

Activated glycoprotein A repetitions predominant (GARP)–expressing regulatory T cells inhibit allergen-induced intestinal inflammation in humanized mice

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Background: Recently, we developed a humanized mouse model of allergen-induced IgE-dependent gut inflammation in PBMC-engrafted immunodeficient mice.

Objective: In the present study, we wanted to investigate the role of regulatory T (Treg) cells and their activation status in this model.

Methods: Nonobese diabetic-severe combined immunodeficiency- $\gamma\text{c}^{-/-}$ mice were injected intraperitoneally with human PBMCs from allergic donors together with the respective allergen or NaCl as control in the presence or absence of different concentrations of CD4⁺CD25⁺ Treg cells of the same donor. After an additional allergen boost 1 week later, mice were challenged with the allergen rectally on day 21 and gut inflammation was monitored by a high-resolution video mini-endoscopic system evaluating translucency, granularity, fibrin production, vascularity, and stool.

Results: Allergen-specific human IgE in mouse sera, which was detectable only in PBMC plus allergen-treated mice, was strongly inhibited by coinjection of Treg cells at a ratio of at least 1:10. Consequently, the presence of Treg cells significantly decreased IgE-dependent allergen-induced gut inflammation after rectal allergen challenge. In addition, Treg cells reduced allergen-specific proliferation and cytokine production of recovered human CD4⁺ T cells *in vitro*. Activation of Treg cells before injection further increased all inhibitory effects.

Prevention of gut inflammation also occurred by the administration of glycoprotein A repetitions predominant, a molecule expressed by activated Treg cells, whereas its blockade completely abrogated inhibition by Treg cells.

Conclusions: These results demonstrate that allergen-specific gut inflammation in human PBMC-engrafted mice can be

avoided by enhancing the numbers or activity of autologous Treg cells, which is of great interest for therapeutic intervention of allergic diseases of the intestine. (J Allergy Clin Immunol 2015;136:159-68.)

Key words: Humanized mice, IgE, allergen, regulatory T cells, colon, inflammation

Tolerance induction in allergic diseases is characterized by the generation of allergen-specific regulatory T (Treg) cells, leading to an inhibition of T_H2 cells, and represents one main mechanism of effective allergen-specific immunotherapy.¹ However, not all patients can be cured by allergen-specific immunotherapy, and there still remains the problem of severe adverse effects, especially the risk of anaphylactic reactions in food allergy.^{2,3} Other therapeutic approaches aiming at decreasing the T_H2 response by blocking critical T_H2 cytokines such as IL-4, IL-5, IL-9, and IL-13 or the effector molecules IgE and CRTH2 have been successfully demonstrated in several mouse models of allergy. Except for the anti-human IgE mAb omalizumab, they failed to show convincing results in human clinical trials or were effective only in specific subsets of patients with asthma.⁴⁻⁸ To overcome these limitations of translating discoveries in rodents into clinical applications, immunodeficient mice engrafted with functional human cells and tissues, termed humanized mice, have been developed. Humanized mice are an important preclinical tool for *in vivo* studies of human pathogens that do not infect mice, such as human immunodeficiency virus type 1 or hepatitis B and C viruses,⁹ and have also been used to study allergic immune responses.¹⁰⁻¹³ However, these first humanized mouse models focused on the analysis of airway hyperresponsiveness and lung inflammation, whereas allergen-induced inflammation of the intestine in the context of pollen-related or other food allergy was not investigated.

Therefore, we recently established a humanized mouse model of allergic gut inflammation by engrafting PBMCs from highly sensitized allergic donors (allergen-specific IgE level of at least ≥ 50 kU/L) in immunodeficient nonobese diabetic (NOD)-severe combined immunodeficiency (*scid*)- $\gamma\text{c}^{-/-}$ mice. Because of a targeted mutation on the IL-2R γ -chain gene, which leads to severe impairments in T- and B-cell development and function and completely prevents natural killer cell development, these mice were superior to all other strains used so far by allowing the survival of human cells even at low cell numbers.^{14,15} Regardless of the allergen used in our model, that is, the aeroallergens birch pollen, alder pollen, hazel pollen, grass pollen, or house dust mite, the food allergens hazelnut or peanut, or insect venom allergens, we were able to measure not only an allergen-specific inflammation of the lung after intranasal allergen challenge,^{16,17} but, for the first time, also an allergen-specific gut inflammation

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This work was supported by Deutsche Forschungsgemeinschaft (grant no. BE 4504/2-2 to I.B.) and the Clinical Research Unit 257 CEDER (grant no. WE 4656/2-2 to B.W.).

Disclosure of potential conflict of interest: B. Weigmann has received research and travel support from the Clinical Research Unit 257-TP07 molecular pathogenesis of IBD. J. Saloga has received research and travel support from University Medical Center Mainz, Research and Education Grants, Federal Country Rhineland-Palatinate, Germany. I. Bellinghausen has received research and travel support from Deutsche Forschungsgemeinschaft (grant no. BE 4504/2-2). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication June 13, 2014; revised March 31, 2015; accepted for publication April 3, 2015.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2015.04.020>

Abbreviations used

DC: Dendritic cell
 GARP: Glycoprotein A repetitions predominant
 IMDM: Iscove modified Dulbecco medium
 NOD: Nonobese diabetic
 SCID: Severe combined immunodeficiency
 Treg: Regulatory T

when the mice were challenged rectally or orally with the respective allergen.¹⁸ Analyzing whether the effector mechanisms being activated are human or murine, we could demonstrate that the allergen-specific inflammation of the colon was primarily dependent on human IgE and IgE receptor-expressing effector cells among PBMCs, and the mediators histamine and platelet-activating factor, while murine mast cells or murine basophils might play a role as bystanders.¹⁸ In the present study, we analyzed the influence of Treg cells in this model and could demonstrate that activated glycoprotein A repetitions predominant (GARP)-expressing Treg cells inhibited allergen-specific gut inflammation in a prophylactic as well as in a therapeutic approach.

METHODS**Patients**

Heparinized blood was obtained from 18 allergic donors suffering from allergic rhinoconjunctivitis and/or asthma to aeroallergens. Some allergic donors also showed cross-reactivity to hazelnut or peanut. Specific sensitization was documented by positive skin prick test result and detection of allergen-specific IgE in the sera (CAP class ≥ 5 measured with the ImmunoCAP specific IgE blood test; Thermo Fisher Scientific, Phadia GmbH, Freiburg, Germany). This study was approved by the local ethics committee. Informed consent was obtained from all subjects before the study.

Mice

NOD.CB17-Prkdc^{scid}/J $\gamma c^{-/-}$ mice (Jackson Laboratory, Bar Harbor, Me) were kept under specific pathogen-free conditions in the Central Animal Facility of the University Medical Center of the Johannes Gutenberg-University in Mainz. Four- to 8-week-old male or female mice were used. Experiments were performed in accordance with current federal, state, and institutional guidelines (authorization no. 23 177-07/G 13-1-011).

