H</sub> cells that do not express hGARP) or T<sub>H</sub> 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-hlgG1 and SA-PE, or with a commercially available mouse anti-hGARP mAb (clone Plato-1) followed by anti-mlgG2b antibodies coupled to AF647.



**Fig. 2. MHG-8 and LHG-10 inhibit production of active TGF- $\beta$ 1 by a human T<sub>reg</sub> clone.** Clone T<sub>reg</sub> 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- $\beta$ 1 production or  $\beta$ -actin as loading control. Bar graphs show quantification of enhanced chemiluminescence (ECL) signals represented as ratios of pSMAD2 to  $\beta$ -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- $\beta$ 1. Accordingly, no surface latent TGF- $\beta$ 1 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 *TGFBI* leads to low surface LAP expression in the absence of GARP. Cotransfection of GARP and *TGFBI* leads to higher surface LAP, corresponding to the presentation of GARP/TGF- $\beta$ 1 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 *TGFBI*. 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- $\beta$ 1 or latent TGF- $\beta$ 1 (fig. S2). A second group ( $n = 17$ ) bound free GARP and GARP/TGF- $\beta$ 1 complexes: they recognized 293T cells transfected with GARP alone or with *TGFBI* (Fig. 3B). They coimmunoprecipitated pro-TGF- $\beta$ 1 and latent TGF- $\beta$ 1 with GARP (fig. S2). A third group ( $n = 6$ ) bound GARP/TGF- $\beta$ 1 complexes but neither free GARP nor free TGF- $\beta$ 1: they recognized cells transfected with GARP and *TGFBI* but not with either GARP alone or *TGFBI* alone (Fig. 3B). They coimmunoprecipitated pro-TGF- $\beta$ 1 and latent TGF- $\beta$ 1 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).

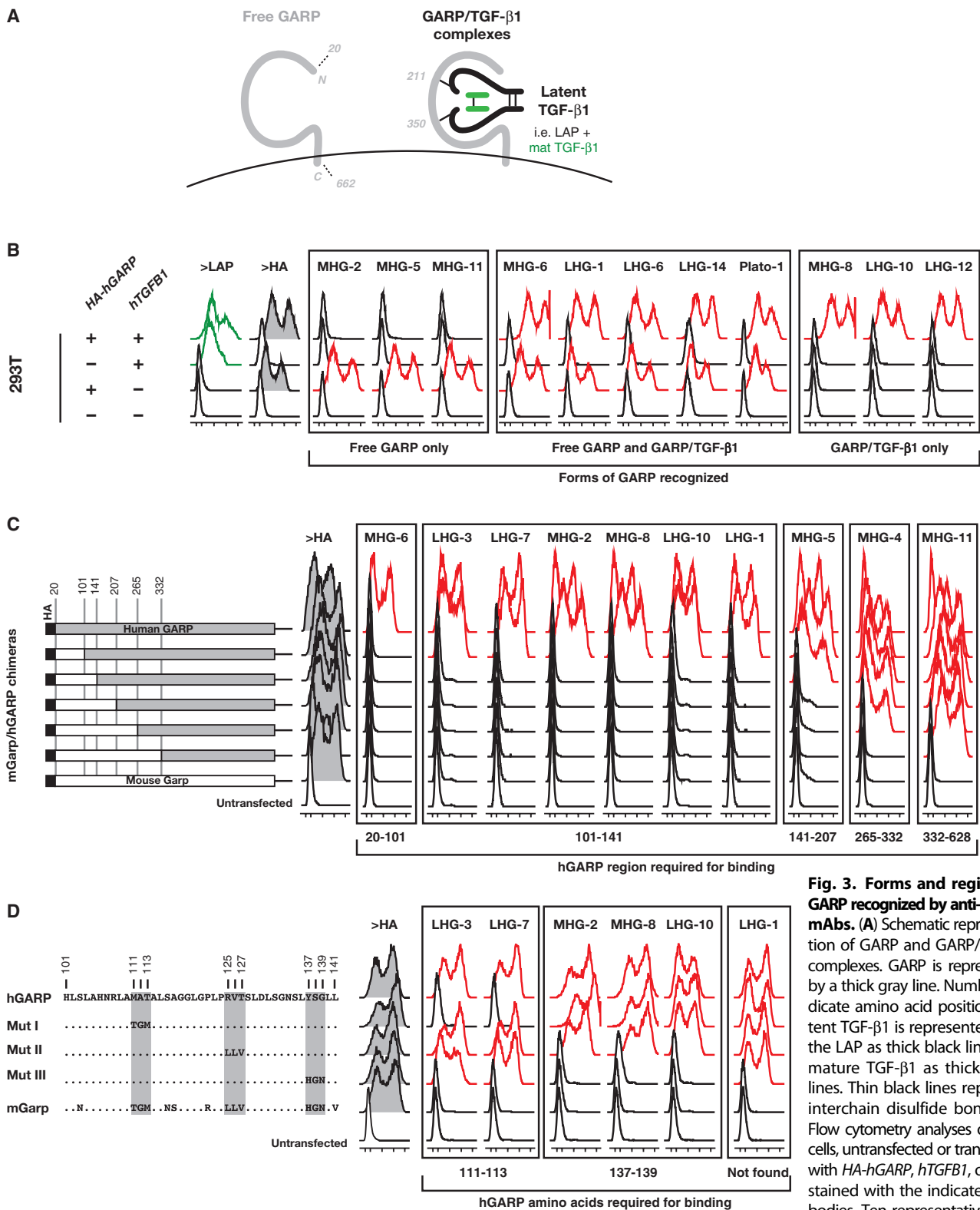
We next defined the GARP regions that were recognized by our antibodies. Because the latter did not recognize mouse GARP (mGarp), we transfected 293T cells with HA-tagged *mGarp/hGARP* chimeras, with or without *TGFBI* depending on the binding requirements determined above. Labeling with anti-HA antibodies 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<sub>20-101</sub>), 15 mAbs required

hGARP<sub>101-141</sub>, 6 required hGARP<sub>141-207</sub>, 1 required hGARP<sub>265-332</sub>, and 4 required hGARP<sub>332-628</sub> (Fig. 3C and Table 1). Both inhibitory antibodies MHG-8 and LHG-10 required hGARP<sub>101-141</sub>, a requirement shared with the four other antibodies that only recognized GARP/TGF- $\beta$  complexes (Table 1).

The sequences of mouse and hGARP<sub>101-141</sub> 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 *hTGFBI* as above. Binding patterns to mutants indicated that five antibodies, including MHG-8 and LHG-10, required hGARP<sub>137-139</sub> (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<sub>reg</sub> TGF- $\beta$ 1 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- $\beta$ 1.

