GARP Dampens Cancer Immunity by Sustaining Function and Accumulation of Regulatory T Cells in the Colon

Mohammad Salem1, Caroline Wallace1, Maria Velegraki1, Anqi Li1, Ephraim Ansa-Addo1, Alessandra Metelli1, Hyunwoo Kwon1, Brian Riesenberg1, Bill Wu1, Yongliang Zhang1, Silvia Guglietta1, Shaoli Sun2, Bei Liu1, and Zihai Li1,3

Abstract

Activated regulatory T (Treg) cells express the surface receptor glycoprotein-A repetitions predominant (GARP), which binds and activates latent TGFβ. How GARP modulates Treg function in inflammation and cancer remains unclear. Here we demonstrate that loss of GARP in Treg cells leads to spontaneous inflammation with highly activated CD4+ and CD8+ T cells and development of enteritis. Treg cells lacking GARP were unable to suppress pathogenic T-cell responses in multiple models of inflammation, including T-cell transfer colitis. GARP-/- Treg cells were significantly reduced in the gut and exhibited a reduction in CD103 expression, a colon-specific migratory marker. In the colitis-associated colon cancer model, GARP on Treg cells dampened immune surveillance, and mice with GARP-/- Treg cells exhibited improved antitumor immunity. Thus, GARP empowers the functionality of Treg cells and their tissue-specific accumulation, highlighting the importance of cell surface TGFβ in Treg function and GARP as a potential therapeutic target for colorectal cancer therapy.

Significance: These findings uncover functions of membrane-bound TGFβ and GARP that tune the activity of Treg cells, highlighting a potential treatment strategy in autoimmune diseases and cancer.

Introduction

To maintain immune homeostasis and uphold tolerance to self-antigens, CD4+ regulatory T (Treg) cells spearhead the adjustment of the immune responses generated by other effector T-cell populations (1). Treg cells are a potent suppressive cell subset that is classically characterized by expression of the master regulatory transcription factor forkhead box protein 3 (Foxp3; ref. 2). Loss-of-function mutations in Foxp3 gene lead to a severe systemic autoimmune disorder characterized by fatal tissue and organ damage (3). In cancer, increased numbers of infiltrating Treg cells into tumor microenvironments suppress antitumor responses and have been clinically associated with poor prognosis (4). Consequently, identifying possible mechanisms that would allow the modulation of Tregs function is urgently needed.

TGFβ is a preeminent cytokine with critical immunomodulatory roles (5). TGFβ is highly implicated in the induction, development, and maintenance of Treg cells. Mice with global TGFβ deletion develop a severe, generalized autoimmune disorder and die within 3–4 weeks after birth (6). Mice lacking TGFβ receptor II on T cells showed similar disease characteristics with aberrant T-cell activation and increased Th1 and Th2 cell populations (7), which suggests that TGFβ plays an important role in immune surveillance and suppression of effector T cells. Lack of TGFβ receptor on CD4+ T cells leads to disappearance of thymic Treg cells during postnatal days 3–5 (8). Moreover, peripheral Tregs are significantly reduced in numbers compared with thymic Tregs in 8- to 10-day-old TGFβ-deficient mice (9). In fact, TGFβ is required to generate peripheral Treg cells (10) by activating the canonical TGFβ/SMADs signaling; mice with Smad2 and Smad3 deletions on CD4+ T cells also developed severe autoimmune disease with reduced Foxp3 expression in the peripheral CD4+ T cells (11). Furthermore, Treg cells with deficient TGFβ signaling failed to home to the inflammatory sites in a T-cell transfer colitis model (12). Despite the increased knowledge of how TGFβ influences the suppressive function of Treg cells, underlying mechanisms that regulate the bioavailability and activation of TGFβ on Treg cells remain unclear.

Glycoprotein-A repetitions predominant (GARP), a transmembrane protein encoded by the gene Leucine rich repeat containing protein 32 (Lrrc32), is a cell surface docking receptor for latent TGFβ, a tetra-peptide complex formed by the TGFβ dimer and two copies of latency-associated peptide (LAP; ref. 13). The release of the biologically active mature TGFβ from this complex can occur by several factors including heat, acidic conditions, and integrons (14). Earlier reports have shown that GARP-deficient CD4+ T cells mice did not impair the suppressive function of Treg cells in vitro (15) and that silencing of its expression by RNA interference does not significantly affect Foxp3 expression in expanded...
GARP can have a beneficial effect in sustaining Treg differentiation in xenogeneic Graft-versus-Host disease (17). Moreover, a mAb directed against the GARP/latent TGFβ complex blocked Treg cell-mediated TGFβ production in the same mouse model (18). However, the roles of GARP in Treg development, lineage stability, and function have not been completely elucidated.

To address the above question, we generated Treg-specific GARP knockout mice and found that GARP plays an important role in immune homeostasis and aged mice with GARP-/- Treg cells develop spontaneous intestinal inflammation. Tregs lacking GARP show reduced ability to suppress inflammatory responses and less accumulation in the intestinal tract. Using in vitro and in vivo settings, we found that GARP modulates the expression of Foxp3, an important molecule that is involved in homing of the T cells to the gut. As a result, deletion of GARP on Treg cells significantly improved the antitumor immunity against colorectal cancer. Overall, our data established that GARP plays an important role in empowering Treg cell function and promoting their accumulation in the colon.