Generation of monocyte-derived allergen-pulsed dendritic cells

PBMCs were isolated from heparinized blood by Ficoll-Paque 1.077 g/mL (PAA Laboratories GmbH, Pasching, Austria) density centrifugation. To enrich CD14⁺ monocytes, 5×10^6 PBMCs per well were incubated for 45 minutes in a 12-well plate (Greiner, Frickenhausen, Germany) in Iscove modified Dulbecco medium (IMDM) containing L-glutamine and 25 mM Hepes (IMDM, PAA Laboratories GmbH) supplemented with an antibiotic-antimycotic solution containing 100 μ g/mL streptomycin, 100 U/mL penicillin, and 250 ng/mL amphotericin B (PAA Laboratories GmbH) and 3% heat-inactivated autologous plasma at 37°C. After washing the nonadherent cells with prewarmed PBS, the remaining monocytes (purity > 90%) were incubated in IMDM (1.5 mL/well) supplemented with 1% heat-inactivated autologous plasma, IL-4 (1000 U/mL; Miltenyi Biotec, Bergisch Gladbach, Germany), and GM-CSF (200 U/mL Leukine; Immunex Corp, Seattle, Wash). On day 6, the resulting immature dendritic cells (DCs) were pulsed with birch pollen, grass pollen, or house dust mite allergen extract (10 μ g/mL; ALK-Abello, Hamburg, Germany), or tetanus toxoid (1 μ g/mL;

Behringwerke, Marburg, Germany), and further stimulated with TNF- α (1000 U/mL; Miltenyi Biotec), IL-1 β (2000 U/mL; Miltenyi Biotec), and prostaglandin E₂ (1 μ g/mL; Cayman Chemical, Ann Arbor, Mich) to induce their full maturation. Mature DCs were harvested 48 hours after stimulation, washed twice, and used for T-cell stimulation assays. Mature DCs expressed more than 90% of CD80, CD83, CD86, and MHC-class II molecules as controlled by flow cytometry.

Purification of T cells

Autologous CD4⁺ T cells were obtained from PBMCs or PBMC-engrafted murine spleens using antibody-coated paramagnetic MultiSort MicroBeads (MACS; Miltenyi Biotec) according to the protocol of the manufacturer. After detaching, CD4⁺ T cells were stained with CD25 MicroBeads (Miltenyi Biotec) and CD4⁺CD25⁺ T cells were positively and CD4⁺CD25⁻ T cells were negatively selected. Separation was controlled by flow cytometry (purity > 98% CD4⁺ T cells, >98% CD4⁺CD25⁻ T cells, and >95% CD4⁺CD25⁺ T cells).

Activation of Treg cells and depletion of GARP-expressing Treg cells

Treg cells were activated with 0.5 μ g/mL anti-human CD3 mAb (UCHT1; R&D Systems, Wiesbaden, Germany) and 1 μ g/mL anti-human CD28 mAb (CD28.2; BD Biosciences, San Jose, Calif) or left untreated for 24 hours before injection into mice. For the experiments analyzing the effect of GARP, Treg cells were activated with autologous allergen-pulsed DCs in a ratio of 10:1 for 48 hours. Then, GARP-expressing cells among activated Treg cells were depleted by 1 μ g of mouse anti-human GARP mAb (7B11; BioLegend, San Diego, Calif) and anti-mouse Dynabeads (Invitrogen, Darmstadt, Germany). In some experiments, mice were also treated intraperitoneally with 10 μ g of 7B11 or 10 μ g of mouse IgG2b isotype control (MPC-11; BioLegend) at the time point of Treg-cell injection.

Reconstitution of mice with PBMCs in the presence or absence of Treg cells and rectal allergen challenge

Mice were injected intraperitoneally with 1×10^7 PBMCs in the presence or absence of Treg cells at a ratio of 1:10 or 1:20 (ie, 1×10^6 or 0.5×10^6 Treg cells) with or without the addition of the respective allergen (20 μ g) in 500 μ L 0.9% NaCl, and, in some experiments, with or without the addition of 4 μ g human recombinant GARP (LRRC32; R&D Systems, Wiesbaden, Germany). Eight days later, mice received an additional intraperitoneal allergen boost of the same amount of allergen in 200 μ L 0.9% NaCl. Control groups received allergen or NaCl alone. In therapeutic experiments, Treg cells were injected not from the beginning of PBMC engraftment but together with the allergen boost. On day 21, sera were collected and stored at -20°C until determination of human allergen-specific and total IgE level as well as human allergen-specific IgG and IgG₄ level by ImmunoCAP specific/total IgE and ImmunoCAP specific IgG/IgG₄ blood test (Thermo Fisher Scientific, Phadia GmbH). Then, mice were anesthetized and examined with a high-resolution video endoscopic "Coloview system" (Karl Storz, Tuttlingen, Germany) before and 2 hours after challenge with 20 μ g allergen extract in 100 μ L 0.9% NaCl rectally as described.^{18,19} Briefly, a 1.9-mm outer diameter mini-endoscope was introduced via the anus and the colon was carefully inflated with an air pump. Endoscopic pictures obtained are of high quality and allow the monitoring and grading of inflammation of the colon up to a depth of 6 cm. Thereafter, endoscopic scoring of 5 parameters (translucent structure, granularity, fibrin, vascularity, and stool) from 0 to 3, resulting in the overall score from 0 (no change) to 15 (severe symptoms), was performed.

Histological analysis of colon sections

Colon was removed and put into formalin for paraffin sections. Staining was performed with hematoxylin and eosin (Sigma-Aldrich, Taufkirchen,

Germany), or mouse anti-human CD45 (2B11 and PD7/26; DakoCytomation, Glostrup, Denmark), or mouse anti-human FoxP3 (236A/E7; Abcam, Cambridge, Mass) and visualized with the Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (DakoCytomation) according to the instructions of the manufacturer. Slides were analyzed with an Olympus BX40 microscope (Olympus, Hamburg, Germany) using the color view soft imaging system camera and software.

T-cell preparation and antigen restimulation *in vitro*

Two hours after rectal challenge and the subsequent endoscopy, mice were killed by means of cervical dislocation. Spleens were removed and dispersed through a 70- μ m nylon cell strainer (BD Biosciences, San Jose, Calif). After lysis of erythrocytes, cells were washed twice with PBS and pooled for isolation of human CD4⁺ T cells as described above. For proliferation or suppression assays, 1×10^5 CD4⁺ or CD4⁺CD25⁻ T cells were cocultured in 96-well plates (Greiner) in triplicates with 1×10^4 autologous unpulsed, allergen-pulsed, or tetanus toxoid-pulsed DCs in 200 μ L IMDM supplemented with 5% heat-inactivated autologous plasma. For suppression assays, 5×10^4 CD4⁺CD25⁺ Treg cells were further added to the culture. After 5 days, the cells were pulsed with 37 kBq/well of [³H]-thymidine (ICN, Irvine, Calif) for 6 hours, and [³H]-thymidine incorporation was evaluated in a beta counter (1205 Betaplate; LKB Wallac, Turku, Finland). For cytokine production assays, 5×10^5 CD4⁺ T cells were cocultured in 48-well plates in the presence of 5×10^4 autologous unpulsed, allergen-pulsed, or tetanus toxoid-pulsed DCs in 1 mL IMDM supplemented with 5% heat-inactivated autologous plasma. On day 7, T cells were restimulated with 5×10^4 newly generated DCs and supernatants were collected 24 hours later.

Flow cytometric analysis

Surface phenotyping was performed by staining 5×10^5 CD4⁺ human T cells, 5×10^5 splenocytes, or 5×10^4 human DCs with specific mAbs for 20 minutes at 4°C, washed, and analyzed by a FACSCalibur (BD Biosciences) equipped with CELLQuest Software. The following mAbs were used: fluorescein isothiocyanate-conjugated anti-human HLA-DR (L243), and anti-human CD3 (UCHT1); phycoerythrin-conjugated anti-human CD80 (L307.4), anti-human CD83 (HB15e), anti-human CD86 (2331(FUN-1)), anti-human CD4 (RPA-T4), and anti-human CD45 (HI30), allophycocyanin-conjugated anti-human CD19 (HIB19), and mouse and rat IgG isotype controls (all BD Biosciences); Alexa Fluor 647-conjugated anti-human CD4 (MT310; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif); allophycocyanin-conjugated anti-human CD8 (MEM-31; EuroBioSciences, Friesoythe, Germany); phycoerythrin-conjugated anti-human CD25 (4 E3, Miltenyi Biotec); fluorescein isothiocyanate-conjugated anti-human FoxP3 (PCH101; eBioscience, San Diego, Calif) and allophycocyanin-conjugated anti-human GARP (7B11; BioLegend).