### Inhibition of human T<sub>reg</sub> function by MHG-8 and LHG-10 in vitro

We previously showed that the production of active TGF- $\beta$ 1 was involved in the immunosuppressive activity of human T<sub>regs</sub> toward other CD4<sup>+</sup> T cells (4). We therefore tested whether MHG-8 and LHG-10 could inhibit human T<sub>reg</sub> function in in vitro suppression assays. We used clone T<sub>reg</sub> A1 as a source of T<sub>regs</sub> and freshly isolated blood CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>hi</sup> cells (T<sub>H</sub> cells) as targets for suppression. Cells were stimulated at a 1 T<sub>reg</sub>/1 T<sub>H</sub> ratio in the presence or absence of anti-hGARP mAbs. The T<sub>reg</sub> clone inhibited the proliferation of T<sub>H</sub> 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<sub>reg</sub> A1 on another T<sub>H</sub> target, clone T<sub>H</sub> A2,



**Fig. 3. Forms and regions of GARP recognized by anti-hGARP mAbs.** (A) Schematic representation of GARP and GARP/TGF- $\beta$ 1 complexes. GARP is represented by a thick gray line. Numbers indicate amino acid positions. Latent TGF- $\beta$ 1 is represented with the LAP as thick black lines and mature TGF- $\beta$ 1 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/hTGFB1 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.**

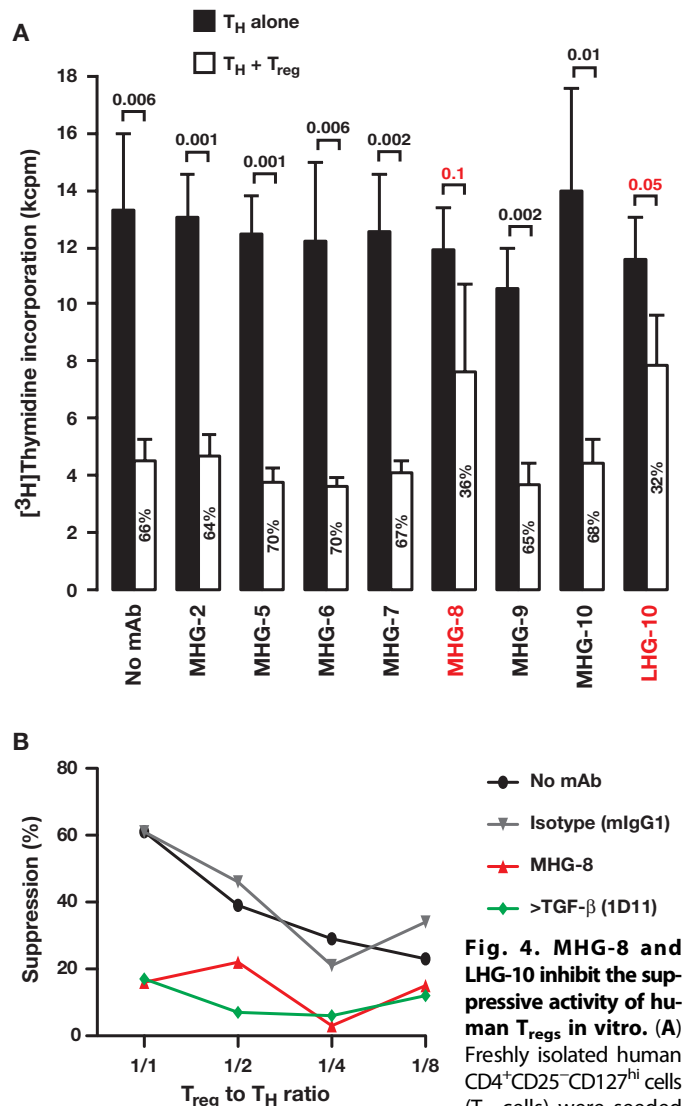
Form of hGARP recognized			hGARP amino acid required	
Free GARP only	Free GARP and GARP/TGF-β1	GARP/TGF-β1 only	Specific amino acid	Region
	MHG-6 MHG-7 MHG-9 LHG-5 LHG-11			20–101
	LHG-7 LHG-9	LHG-3	111–113	101–141
			125–127	
MHG-2 MHG-3	LHG-17	<b>MHG-8</b> <b>LHG-10</b>	137–139	
	LHG-1 LHG-2 LHG-4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

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<sup>-/-</sup>* (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).

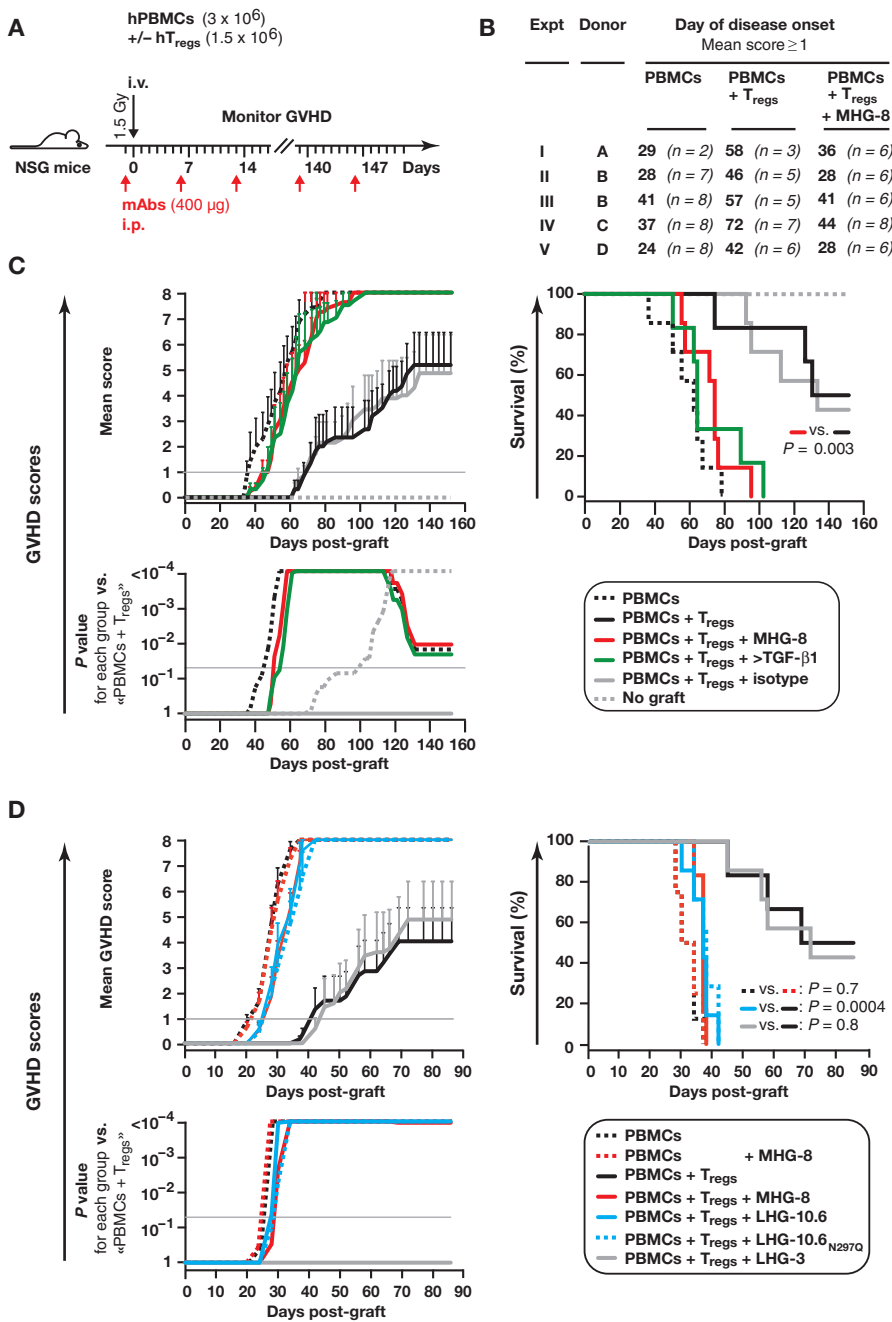
We transferred human PBMCs into NSG mice with or without autologous  $T_{regs}$ , namely, blood  $CD4^+CD25^{hi}CD127^{lo}$  cells shortly