Quantification of cytokine production by ELISA

Human IgG, IgG₄, IL-4, IL-10, IL-13, and IFN- γ were measured by ELISA according to the instructions of the manufacturer (BD Biosciences).

Statistics

ANOVA was used for analysis of variance between different experimental groups. Student 2-tailed *t* test and the Mann-Whitney *U* test were used to test the statistical significance of the results; *P* $\leq$.05 was considered significant.

RESULTS

Inhibition of human allergen-specific IgE production in PBMC-engrafted immunodeficient mice by coinjection of autologous Treg cells

Previously, we have developed a humanized mouse model of allergy and IgE-dependent allergen-induced gut inflammation in

immunodeficient NOD-*scid- γ* ^{-/-} mice by injection of PBMCs from highly sensitized allergic donors (allergen-specific IgE of at least ≥ 50 kU/L) together with the respective allergen or not. In this model, we could demonstrate that human allergen-specific IgE in murine sera was detectable only in PBMC plus allergen-treated mice while human total IgE was also present in PBMC-engrafted mice that were not treated with the respective allergen (Weigmann et al¹⁸ and Fig 1).

To analyze whether enhancement of Treg-cell frequency in these mice would inhibit allergen-specific IgE production and subsequent gut inflammation, we coinjected freshly isolated or *in vitro*-activated autologous CD4⁺CD25⁺FoxP3⁺ Treg cells together with PBMCs. Because the regulatory activity of Treg cells from allergic subjects is impaired in some donors,²⁰⁻²³ isolated Treg cells were primarily characterized by their capacity to suppress the proliferation of autologous CD4⁺CD25⁻ effector T cells stimulated with allergen-pulsed autologous DCs *in vitro*, and only those donors that had functionally active Treg cells were included into the study. The presence of a higher number of such functionally active Treg cells at a ratio of 1:10 significantly inhibited total as well as allergen-specific human IgE production. Activation of Treg cells with anti-CD3/CD28 mAb before injection further increased this inhibition (Fig 1). Besides IgE, the production of allergen-specific human IgG and IgG₄ was similarly reduced by coinjection of Treg cells while the total IgG and IgG₄ production was not affected (Fig 1).

Treg cells inhibit allergen-specific gut inflammation in human PBMC-engrafted NOD-*scid- γ* ^{-/-} mice

Next, we investigated the influence of Treg cells on allergen-induced gut inflammation, which can be initiated by rectal allergen challenge 3 weeks after the engraftment of PBMCs from allergic donors and measured by video mini-endoscopy evaluating translucent structure, granularity, fibrin formation, vascularity, and stool on the basis of a score ranging from 0 to 3, reaching a maximal score of 15. As already known from our previous study, no inflammation was observed in the colon before challenge.¹⁸ Two hours after rectal allergen challenge, all mice that had received PBMCs from allergic donors together with the respective allergen showed a significantly enhanced endoscopic score than did mice that had received no PBMCs or PBMCs without allergen (Fig 2, A and B). Injection of freshly isolated autologous Treg cells at a ratio of 1:10 together with PBMCs and the allergen significantly inhibited the inflammation of the colon. The inhibition was further enhanced by *in vitro* activation of Treg cells with anti-CD3/CD28 mAb especially at a lower Treg-cell ratio of 1:20 where nonactivated Treg cells did not significantly inhibit the inflammation (Fig 2, A-C).

Histology of the colon tissue showed early inflammatory changes such as mucosal hypertrophy and infiltration of the mucosa with mononuclear cells in PBMC plus allergen-treated mice, which was abolished by coinjection of Treg cells (Fig 2, A). Further immunohistologic staining revealed that infiltration of the colon with human CD45⁺ cells, mainly consisting of CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells, was also strongly inhibited by activated Treg cells, while the number of FoxP3⁺ cells was enhanced (Fig 2, D). Similarly, a reduced number of human CD45⁺, CD3⁺, CD4⁺, and CD8⁺ cells together with a significant increase in CD4⁺FoxP3⁺ cells in the presence of a higher number of Treg cells was observed in the spleen (Fig 2, E).

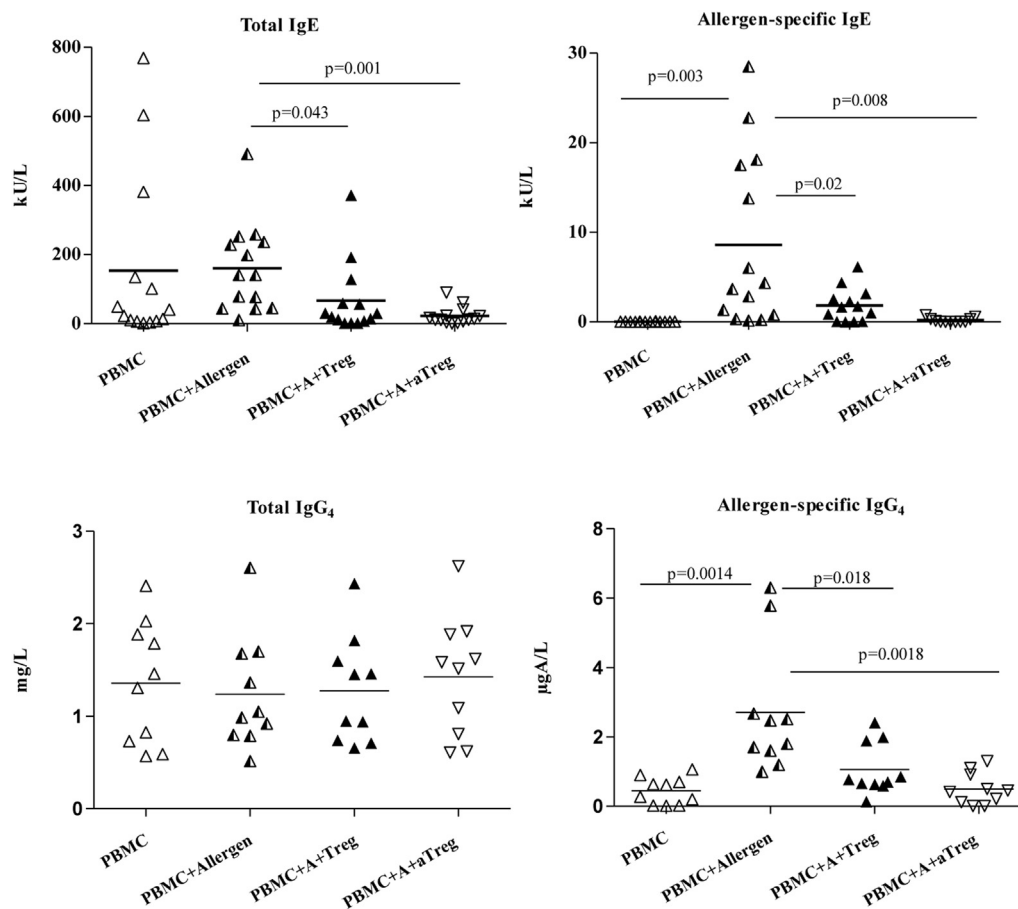


FIG 1. Inhibition of total and allergen-specific human IgE production in humanized NOD-scid- $\gamma c^{-/-}$ mice by coinjection of autologous Treg cells. 1×10^7 PBMCs were injected intraperitoneally with or without the respective allergen in the presence or absence of $(0.5-1) \times 10^6$ autologous CD4⁺CD25⁺ Treg cells. Three weeks later, total and allergen-specific human IgE and IgG₄ level was measured in murine sera by ImmunoCAP test or ELISA. Shown are the single values + mean of 14 (10 for IgG₄) experiments. A, Allergen; aTreg, activated Treg.