**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. [ $^3H$ ]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:  $0.5 \pm 0.04$  kcpm). Suppression of  $T_H$  proliferation in the presence of  $T_{regs}$  is indicated inside white bars and is calculated as follows: % suppression =  $1 - [kcpm (T_H \text{ alone})/kcpm (T_H + T_{reg})]$ . 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).

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 bi-weekly to establish a disease score. We tested MHG-8 in five independent



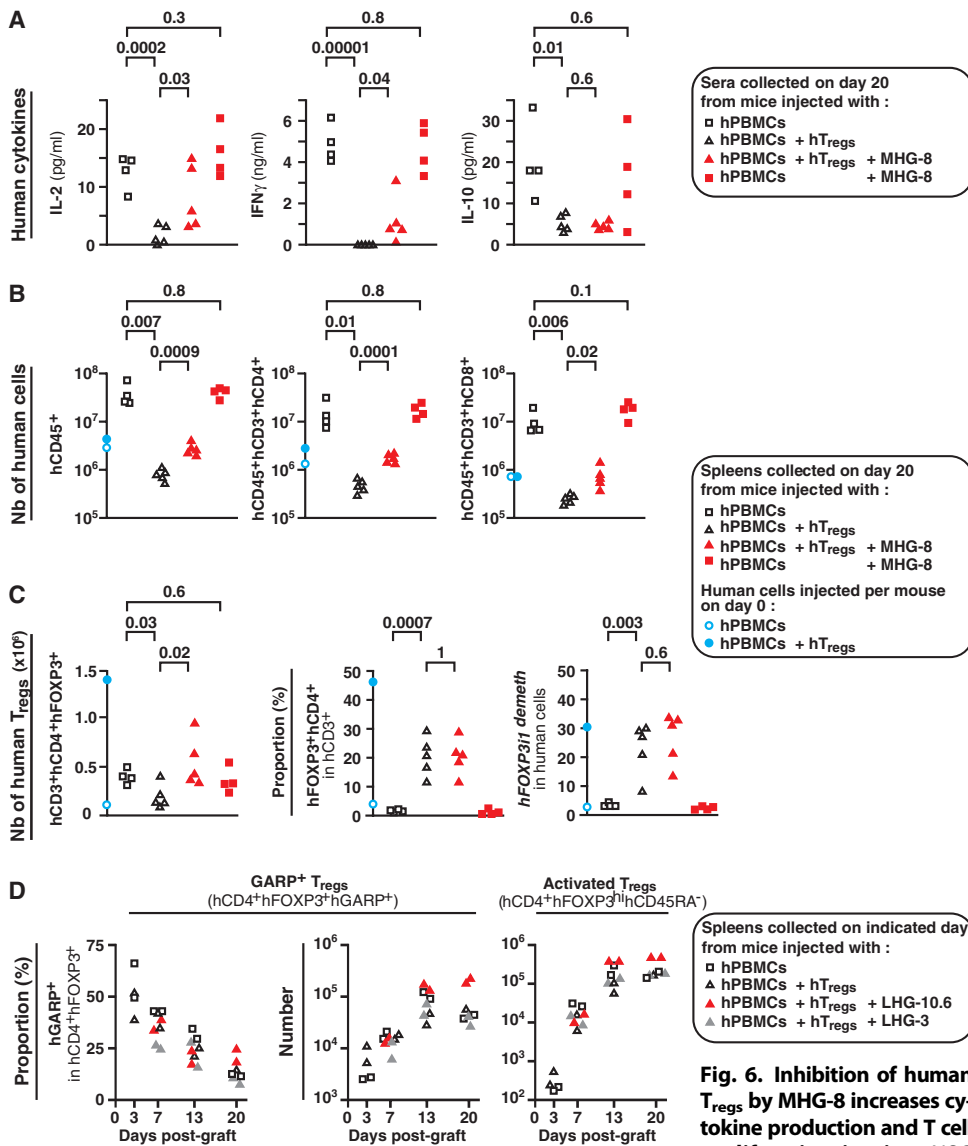
**Fig. 5. Anti-hGARP mAbs that block TGF- $\beta$  production inhibit suppression by human T<sub>regs</sub> in vivo.** (A) Experimental setup. On day 0, PBMCs and autologous T<sub>regs</sub> were injected intravenously in preconditioned NSG mice (1.5 Gy on day -1). The mice received weekly intraperitoneal 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  $\geq 1$ . *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 with SEM; bottom graphs show *P* values of comparisons between each group and the group receiving PBMCs + T<sub>regs</sub> 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<sub>regs</sub> delayed GVHD onset from 24 to 41 days to 42 to 72 days, confirming the protective activity of T<sub>regs</sub>. 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<sub>regs</sub> was also observed with a neutralizing anti-TGF- $\beta$ 1 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<sub>regs</sub> and its abrogation by MHG-8 were also seen on survival curves (Fig. 5C). Whereas 50% of mice grafted with PBMCs and T<sub>regs</sub> showed long-term survival (>152 days), all mice either grafted with PBMCs only or grafted with PBMCs and T<sub>regs</sub> and treated with MHG-8 or anti-TGF- $\beta$ 1 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<sub>regs</sub> (Fig. 5D). We also examined whether abrogation of T<sub>reg</sub> protection by MHG-8 depended on its ability to block TGF- $\beta$  production. We thus compared MHG-8 to LHG-10.6, which also blocks TGF- $\beta$  production by T<sub>regs</sub>, and to LHG-3, which does not. Antibody LHG-10.6 is a variant of LHG-10 with increased affinity for GARP/TGF- $\beta$ 1 complexes that was selected by phage display from Fabs in which the heavy chain of LHG-10 was combined to the V <sub>$\kappa$</sub>  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<sub>reg</sub> protection by blocking T<sub>reg</sub> production of TGF- $\beta$ 1 and not by inducing T<sub>reg</sub> depletion. To further exclude the latter possibility, we also tested a mutated version of LHG-10.6, named LHG-10.6<sub>N297Q</sub>. 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<sub>N297Q</sub> aggravated GVHD in mice grafted with PBMCs and T<sub>regs</sub> as potently as LHG-10.6, confirming that anti-GARP antibodies do not act by depleting T<sub>regs</sub> (Fig. 5D).

We measured human cytokines in the serum of mice 20 days after cell transfer (Fig. 6A). Human IL-2 and interferon- $\gamma$  (IFN $\gamma$ ) 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<sub>regs</sub> 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<sub>regs</sub>, confirming suppressive activity. MHG-8 decreased the suppression by T<sub>regs</sub> 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<sub>regs</sub>, suggesting that T<sub>regs</sub> 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<sup>+</sup>) comprised mostly T lymphocytes (CD4<sup>+</sup> and CD8<sup>+</sup>), which had considerably proliferated in mice grafted with PBMCs alone. This proliferation was inhibited by the cotransfer of T<sub>regs</sub>, an effect that was decreased by MHG-8 (Fig. 6B). Notably, we observed that numbers and proportions of T<sub>regs</sub> (hCD3<sup>+</sup>hCD4<sup>+</sup>hiFOXP3<sup>+</sup> cells or cells with a demethylated *FOXP3i1* allele) were not reduced in mice treated with MHG-8. On the contrary, T<sub>reg</sub> numbers were significantly increased in mice transferred with T<sub>regs</sub> and treated with MHG-8 as compared to untreated mice (Fig. 6C). This suggests that the MHG-8-mediated blockade of autocrine TGF- $\beta$ 1 activity favors T<sub>reg</sub> proliferation while concomitantly inhibiting T<sub>reg</sub> function. It also supports our hypothesis that inhibitory anti-GARP mAbs do not act by depleting T<sub>regs</sub>.

However, it could still be that inhibitory mAbs deplete a minor subpopulation of T<sub>regs</sub> without affecting total T<sub>reg</sub> numbers. For example, this could occur if only a small proportion of T<sub>regs</sub> expressed GARP as a result of activation in this model. In vitro, GARP was shown to be expressed only on activated T<sub>regs</sub> (19, 23). First, we thus measured the proportions and numbers of GARP<sup>+</sup> T<sub>regs</sub> at several time points after the transfer of human PBMCs  $\pm$  T<sub>regs</sub> in NSG mice (Fig. 6D and fig. S5). Three days after transfer, about 50% of CD4<sup>+</sup>FOXP3<sup>+</sup> cells expressed GARP, indicating that GARP<sup>+</sup> cells do not represent a minor subpopulation of T<sub>regs</sub>. Proportions of GARP<sup>+</sup> cells decreased progressively to 10 to 20% of CD4<sup>+</sup>FOXP3<sup>+</sup> 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<sup>+</sup> T<sub>regs</sub> 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<sub>regs</sub>, as proposed by Miyara *et al.* (31), who defined these cells as a subset of FOXP3<sup>+</sup> T<sub>reg</sub> cells characterized by high FOXP3 levels and no CD45RA expression (CD4<sup>+</sup>FOXP3<sup>hi</sup>CD45RA<sup>-</sup> cells). A subpopulation of human FOXP3<sup>hi</sup> cells appeared in mice 7 days after the transfer of human PBMCs  $\pm$  T<sub>regs</sub>. FOXP3<sup>hi</sup> cells expressed the highest levels of GARP and most were CD45RA<sup>-</sup> (fig. S5). Therefore, in vivo, GARP expression was maximal in the activated human CD4<sup>+</sup>FOXP3<sup>hi</sup>CD45RA<sup>-</sup> T<sub>regs</sub>. The numbers of CD4<sup>+</sup>FOXP3<sup>hi</sup>CD45RA<sup>-</sup> 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<sub>regs</sub> in vivo without inducing T<sub>reg</sub> depletion.

## DISCUSSION

GARP is expressed on stimulated T<sub>regs</sub> but not on other types of T lymphocytes. It has been shown to play a role in TGF- $\beta$ 1 processing by T<sub>regs</sub> by forming disulfide bonds with pro-TGF- $\beta$ 1, favoring its cleavage into latent TGF- $\beta$ 1 and inducing the secretion and surface presentation of GARP/latent TGF- $\beta$ 1 complexes (19, 23, 32, 33). Several results suggested that GARP mediated the activation of latent TGF- $\beta$ 1 by T<sub>regs</sub> (4, 32, 34), but direct evidence was lacking. Mouse *Garp*<sup>-/-</sup> T<sub>regs</sub>, in contrast to wild-type T<sub>regs</sub>, could not induce the differentiation of cocultured naïve CD4<sup>+</sup> T lymphocytes into T<sub>H</sub>17 or iT<sub>regs</sub>, two processes known to depend upon active TGF- $\beta$ 1 (34). In transfected 293T cells,  $\alpha_V\beta_6$  and  $\alpha_V\beta_8$  integrins were shown to activate latent TGF- $\beta$ 1 bound to GARP (32). On the other hand, lentiviral-mediated expression of GARP was not sufficient to induce the production of active TGF- $\beta$ 1 by human T<sub>H</sub> cells (4). Our results with anti-hGARP mAbs are the first demonstrating that GARP is required for the production of active TGF- $\beta$ 1 by stimulated human T<sub>regs</sub>. Note that at least one other T<sub>reg</sub>-specific molecule must cooperate with GARP to activate TGF- $\beta$ 1, because GARP alone is not sufficient. Integrin  $\alpha_V\beta_8$  appears to be the GARP partner for T<sub>reg</sub> TGF- $\beta$  activation in mouse T<sub>regs</sub> (35). If this is true in human T<sub>regs</sub> also, blocking anti-hGARP mAbs could function by disrupting the interaction between GARP/latent TGF- $\beta$ 1 complexes and integrins.