Coinjection of Treg cells inhibit allergen-specific proliferation and cytokine production by human CD4⁺ T cells recovered from the spleens of PBMC-engrafted mice

After the measurement of allergen-induced gut inflammation, human CD4⁺ T cells were isolated from murine spleens and restimulated *in vitro* with autologous DCs that were pulsed with the same allergen being present in the mice or with tetanus toxoid as control antigen. Freshly isolated CD4⁺ T cells from the same donor were used as control. Fig 3 shows allergen-specific proliferation and cytokine production (IL-4, IL-10, IL-13, and IFN- γ) of freshly isolated CD4⁺ T cells and of those that had been isolated from PBMC plus allergen-treated mice whereas CD4⁺ T cells from mice that had received no allergen remained unresponsive to allergen-pulsed DCs. Allergen-specific proliferation as well as production of IL-4, IL-13, and IFN- γ was inhibited by coinjection of autologous Treg cells at a ratio of 1:10 together with PBMCs plus allergen reaching significance using *in vitro* anti-CD3/CD28-activated Treg cells, while the production of IL-10 was not significantly altered. Stimulation with tetanus toxoid-pulsed DCs led to proliferation and cytokine production only in freshly isolated CD4⁺ T cells, implying that in the absence of

stimulation tetanus toxoid-specific cells do not survive in the mice or become anergic.

Inhibition of gut inflammation by activated Treg cells also occurs in a therapeutic setting and is abolished by the blockade of GARP

To analyze whether the administration of Treg cells at a later time point mimicking a therapeutic approach would still lead to an inhibition of allergen-specific gut inflammation, coinjection of *in vitro*-activated autologous Treg cells at a ratio of 1:20 was performed either on day 0 or during allergen boost 8 days after PBMC engraftment. In these sets of experiments, we further analyzed the effect of blockade of GARP, a protein that has recently been reported to be upregulated on activated Treg cells and to play an important role in their regulatory function.^{24,25} As we observed in preliminary kinetic experiments that the highest upregulation of GARP occurred 48 hours after the stimulation of CD4⁺ T cells with autologous allergen-pulsed DCs compared with anti-CD3/CD28 treatment (Fig 4, A), allergen-pulsed DC-activated Treg cells were used in these experiments. Of note, the suppression of allergen-induced gut

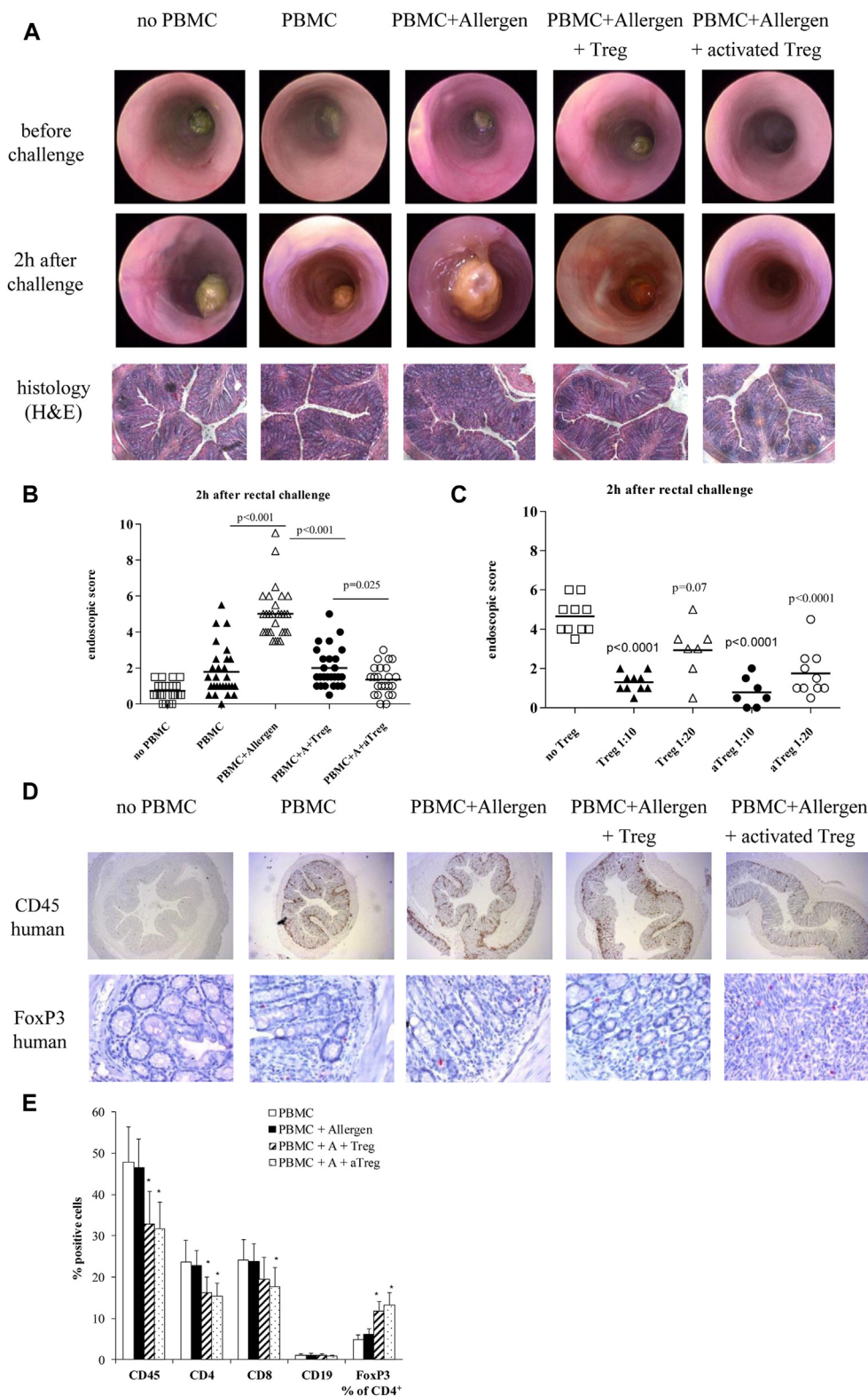


FIG 2. Allergen-specific gut inflammation in human PBMC-engrafted NOD-*scid*- γ γ ^{-/-} mice is inhibited by coinjection of autologous Treg cells. Three weeks after PBMC $\pm$ Treg-cell engraftment, mice were examined with a video mini-endoscope before and after rectal allergen challenge. Shown are the endoscopic pictures and the histology (magnification $\times 100$) of a representative result (**A**) and the single values + mean of the endoscopic score out of 14 (**B**) or 5 (**C**) experiments with generally 2 mice per group. **D**, Staining of the colon with anti-human CD45 (magnification $\times 40$) or anti-human FoxP3 (magnification $\times 400$) after rectal challenge. **E**, Staining of the spleen with the indicated markers, mean $\pm$ SEM of 13 experiments. A, Allergen; aTreg, activated Treg; H&E, hematoxylin and eosin. * $P \leq .05$ compared with PBMC + allergen.

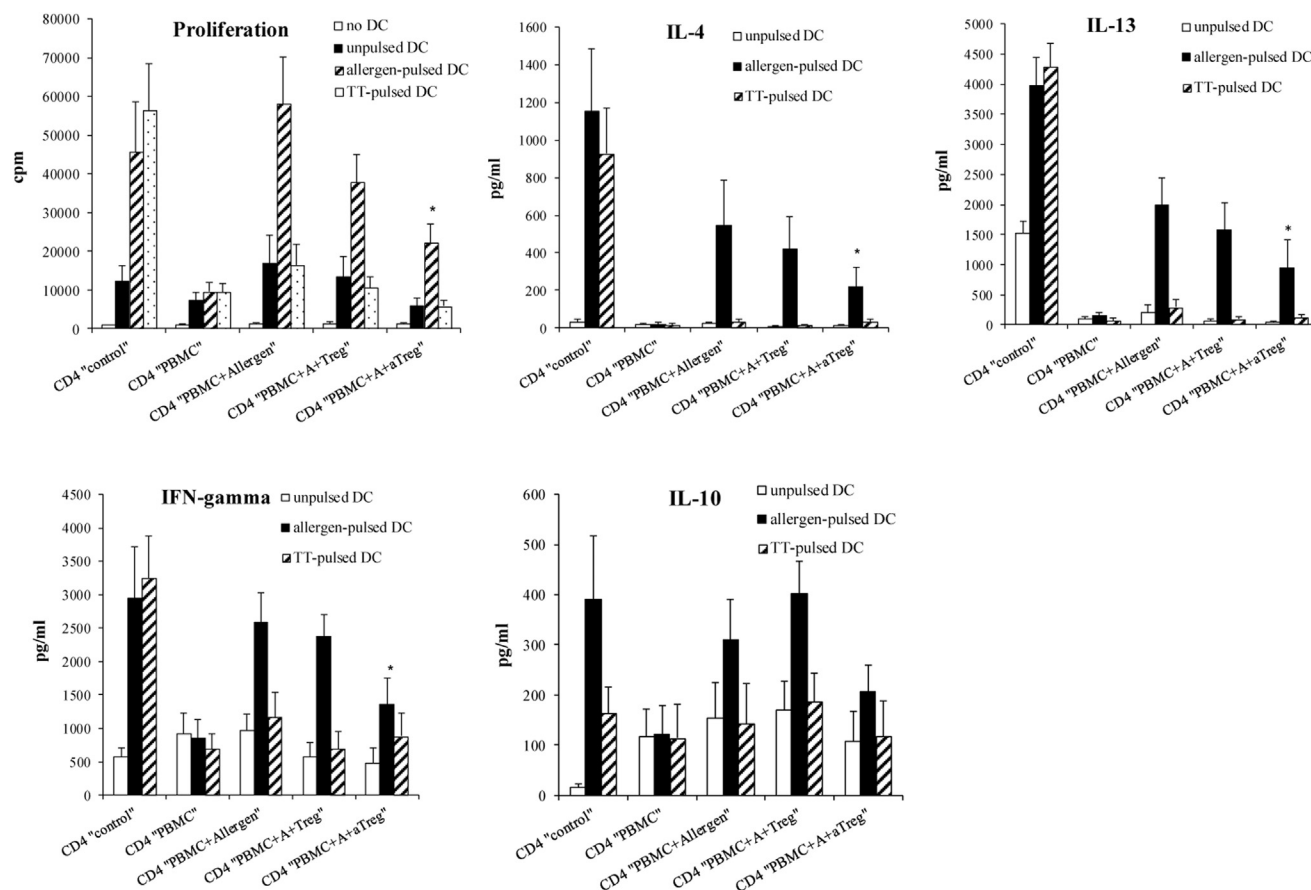


FIG 3. Coinjection of autologous Treg cells inhibits allergen-specific proliferation and cytokine production *in vitro*. Three weeks after PBMC $\pm$ Treg-cell engraftment, human CD4⁺ T cells were recovered from spleens and stimulated with autologous unpulsed (DC medium), allergen-pulsed (DC allergen), or tetanus toxoid-pulsed DC (DC TT) to analyze their proliferation and cytokine production. Shown is the mean $\pm$ SEM of 8 experiments. A, Allergen; aTreg, activated Treg. * $P \leq .05$ compared with PBMC + Allergen.

inflammation by allergen-pulsed DC-activated Treg cells was comparable to that of anti-CD3/CD28-activated Treg cells (Fig 4, B). Furthermore, depletion of GARP-expressing cells among allergen-pulsed DC-activated Treg cells using the anti-human GARP mAb 7B11 (Fig 4, C) significantly inhibited their suppressive activity *in vitro*, while depletion of GARP-expressing cells among anti-CD3/CD28-activated Treg cells could not completely restore T-effector cell proliferation (Fig 4, D).

In vivo depletion of GARP-expressing cells among DC-activated Treg cells before their injection also abolished their suppressive function on human total and allergen-specific IgE production in murine sera (Fig 5, A) as well as on subsequent allergen-induced gut inflammation (Fig 5, B and C). Importantly, in the therapeutic setting, allergen-induced inflammation of the intestine was also significantly inhibited by injection of DC-activated Treg cells, but somewhat less pronounced compared with the presence of Treg cells at the beginning of the experiment (Fig 5, B and C). The inhibition of gut inflammation was completely abrogated when the expression of human GARP was also blocked in the PBMC-engrafted mice by intraperitoneal injection of 7B11 (Fig 5, D). Again, hematoxylin and eosin staining of the colon tissues showed more early inflammatory changes and mononuclear cell infiltration in PBMC plus

allergen-treated mice receiving no Treg cells or GARP-depleted Treg cells (Fig 5, B).

Prevention of allergen-specific gut inflammation by administration of recombinant human GARP during PBMC engraftment

Because GARP was shown to have an anti-inflammatory effect on human CD4⁺ T cells *in vitro* and *in vivo* by enhancing Treg-cell activity,²⁶ we investigated whether the administration of GARP might be protective in our humanized mouse model of allergy, too. In Fig 6, A to C, it is demonstrated that coinjection of recombinant human GARP together with PBMC engraftment reduced the number of migrated CD45⁺ human cells in mice. Furthermore, an increased amount of FoxP3⁺ cells could be observed especially regarding the overall amount of human CD45⁺ cells, leading to an almost as strong prevention of human IgE production and allergen-induced gut inflammation as shown for activated Treg cells.

DISCUSSION

In the present study, we have analyzed the modulation of our recently developed human PBMC-engrafted murine model of