GARP is present on other cells than T<sub>regs</sub>, for example, megakaryocytes, platelets, and some endothelial or fibroblast cell lines (36–39), but does not appear to activate latent TGF- $\beta$ 1 in these cells (fig. S6). Therefore, in contrast to anti-TGF- $\beta$  antibodies, anti-hGARP antibodies like MHG-8 and LHG-10 might be T<sub>reg</sub>-specific TGF- $\beta$  inhibitors. This should greatly reduce the adverse events resulting from the inhibition of tumor suppression by TGF- $\beta$ 1.

The role of active TGF- $\beta$ 1 production in T<sub>reg</sub> suppression is still controversial. In murine models, *Tgfb1*<sup>-/-</sup> or *Garp*<sup>-/-</sup> T<sub>regs</sub> 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<sub>regs</sub> failed to inhibit disease development (41). Moreover, naïve T cells that resist TGF- $\beta$ 1 signaling because of the expression of a dominant-negative TGF- $\beta$ RII or to a T cell-specific deletion of TGF- $\beta$ RII cannot be suppressed by wild-type T<sub>regs</sub> (42–44). Together, these results support a role for TGF- $\beta$ 1 in suppression by murine T<sub>regs</sub> in vivo. It must be noted that in contrast to results mentioned above (41), others reported that *Tgfb1*-deficient T<sub>regs</sub> do inhibit colitis induced by the transfer of wild-type naïve T cells (42). In this latter study, however, suppression by *Tgfb1*-deficient T<sub>regs</sub> was not as efficient as that by wild-type T<sub>regs</sub> and was abrogated by anti-TGF- $\beta$ 1 antibodies. We suggest that *Tgfb1*-deficient T<sub>regs</sub> inhibited autoimmune colitis by activating latent TGF- $\beta$ 1 produced by a non-T<sub>reg</sub> cell type. In support of this hypothesis, exogenously added latent TGF- $\beta$ 1 was shown to bind GARP present on human T<sub>regs</sub> treated with *sTGFB1* (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<sub>reg</sub> function in specific pathologies in mice or humans. Little is known regarding the T<sub>reg</sub> suppressive mechanisms in humans. We show here that the in vivo administration of anti-hGARP mAbs blocking TGF- $\beta$ 1 production by human T<sub>regs</sub> abrogates their ability to suppress xenogeneic GVHD. We also provide evidence that their mode of action is based on the inhibition of T<sub>reg</sub> function but not on T<sub>reg</sub> depletion. Accordingly, we observed an increase instead of a reduction in T<sub>reg</sub> numbers upon treatment with MHG-8. Increased T<sub>reg</sub> numbers may result from the blockade of autocrine TGF- $\beta$ 1 cytostatic activity in T<sub>regs</sub>. In line with this, increased T<sub>reg</sub> 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- $\beta$ 1 by human T<sub>regs</sub> can be inhibited with anti-GARP mAbs in a model of xenogeneic GVHD, an important limitation to our study is that T<sub>regs</sub> exert a desirable immune-suppressive activity in the GVHD model, and that reduction of T<sub>reg</sub> 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<sub>reg</sub> function in murine models of cancer or chronic infection, where T<sub>regs</sub> exert adverse immune suppression.

Inhibition of T<sub>reg</sub> 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<sub>regs</sub> (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<sub>reg</sub> 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- $\beta$ 1 production by human T<sub>regs</sub> 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- $\beta$ 1 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<sub>regs</sub>, providing a means to test the ability of anti-GARP mAbs to inhibit human T<sub>reg</sub> function. Sample sizes of in vivo experiments were defined on the basis of the number of available human autologous T<sub>regs</sub>. T<sub>regs</sub> 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<sub>reg</sub> numbers must be as short as possible.



This considerably limits the number of  $T_{\text{regs}}$  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<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/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  $\mu\text{g}/\text{ml}$ ) under limiting dilution conditions. Two clones expressing high surface hGARP (P1.HTR + hGARP) were isolated and used to immunize H-2<sup>d</sup> 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- $\beta$ 1 and selected in neomycin (3 mg/ml) under limiting dilution conditions. A subclone expressing high levels of surface hGARP/hTGF- $\beta$ 1 complexes (BW5147 + hGARP + hTGFB1) was isolated and used to immunize llamas.

Human  $T_{\text{reg}}$  and  $T_{\text{H}}$  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  $T_{\text{regs}}$  were obtained by sorting CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> 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- $\beta$ 1-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  $1 \times 10^7$  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- $\beta$ 1 complexes by enzyme-linked immunosorbent assay (ELISA) using immobilized re-

combinant GARP/TGF- $\beta$ 1 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  $\mu\text{g}$  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 ( $V_{\text{H}}$ ,  $V_{\lambda}$ , and  $V_{\kappa}$ ). Two independent phagemid libraries, coding for  $V_{\text{H}}/V_{\lambda}$  and  $V_{\text{H}}/V_{\kappa}$  Fabs, respectively, were constructed as previously described (26) to obtain a diversity of  $1 \times 10^8$  to  $7 \times 10^8$  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- $\beta$ 1, washing, and elution with trypsin. In some instances, counter selections with soluble hGARP (hGARP<sub>1-628</sub> fused to a TEV-3 $\times$  Strep-tag produced in 293E cells) and soluble latent TGF- $\beta$ 1 were used to enrich for Fabs binding hGARP/TGF- $\beta$ 1 complexes only. Individual colonies were isolated and periplasmic fractions containing soluble Fabs were produced by isopropyl- $\beta$ -D-thiogalactoside induction. Fabs in periplasmic fractions were then screened by ELISA for binding to immobilized hGARP/TGF- $\beta$ 1.  $V_{\text{H}}$  and  $V_{\text{L}}$  regions of Fab clones binding to hGARP/TGF- $\beta$ 1 complexes were sequenced. Fab clones were divided into 17 families on the basis of similarities in the sequences coding for the  $V_{\text{H}}$  CDR3 region.  $V_{\text{H}}$  and  $V_{\text{L}}$  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  $T_{\text{reg}}$  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: AAACC-TACTACAAAACAACAAC/GGAGGAAGAGAAGAGGGTA/CCTATAAAATAAAATATCTACCCTC; demethylated FOXP3i1 alleles: TCTACCCTCTTCTCTTCTCCTCCA/GATTTTTTTTGTATT-GATGTTATGGT/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-hCD8 $\alpha$ -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- $\beta$ 1 production by human T<sub>reg</sub> cells

A human T<sub>reg</sub> clone ( $1 \times 10^6$  cells/ml) was stimulated in serum-free medium with coated anti-CD3 (Orthoclone OKT3; Janssen-Cilag, 1  $\mu$ g/ml) and soluble anti-CD28 (BD Biosciences; 1  $\mu$ g/ml) in the presence or absence of an anti-hGARP mAb (10  $\mu$ g/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- $\beta$  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  $\beta$ -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- $\beta$ 1 by the stimulated T<sub>reg</sub> clone. ECL signals were quantified by measuring the density of the 55-kD pSMAD2 and 40-kD  $\beta$ -actin bands on autoradiographs using the ImageJ software. Source data for Western blot analyses are shown in fig. S8.