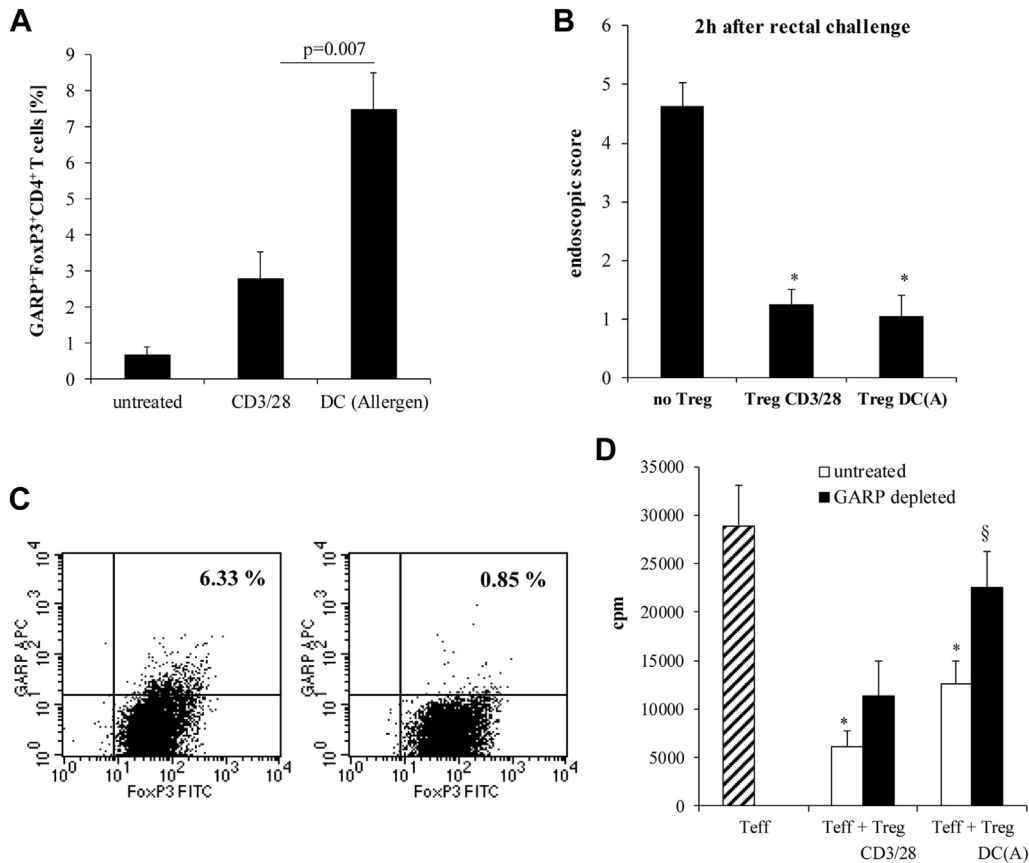


FIG 4. Comparison of GARP expression and the effect of its depletion on differently activated Treg cells. **A**, CD4⁺ T cells were activated with anti-human CD3/CD28 or allergen-pulsed DC for 48 hours. Shown is the mean $\pm$ SEM of FoxP3 and GARP expression of CD25⁺CD4⁺ T cells (n = 5). **B**, Endoscopic score of PBMC $\pm$ Treg-cell-engrafted mice after rectal allergen challenge. Shown is the mean $\pm$ SEM of 6 experiments, *P $\leq$.001 compared with no Treg cell. **C**, Staining of DC-activated Treg cells before (left panel) and after depletion of GARP (right panel). **D**, Suppression of DC-stimulated CD4⁺CD25⁺ effector T-cell (T_{eff}) proliferation by differentially activated Treg cells. Shown is the mean $\pm$ SEM of 4 experiments. A, Allergen; FITC, fluorescein isothiocyanate. *P $\leq$.01 compared with T_{eff}. §P $\leq$.05 compared with untreated Treg cells.

allergen-induced IgE-dependent inflammation of the intestine in immunodeficient NOD-*scid*- γ c^{-/-} mice by enhancing the frequency of autologous Treg cells. We could demonstrate that co-injection of autologous Treg cells at a ratio of at least 1:10 together with PBMCs from the beginning of the experiment as well as during allergen boost on day 8 significantly inhibited the production of human total and allergen-specific IgE among other immunoglobulins and the subsequent allergen-driven IgE-dependent gut inflammation. Furthermore, proliferation and cytokine production of human CD4⁺ T cells recovered from the spleens of PBMC-engrafted mice after restimulation with allergen-pulsed autologous DC was inhibited by the presence of higher Treg-cell numbers in the mice. Injection of Treg cells at ratios lower than 1:10 did not lead to a significant reduction in gut inflammation. However, when Treg cells were preactivated *in vitro*, either with anti-CD3/CD28 mAb or with allergen-pulsed autologous DCs, all inhibitory effects were enhanced, reaching significance even at a ratio of 1:20. Importantly, depletion of GARP-expressing cells, a recently defined marker on activated Treg cells,^{24,25} completely abrogated the inhibition of human allergen-specific IgE and subsequent IgE-

mediated intestinal inflammation. Conversely, administration of recombinant human GARP during PBMC engraftment increased the amount of Treg cells, and consequently prevented gut inflammation.

Our findings are in accordance with several studies analyzing the important role of Treg cells and IL-10-producing type 1 Treg cells for suppression of T_H2-mediated inflammation and the maintenance of immune tolerance to allergens either in animal models of allergy or in *in vitro* settings of allergen-specific human immune cells.^{5,20,27-30} Besides the inhibition of T_H2 effector cells, Treg cells or Treg-cell-derived cytokines contribute to a reduced mediator release by mast cells and basophils.^{1,30-33} Furthermore, it has been shown for a long time that both populations are able to prevent colitis induced in immunodeficient mice by transfer of pathogenic CD4⁺CD45RB(high) splenic T cells.³³⁻³⁶ More recently, the suppressive capacity of antigen-specific and polyclonal Treg cells in a mouse model of autoimmune gastritis has also been reported.^{37,38} In the study presented here, we demonstrate for the first time the inhibition of allergen-induced colitis by coinjection of autologous Treg cells or the Treg-cell-inducing protein GARP in a humanized mouse model

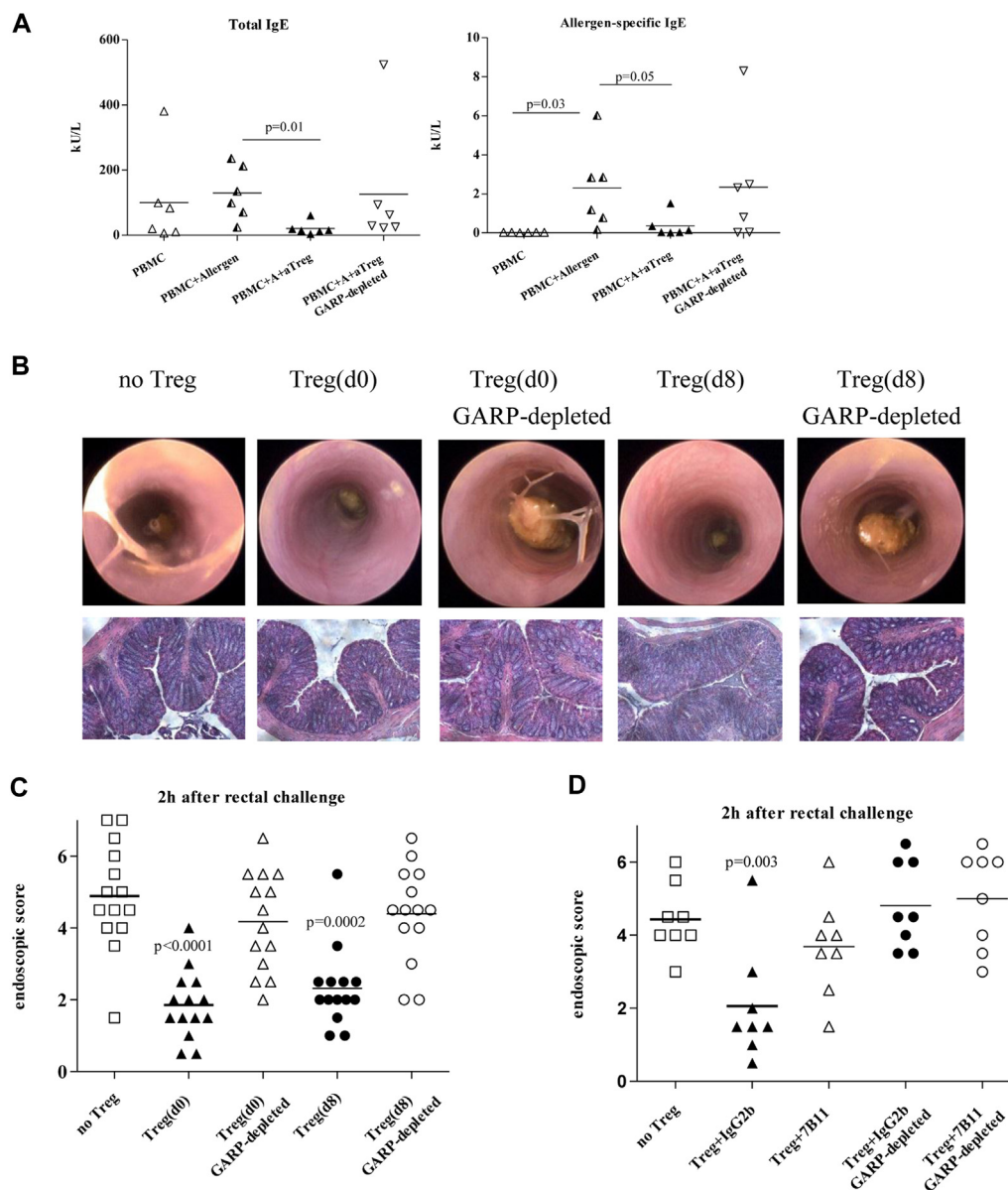


FIG 5. Depletion of GARP-expressing Treg cells after their activation abolishes inhibition of allergen-specific gut inflammation. Mice were treated with PBMCs $\pm$ activated Treg cells or GARP-depleted Treg cells on day 0 or during allergen boost on day 8 and challenged as described in Fig 2. Anti-human GARP mAb 7B11 or mouse IgG2b isotype control was also applied together with Treg-cell injection. Shown are the single values + mean of total and allergen-specific human IgE in murine sera out of 6 experiments (**A**), the endoscopic pictures and hematoxylin and eosin staining (magnification $\times 100$) of a representative result (**B**), and the single values + mean (**C** and **D**) of the endoscopic score out of 7 (4 for **D**) experiments. A, Allergen; aTreg, activated Treg.

of allergy. Humanized mouse models more closely recapitulate human biological systems and have been developed to circumvent the limitations of species-specific differences in the immune system between mice and men at least partially and therefore may allow a more predictable translation of the results into human clinical trials.¹⁴

Like in our previous *in vitro* studies,^{20,27} the inhibitory capacity of Treg cells was not antigen-specific because we observed no difference between anti-CD3/CD28-activated Treg cells or autologous allergen-pulsed DC-activated Treg cells (Fig 4, B) or Treg cells activated with DCs pulsed with

another allergen as used for injection into the mice (data not shown). Depletion of GARP-expressing cells, however, completely abrogated the inhibition, regardless of the Treg-cell activation condition.

In our humanized mouse model, allergen-specific pulmonary inflammation after intranasal allergen challenge could also be prevented by CD4-mediated activation of Treg cells via the HIV-1 surface protein gp120 published by us earlier.¹⁷ Activation of Treg cells by gp120 has formerly been shown in a xenogeneic graft-versus-host disease model in humanized mice, where a single dose of gp120 completely inhibited a lethal graft-versus-host

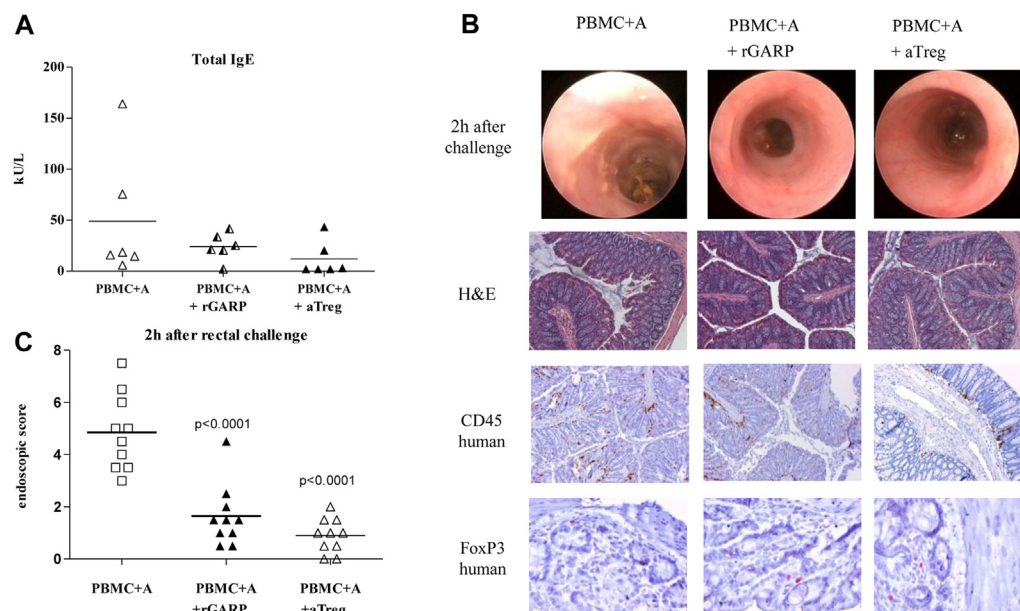


FIG 6. Administration of GARP enhances Treg-cell numbers and prevents allergen-specific gut inflammation. Mice were treated with PBMCs $\pm$ recombinant GARP or activated Treg cells and challenged as described in Fig 2. Shown are the single values + mean of total human IgE (**A**), the endoscopic pictures, hematoxylin and eosin (magnification $\times 100$), anti-human CD45 (magnification $\times 100$), and anti-human FoxP3 staining of the colon (magnification $\times 400$) of a representative result (**B**), and the single values + mean of the endoscopic score (**C**) out of 5 experiments. A, Allergen; aTreg, activated Treg; H&E, hematoxylin and eosin.

disease.³⁹ Accordingly, Hahn et al²⁶ have shown that repetitive administration of soluble GARP also prevented the onset of graft-versus-host disease in this model by enhancing Treg-cell and inhibiting T-effector cell activity, suggesting a critical role of GARP in the modulation of peripheral tolerance. This assumption could be confirmed by us as coinjection of recombinant GARP during PBMC engraftment protected from allergen-specific gut inflammation via Treg-cell induction, too. Moreover, depletion of GARP-positive cells on activated Treg cells completely restored the allergen-induced IgE-mediated inflammation of the intestine. To this end, we have previously demonstrated that inhibition of allergic airway reactivity by the chemokine CCL18 is associated with a strengthened migration of human GARP⁺ Treg cells into the lung.¹⁵

The finding that the blockade of GARP diminished the suppressive capacity of Treg cells might also be exploited in cancer therapy where Treg cells are responsible for ineffective antitumor immune responses.⁴⁰ Indeed, higher frequencies of GARP-expressing Treg cells have recently been found in patients with hepatocellular carcinoma being associated with impaired T-cell functionality.⁴¹

Taken together, our results indicate that Treg cells not only inhibit T_H2-driven inflammatory processes *in vitro* or in murine models of allergy *in vivo* (analyzing mainly airway hyperreactivity) but also suppress allergen-induced inflammation of the intestine in our recently developed humanized mouse model of allergy. Furthermore, it has become evident that the inhibitory function of Treg cells is dependent on their activation status characterized by the expression of GARP. Thus, activation of Treg cells by GARP, on the one hand, or blockade of GARP-expressing Treg cells, on the other hand, may represent promising tools in the treatment of allergy and autoimmunity or cancer.

We thank Mrs Claudia Braun and Mrs Ulrike Hilbig for excellent technical assistance.

Clinical implications: Allergen-induced IgE-mediated inflammation of the intestine is inhibited by activated GARP-expressing Treg cells, indicating that soluble GARP may be exploited for the treatment of allergy, while blocking of GARP may be useful for cancer immunotherapy.