### In vitro suppression assays

T<sub>H</sub> cells ( $2 \times 10^4$ ) were seeded alone or with the indicated numbers of T<sub>regs</sub> and stimulated with coated anti-CD3 (Orthoclone OKT3, Janssen-Cilag, 1  $\mu$ g/ml) and soluble anti-CD28 (BD Biosciences, 1  $\mu$ g/ml) in the presence or absence of an anti-hGARP mAb (MHG or LHG), an anti-TGF- $\beta$  antibody (clone 1D11, R&D Systems), or an isotype control (mIgG1 clone 11711, R&D Systems) (10  $\mu$ g/ml). [Methyl-<sup>3</sup>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 ( $3 \times 10^6$  per mouse) alone or mixed with autologous polyclonal T<sub>regs</sub> ( $1.5 \times 10^6$  per mouse). One day before graft and weekly thereafter, mice received intraperitoneal injections of PBS or 400  $\mu$ g of MHG-8 (mIgG1), an anti-TGF- $\beta$ 1 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  $\geq 10\%$ ; 2 if  $\geq 20\%$ ); 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  $\geq 6$ . Death corresponds to a maximum score of 8.

### Cytokine concentrations in sera

Concentrations of human IL-2, IL-10, and IFN $\gamma$  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 IFN $\gamma$ , 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 ( $\alpha$  level for all statistical analyses: 0.05).

## SUPPLEMENTARY MATERIALS

[www.sciencetranslationalmedicine.org/cgi/content/full/7/284/284ra56/DC1](http://www.sciencetranslationalmedicine.org/cgi/content/full/7/284/284ra56/DC1)

- Fig. S1. MHG-8 and LHG-10 inhibit production of active TGF- $\beta$ 1 by human polyclonal T<sub>regs</sub>.  
 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  $\pm$  T<sub>regs</sub>.  
 Fig. S6. Human fibroblasts express GARP but do not produce active TGF- $\beta$ 1.  
 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<sub>regs</sub>.

## REFERENCES AND NOTES

1. Y. Zheng, A. Y. Rudensky, Foxp3 in control of the regulatory T cell lineage. *Nat. Immunol.* **8**, 457–462 (2007).
2. S. Sakaguchi, M. Miyara, C. M. Costantino, D. A. Hafler, FOXP3<sup>+</sup> regulatory T cells in the human immune system. *Nat. Rev. Immunol.* **10**, 490–500 (2010).
3. Y. Zheng, S. Josefowicz, A. Chaudhry, X. P. Peng, K. Forbush, A. Y. Rudensky, Role of conserved non-coding DNA elements in the *Foxp3* gene in regulatory T-cell fate. *Nature* **463**, 808–812 (2010).
4. J. Stockis, W. Fink, V. François, T. Connerotte, C. de Smet, L. Knoops, P. van der Bruggen, T. Boon, P. G. Coulie, S. Lucas, Comparison of stable human Treg and Th clones by transcriptional profiling. *Eur. J. Immunol.* **39**, 869–882 (2009).
5. S. Floess, J. Freyer, C. Siewert, U. Baron, S. Olek, J. Polanski, K. Schlawe, H.-D. Chang, T. Bopp, E. Schmitt, S. Klein-Hessling, E. Serfling, A. Hamann, J. Huehn, Epigenetic control of the *foxp3* locus in regulatory T cells. *PLOS Biol.* **5**, e38 (2007).

6. G. Wieczorek, A. Asemissen, F. Model, I. Turbachova, S. Floess, V. Liebenberg, U. Baron, D. Stauch, K. Kotsch, J. Pratschke, A. Hamann, C. Loddenkemper, H. Stein, H. D. Volk, U. Hoffmüller, A. Grützkau, A. Mustea, J. Huehn, C. Scheibenbogen, S. Olek, Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res.* **69**, 599–608 (2009).
7. I. J. de Vries, C. Castelli, C. Huygens, J. F. Jacobs, J. Stockis, B. Schuler-Thurner, G. J. Adema, C. J. Punt, L. Rivoltini, G. Schuler, P. G. Coulie, S. Lucas, Frequency of circulating Tregs with demethylated FOXP3 intron 1 in melanoma patients receiving tumor vaccines and potentially Treg-depleting agents. *Clin. Cancer Res.* **17**, 841–848 (2011).
8. U. Baron, S. Floess, G. Wieczorek, K. Baumann, A. Grützkau, J. Dong, A. Thiel, T. J. Boeld, P. Hoffmann, M. Edinger, I. Turbachova, A. Hamann, S. Olek, J. Huehn, DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3<sup>+</sup> conventional T cells. *Eur. J. Immunol.* **37**, 2378–2389 (2007).
9. M. Miyara, Y. Ito, S. Sakaguchi, T<sub>REG</sub>-cell therapies for autoimmune rheumatic diseases. *Nat. Rev. Rheumatol.* **10**, 543–551 (2014).
10. C. Ménétrier-Caux, T. Curiel, J. Faget, M. Manuel, C. Caux, W. Zou, Targeting regulatory T cells. *Target. Oncol.* **7**, 15–28 (2012).
11. J. F. M. Jacobs, S. Nierkens, C. G. Figdor, I. J. M. de Vries, G. J. Adema, Regulatory T cells in melanoma: The final hurdle towards effective immunotherapy? *Lancet Oncol.* **13**, e32–e42 (2012).
12. S. Z. Josefowicz, L.-F. Lu, A. Y. Rudensky, Regulatory T cells: Mechanisms of differentiation and function. *Annu. Rev. Immunol.* **30**, 531–564 (2012).
13. M. O. Li, Y. Y. Wan, S. Sanjabi, A.-K. Robertson, R. A. Flavell, Transforming growth factor- $\beta$  regulation of immune responses. *Annu. Rev. Immunol.* **24**, 99–146 (2006).
14. M. M. Shull, I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, N. Annunziata, T. Doetschman, Targeted disruption of the mouse transforming growth factor- $\beta$  gene results in multifocal inflammatory disease. *Nature* **359**, 693–699 (1992).
15. J. Massague, TGF $\beta$  in cancer. *Cell* **134**, 215–230 (2008).
16. J. C. Morris, A. R. Tan, T. E. Olencki, G. I. Shapiro, B. J. Dezube, M. Reiss, F. J. Hsu, J. A. Berzofsky, D. P. Lawrence, Phase I study of GC1008 (fresolimumab): A human anti-transforming growth factor-beta (TGF $\beta$ ) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma. *PLoS One* **9**, e90353 (2014).
17. S. Lonning, J. Mannick, J. M. McPherson, Antibody targeting of TGF- $\beta$  in cancer patients. *Curr. Pharm. Biotechnol.* **12**, 2176–2189 (2011).
18. M. A. Travis, D. Sheppard, TGF- $\beta$  activation and function in immunity. *Annu. Rev. Immunol.* **32**, 51–82 (2014).
19. J. C. Stockis, D. Colau, P. G. Coulie, S. Lucas, Membrane protein GARP is a receptor for latent TGF- $\beta$  on the surface of activated human Treg. *Eur. J. Immunol.* **39**, 3315–3322 (2009).
20. J. H. Kehrl, L. M. Wakefield, A. B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, M. B. Sporn, A. S. Fauci, Production of transforming growth factor  $\beta$  by human T lymphocytes and its potential role in the regulation of T cell growth. *J. Exp. Med.* **163**, 1037–1050 (1986).
21. K. Nakamura, A. Kitani, W. Strober, Cell contact-dependent immunosuppression by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells is mediated by cell surface-bound transforming growth factor  $\beta$ . *J. Exp. Med.* **194**, 629–644 (2001).
22. K. Nakamura, A. Kitani, I. Fuss, A. Pedersen, N. Harada, H. Nawata, W. Strober, TGF- $\beta$ 1 plays an important role in the mechanism of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell activity in both humans and mice. *J. Immunol.* **172**, 834–842 (2004).
23. D. Q. Tran, J. Andersson, R. Wang, H. Ramsey, D. Unutmaz, E. M. Shevach, GARP (LRRC32) is essential for the surface expression of latent TGF- $\beta$  on platelets and activated FOXP3<sup>+</sup> regulatory T cells. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 13445–13450 (2009).
24. R. Wang, Q. Wan, L. Kozhaya, H. Fujii, D. Unutmaz, Identification of a regulatory T cell specific cell surface molecule that mediates suppressive signals and induces Foxp3 expression. *PLoS One* **3**, e2705 (2008).
25. M. Probst-Keppler, R. Geffers, A. Kröger, N. Viegas, C. Erck, H.-J. Hecht, H. Lünsdorf, R. Roubin, D. Moharegh-Khiabani, K. Wagner, F. Ocklenburg, A. Jeron, H. Garritsen, T. P. Arstila, E. Kekäläinen, R. Balling, H. Hauser, J. Buer, S. Weiss, GARP: A key receptor controlling FOXP3 in human regulatory T cells. *J. Cell. Mol. Med.* **13**, 3343–3357 (2009).
26. C. Basilio, A. Hultberg, C. Blanchetot, N. de Jonge, E. Festjens, V. Hanssens, S.-I. Osepa, G. De Boeck, A. Mira, M. Cazzanti, V. Morello, T. Dreier, M. Saunders, H. de Haard, P. Michieli, Four individually druggable MET hotspots mediate HGF-driven tumor progression. *J. Clin. Invest.* **124**, 3172–3186 (2014).
27. L. D. Shultz, M. A. Brehm, J. V. Garcia-Martinez, D. L. Greiner, Humanized mice for immune system investigation: Progress, promise and challenges. *Nat. Rev. Immunol.* **12**, 786–798 (2012).
28. M. Hannon, C. Lechanteur, S. Lucas, J. Somja, L. Seidel, L. Belle, F. Bruck, E. Baudoux, O. Giet, A.-M. Chantillon, P. Delvenne, P. Drion, Y. Beguin, S. Humblet-Baron, F. Baron, Infusion of clinical-grade enriched regulatory T cells delays experimental xenogeneic graft-versus-host disease. *Transfusion* **54**, 353–363 (2014).
29. M. B. Overdijk, S. Verploegen, A. Ortiz Buijse, T. Vink, J. H. W. Leusen, W. K. Bleeker, P. W. H. I. Parren, Crosstalk between human IgG isotypes and murine effector cells. *J. Immunol.* **189**, 3430–3438 (2012).
30. M. H. Tao, S. L. Morrison, Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J. Immunol.* **143**, 2595–2601 (1989).
31. M. Miyara, Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Tafin, T. Heike, D. Valeyre, A. Mathian, T. Nakahata, T. Yamaguchi, T. Nomura, M. Ono, Z. Amoura, G. Gorochov, S. Sakaguchi, Functional delineation and differentiation dynamics of human CD4<sup>+</sup> T cells expressing the FoxP3 transcription factor. *Immunity* **30**, 899–911 (2009).
32. R. Wang, J. Zhu, X. Dong, M. Shi, C. Lu, T. A. Springer, GARP regulates the bioavailability and activation of TGF $\beta$ . *Mol. Biol. Cell* **23**, 1129–1139 (2012).
33. E. Gauthy, J. Cuende, J. Stockis, C. Huygens, B. Lethé, J.-F. Collet, G. Bommer, P. G. Coulie, S. Lucas, GARP is regulated by miRNAs and controls latent TGF- $\beta$ 1 production by human regulatory T cells. *PLoS One* **8**, e76186 (2013).
34. J. P. Edwards, H. Fujii, A. X. Zhou, J. Creemers, D. Unutmaz, E. M. Shevach, Regulation of the expression of GARP/latent TGF- $\beta$ 1 complexes on mouse T cells and their role in regulatory T cell and Th17 differentiation. *J. Immunol.* **190**, 5506–5515 (2013).
35. J. P. Edwards, A. M. Thornton, E. M. Shevach, Release of active TGF- $\beta$ 1 from the latent TGF- $\beta$ 1/GARP complex on T regulatory cells is mediated by integrin  $\beta$ <sub>8</sub>. *J. Immunol.* **193**, 2843–2849 (2014).
36. R. Roubin, S. Pizette, V. Ollendorff, J. Planche, D. Birnbaum, O. Delapeyriere, Structure and developmental expression of mouse Garp, a gene encoding a new leucine-rich repeat-containing protein. *Int. J. Dev. Biol.* **40**, 545–555 (1996).
37. M. N. O'Connor, I. I. Sallas, A. Cvejic, N. A. Watkins, A. Walker, S. F. Garner, C. I. Jones, I. C. Macaulay, M. Steward, J.-J. Zwaginga, S. L. Bray, F. Dudbridge, B. de Bono, A. H. Goodall, H. Deckmyn, D. L. Stemple, W. H. Ouwehand, Functional genomics in zebrafish permits rapid characterization of novel platelet membrane proteins. *Blood* **113**, 4754–4762 (2009).
38. I. C. Macaulay, M. R. Tijssen, D. C. Thijssen-Timmer, A. Gusnanto, M. Steward, P. Burns, C. F. Langford, P. D. Ellis, F. Dudbridge, J.-J. Zwaginga, N. A. Watkins, C. E. van der Schoot, W. H. Ouwehand, Comparative gene expression profiling of in vitro differentiated megakaryocytes and erythroblasts identifies novel activatory and inhibitory platelet membrane proteins. *Blood* **109**, 3260–3269 (2007).
39. A. Kehrmann, H. Truong, A. Repenning, R. Boger, L. Klein-Hitpass, U. Pascheberg, A. Beckmann, B. Opalka, K. Kleine-Lowinski, Complementation of non-tumorigenicity of HPV18-positive cervical carcinoma cells involves differential mRNA expression of cellular genes including potential tumor suppressor genes on chromosome 11q13. *Cancer Genet.* **206**, 279–292 (2013).
40. C. A. Piccirillo, J. J. Letterio, A. M. Thornton, R. S. McHugh, M. Mamura, H. Mizuhara, E. M. Shevach, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells can mediate suppressor function in the absence of transforming growth factor  $\beta$ 1 production and responsiveness. *J. Exp. Med.* **196**, 237–246 (2002).
41. M. O. Li, Y. Y. Wan, R. A. Flavell, T cell-produced transforming growth factor- $\beta$ 1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* **26**, 579–591 (2007).
42. L. Fahlén, S. Read, L. Gorelik, S. D. Hurst, R. L. Coffman, R. A. Flavell, F. Powrie, T cells that cannot respond to TGF- $\beta$  escape control by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. *J. Exp. Med.* **201**, 737–746 (2005).
43. J. C. Marie, D. Liggitt, A. Y. Rudensky, Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor- $\beta$  receptor. *Immunity* **25**, 441–454 (2006).
44. M. O. Li, S. Sanjabi, R. A. Flavell, Transforming growth factor- $\beta$  controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* **25**, 455–471 (2006).
45. Y. Liu, P. Zhang, J. Li, A. B. Kulkarni, S. Perruche, W. Chen, A critical function for TGF- $\beta$  signaling in the development of natural CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells. *Nat. Immunol.* **9**, 632–640 (2008).
46. D. B. Page, M. A. Postow, M. K. Callahan, J. P. Allison, J. D. Wolchok, Immune modulation in cancer with antibodies. *Annu. Rev. Med.* **65**, 185–202 (2014).
47. T. R. Simpson, F. Li, W. Montalvo-Ortiz, M. A. Sepulveda, K. Bergerhoff, F. Arce, C. Roddie, J. Y. Henry, H. Yagita, J. D. Wolchok, K. S. Peggs, J. V. Ravetch, J. P. Allison, S. A. Quezada, Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J. Exp. Med.* **210**, 1695–1710 (2013).
48. M. J. Selby, J. J. Engelhardt, M. Quigley, K. A. Henning, T. Chen, M. Srinivasan, A. J. Korman, Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol. Res.* **1**, 32–42 (2013).
49. J. D. Wolchok, H. Kluger, M. K. Callahan, M. A. Postow, N. A. Rizvi, A. M. Lesokhin, N. H. Segal, C. E. Ariyan, R.-A. Gordon, K. Reed, M. M. Burke, A. Caldwell, S. A. Kronenberg, B. U. Agunwamba, X. Zhang, I. Lowy, H. D. Inzunza, W. Feely, C. E. Horak, Q. Hong, A. J. Korman, J. M. Wigginton, A. Gupta, M. Sznol, Nivolumab plus ipilimumab in advanced melanoma. *N. Engl. J. Med.* **369**, 122–133 (2013).
50. C. Robert, L. Thomas, I. Bondarenko, S. O'Day, J. Weber, C. Garbe, C. Lebbe, J.-F. Baurain, A. Testori, J.-J. Grob, N. Davidson, J. Richards, M. Maio, A. Hauschild, W. H. Miller Jr., P. Gascon, M. Lotem, K. Harmanakaya, R. Ibrahim, S. Francis, T.-T. Chen, R. Humphrey, A. Hoos, J. D. Wolchok, Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N. Engl. J. Med.* **364**, 2517–2526 (2011).