REFERENCES

1. Akdis CA. Therapies for allergic inflammation: refining strategies to induce tolerance. *Nat Med* 2012;18:736-49.
2. Burks AW, Calderon MA, Casale T, Cox L, Demoly P, Jutel M, et al. Update on allergy immunotherapy: American Academy of Allergy, Asthma & Immunology/European Academy of Allergy and Clinical Immunology/PRACTALL consensus report. *J Allergy Clin Immunol* 2013;131:1288-96.
3. Sicherer SH, Leung DY. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects in 2012. *J Allergy Clin Immunol* 2013;131:55-66.
4. Takeda K, Gelfand EW. Mouse models of allergic diseases. *Curr Opin Immunol* 2009;21:660-5.
5. Wisniewski J, Agrawal R, Woodfolk JA. Mechanisms of tolerance induction in allergic disease: integrating current and emerging concepts. *Clin Exp Allergy* 2013;43:164-76.
6. Nguyen TH, Casale TB. Immune modulation for treatment of allergic disease. *Immunol Rev* 2011;242:258-71.
7. Wenzel S, Ford L, Pearlman D, Spector S, Sher L, Skobieranda F, et al. Dupilumab in persistent asthma with elevated eosinophil levels. *N Engl J Med* 2013;368:2455-66.
8. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, et al. Lebrikizumab treatment in adults with asthma. *N Engl J Med* 2011;365:1088-98.
9. Brehm MA, Jouvet N, Greiner DL, Shultz LD. Humanized mice for the study of infectious diseases. *Curr Opin Immunol* 2013;25:428-35.
10. Gagnon R, Boutin Y, Hebert J. Lol p I-specific IgE and IgG synthesis by peripheral blood mononuclear cells from atopic subjects in SCID mice. *J Allergy Clin Immunol* 1995;95:1268-75.

11. Herz U, Botchkarev VA, Paus R, Renz H. Increased airway responsiveness, allergy-type-I skin responses and systemic anaphylaxis in a humanized-severe combined immuno-deficiency mouse model. *Clin Exp Allergy* 2004;34:478-87.
12. Perros F, Hoogsteden HC, Coyle AJ, Lambrecht BN, Hammad H. Blockade of CCR4 in a humanized model of asthma reveals a critical role for DC-derived CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation. *Allergy* 2009;64:995-1002.
13. Sonar SS, Hsu YM, Conrad ML, Majeau GR, Kilic A, Garber E, et al. Antagonism of TIM-1 blocks the development of disease in a humanized mouse model of allergic asthma. *J Clin Invest* 2010;120:2767-81.
14. Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL. Humanized mice for immune system investigation: progress, promise and challenges. *Nat Rev Immunol* 2012;12:786-98.
15. King M, Pearson T, Shultz LD, Leif J, Bottino R, Trucco M, et al. A new Hu-PBL model for the study of human islet alloreactivity based on NOD-scid mice bearing a targeted mutation in the IL-2 receptor gamma chain gene. *Clin Immunol* 2008;126:303-14.
16. Bellinghausen I, Reuter S, Martin H, Maxeiner J, Luxemburger U, Tureci O, et al. Enhanced production of CCL18 by tolerogenic dendritic cells is associated with inhibition of allergic airway reactivity. *J Allergy Clin Immunol* 2012;130:1384-93.
17. Martin H, Reuter S, Dehzad N, Heinz A, Bellinghausen I, Saloga J, et al. CD4-mediated regulatory T-cell activation inhibits the development of disease in a humanized mouse model of allergic airway disease. *J Allergy Clin Immunol* 2012;129:521-8.
18. Weigmann B, Schughart N, Wiebe C, Sudowe S, Lehr HA, Jonuleit H, et al. Allergen-induced IgE-dependent gut inflammation in a human PBMC-engrafted murine model of allergy. *J Allergy Clin Immunol* 2012;129:1126-35.
19. Becker C, Fantini MC, Neurath MF. High resolution colonoscopy in live mice. *Nat Protoc* 2006;1:2900-4.
20. Bellinghausen I, Klostermann B, Knop J, Saloga J. Human CD4+CD25+ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. *J Allergy Clin Immunol* 2003;111:862-8.
21. Bellinghausen I, König B, Böttcher I, Knop J, Saloga J. Regulatory activity of human CD4+CD25+ T cells depends on allergen concentration, type of allergen and atopy status of the donor. *Immunology* 2005;116:103-11.
22. Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergics during birch pollen season. *Clin Exp Allergy* 2004;34:1364-72.
23. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, et al. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;363:608-15.
24. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, Unutmaz D. Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* 2009;106:13439-44.
25. Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D, Shevach EM. GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* 2009;106:13445-50.
26. Hahn SA, Stahl HF, Becker C, Correll A, Schneider FJ, Tuettenberg A, et al. Soluble GARP has potent antiinflammatory and immunomodulatory impact on human CD4(+) T cells. *Blood* 2013;122:1182-91.
27. Bellinghausen I, König B, Böttcher I, Knop J, Saloga J. Inhibition of human allergic T-helper type 2 immune responses by induced regulatory T cells requires the combination of interleukin-10-treated dendritic cells and transforming growth factor-beta for their induction. *Clin Exp Allergy* 2006;36:1546-55.
28. Maazi H, Shirinbak S, Willart M, Hammad HM, Cabanski M, Boon L, et al. Contribution of regulatory T cells to alleviation of experimental allergic asthma after specific immunotherapy. *Clin Exp Allergy* 2012;42:1519-28.
29. Pellerin L, Jenks JA, Begin P, Bacchetta R, Nadeau KC. Regulatory T cells and their roles in immune dysregulation and allergy. *Immunol Res* 2014;58:358-68.
30. Soyer OU, Akdis M, Ring J, Behrendt H, Cramer R, Lauener R, et al. Mechanisms of peripheral tolerance to allergens. *Allergy* 2013;68:161-70.
31. Gri G, Piconese S, Frossi B, Manfroi V, Merluzzi S, Tripodo C, et al. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity* 2008;29:771-81.
32. Pierkes M, Bellinghausen I, Hultsch T, Metz G, Knop J, Saloga J. Decreased release of histamine and sulfidoleukotrienes by human peripheral blood leukocytes after wasp venom immunotherapy is partially due to induction of IL-10 and IFN-gamma production of T cells. *J Allergy Clin Immunol* 1999;103:326-32.
33. Kanjarawi R, Dy M, Bardel E, Sparwasser T, Dubois B, Mecheri S, et al. Regulatory CD4+Foxp3+ T cells control the severity of anaphylaxis. *PLoS One* 2013;8:e69183.
34. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997;389:737-42.
35. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008;133:775-87.
36. Shale M, Schiering C, Powrie F. CD4(+) T-cell subsets in intestinal inflammation. *Immunol Rev* 2013;252:164-82.
37. Stummvoll GH, DiPaolo RJ, Huter EN, Davidson TS, Glass D, Ward JM, et al. Th1, Th2, and Th17 effector T cell-induced autoimmune gastritis differs in pathological pattern and in susceptibility to suppression by regulatory T cells. *J Immunol* 2008;181:1908-16.
38. Huter EN, Stummvoll GH, DiPaolo RJ, Glass DD, Shevach EM. Pre-differentiated Th1 and Th17 effector T cells in autoimmune gastritis: Ag-specific regulatory T cells are more potent suppressors than polyclonal regulatory T cells. *Int Immunopharmacol* 2009;9:540-5.
39. Becker C, Taube C, Bopp T, Becker C, Michel K, Kubach J, et al. Protection from graft-versus-host disease by HIV-1 envelope protein gp120-mediated activation of human CD4+CD25+ regulatory T cells. *Blood* 2009;114:1263-9.
40. Nishikawa H, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Curr Opin Immunol* 2014;27:1-7.
41. Kalathil S, Lugade AA, Miller A, Iyer R, Thanavala Y. Higher frequencies of GARP(+)-CTLA-4(+)-Foxp3(+) T regulatory cells and myeloid-derived suppressor cells in hepatocellular carcinoma patients are associated with impaired T-cell functionality. *Cancer Res* 2013;73:2435-44.