51. F. S. Hodi, S. J. O'Day, D. F. McDermott, R. W. Weber, J. A. Sosman, J. B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J. C. Hassel, W. Akerley, A. J. M. van den Eertwegh, J. Lutzky, P. Lorigan, J. M. Vaubel, G. P. Linette, D. Hogg, C. H. Ottensmeier, C. Lebbé, C. Peschel, I. Quirt, J. I. Clark, J. D. Wolchok, J. S. Weber, J. Tian, M. J. Yellin, G. M. Nichol, A. Hoos, W. J. Urb, Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.* **363**, 711–723 (2010).
52. A. Van Pel, E. De Plaen, T. Boon, Selection of highly transfectable variant from mouse mastocytoma P815. *Somat. Cell Mol. Genet.* **11**, 467–475 (1985).
53. M. M. Lemaire, A. Vanhaunderde, Y. Nizet, L. Dumoutier, J.-C. Renault, Induction of autoantibodies against mouse soluble proteins after immunization with living cells presenting the autoantigen at the cell surface in fusion with a human type 2 transmembrane protein. *J. Immunol.* **367**, 56–62 (2011).
54. L. P. Daley, L. F. Gagliardo, M. S. Duffy, M. C. Smith, J. A. Appleton, Application of monoclonal antibodies in functional and comparative investigations of heavy-chain immunoglobulins in new world camelids. *Clin. Diagn. Lab. Immunol.* **12**, 380–386 (2005).
55. P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159 (1987).
56. C. Uyttenhove, R. G. Marillier, F. Tacchini-Cottier, M. Charmoy, R. R. Caspi, J. M. Damsker, S. Goriely, D. Su, J. Van Damme, S. Struyf, G. Opendakker, J. Van Snick, Amine-reactive OVA multimers for auto-vaccination against cytokines and other mediators: Perspectives illustrated for GCP-2 in L. major infection. *J. Leukoc. Biol.* **89**, 1001–1007 (2011).
57. P. G. Coulie, J. Van Snick, Enhancement of IgG anti-carrier responses by IgG2 anti-hapten antibodies in mice. *Eur. J. Immunol.* **15**, 793–798 (1985).

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