Materials and Methods

Animals

Lrc32(2lox)Foxp3(YFP)-Cre+ mice were generated in house. Lrc32(2lox) mice were obtained from Riken (15). Foxp3(2lox)Cre-, and Rag2(-/-) mice were from Jackson Laboratory. Inducible GARP OE and global GARP deletion (R26-creERT2-GARP) mice were described previously (19, 20). GARP OE was induced with doxycycline (50 μg/ml; Sigma) in 1% sucrose drinking water. GARP deletion in R26-creERT2-GARP cells was induced in vitro using 250 nmol/L 4-Hydroxytamoxifen (4-HT). All mice were on a pure C57BL6/J background. The animal procedures were conducted on an approved protocol by the Medical University of South Carolina Institutional Animal Care and Use Committee.

Isolation of immune cells from tissues

Thymus, spleen, mesenteric lymph nodes (mLNs), and peripheral lymph nodes (pLN)s, were dissociated into a single-cell suspension and RBC lysis buffer (Sigma) was used to remove red blood cells. For colons, tissues were dissected and incubated for 30 minutes at 37°C with collagenase D (1 mg/ml; Roche), dispase (0.05 U/ml; Worthington), and DNase I (100 mg/ml; Sigma-Aldrich). Lymphocytes were collected from the interface of a 40%/80% Percoll gradient (GE Healthcare).

Flow cytometry

For surface staining, after Fixable Viability Dye (Affymetrix) and Fc-receptor blocking, cells were stained for surface markers. Antibodies against mouse CCR9 (eBioCW-1.2), CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70), CD44 (IM7), CD62L (MEL-14), CTLA-4 (UC10-4B9), Foxp3 (FJK-16), GARP (YGIC86), GILT (DTA-1), GL7 (GL7), and KLRG1 (2F1) were from Thermo Fisher Scientific. Those recognizing, CCR2 (48607), CD25 (PC61), Gr1 (RB6-8C5), ICOS (7E.L7G9), IFNy (XMG1.2), K67 (SolA15), and TNF (MP6-XT22) were from BD Biosciences. B220 (RA3-6B2) antibody was purchased from BioLegend. Antibodies against human CD4 (RPA-T4), CD25 (M-A251), and CD103 (Ber-ACT8) were purchased from BD Biosciences. Antibodies against human Foxp3 (236A/E7) and GARP (7B11) were purchased from eBioscience and BioLegend, respectively.

For intracellular staining for transcription factors, Foxp3/Transcription Factor Staining Buffer Set (Affymetrix) was used. To assess the expression of the intracellular cytokines, cells were stimulated for 2 hours with 50 ng/ml PMA (phorbol 12-myristate-13-acetate) and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of 5 μg/ml brefeldin A (BD Biosciences). The staining was then performed using BD Cytofix/Cytoperm Kit according to the manufacturer’s protocol (BD Biosciences). Samples were analyzed immediately on BD FACS DIVa, and data analysis was performed using FlowJo Software (Tree Star).

Generation of in vitro-inducible Treg cells and anti-GARP treatment

CD4^+CD25^+T cells were isolated from 8- to 10-week-old WT or if/cre^+ mice or from peripheral blood mononuclear cells of healthy individuals using MACS columns (Miltenyi Biotec). A total of 2 × 10^6 mouse or 4 × 10^5 human cells were seeded per well in a 24- or 96-well plate, respectively, and stimulated with 5 μg/ml of plate-bound anti-CD3 (145-2C11; BD Biosciences) in the presence of 5 × 10^8 Rag2(-/-) splenocytes. WT and GARP-/- Treg cells were added in 1:1 to 1:8 Treg/Teff cell ratio. Activated CD4^+CD25^-T cells only, without Treg cells, were used as a positive control for T-cell proliferation. Three days poststimulation, CFSE dilutions of T cells were analyzed and quantified by flow cytometry.

Lupus induction

Female WT and if/cre^+ mice were given a single intraperitoneal injection of pristane (500 μl/20 g mouse; Sigma). Mice were sacrificed after 4 months and peripheral blood, spleen, and lung were collected for further analysis.

T-cell transfer colitis

Naive T cells (CD4^+CD45RB^hiCD25^-) from WT mice were sorted using FACSAria II (BD Biosciences). A total of 3.5 × 10^6 CD4^+CD45RB^hiCD25^- T cells per mouse, alone or in combination with 1.5 × 10^6 WT or GARP-/- Treg cells, were intraperitoneally injected into Rag2(-/-) mice. After T-cell reconstitution, mice were weighed weekly and monitored for signs of disease. At the end of the experiment, mice were sacrificed and their organs were examined histologically and by flow cytometry for evidence of colitis and inflammation. Standard hematoxylin and eosin-stained sections were examined and scored by an experienced pathologist (S. Sun) in a blinded fashion. Grading was based on...
acute inflammation as absent (0), minimal (1), mild (2), moderate (3), or severe (4).

Colitis-associated colon cancer
A total of 7–12 mice per group were given 12.5 mg/kg body weight azoxymethane (AOM) with intraperitoneal injection on day 1. Dextran sodium sulfate (DSS) was added to drinking water was given at 2.5% for 5 days in weeks 2 and 5, and then at 2% for 4 days on week 8. Mice were weighed and monitored weekly for overall health. Mice were euthanized at 12 weeks. Tumors were counted and measured. Tumor-infiltrating lymphocytes (TIL) were isolated by collagenase D (Sigma) digestion followed by Histopaque-1083 (Sigma) mediated density separation.

Generation of human GARP-Jurkat cells
Human GARP coding sequence was inserted into the BLR retroviral vector via BglII and EcoRI sites (Addgene). The retrovirus was generated in Platinum-A cells (Cell Biolabs) and then transduced into Jurkat cell. Human GARP-Jurkat cells were further selected by blasticidin treatment (Invivogen).

ELISA
For active and total TGFβ, ELISA plates (Corning) were coated overnight at 4°C with anti-mouse/human TGFβ1 capture antibody (BioLegend), followed by blocking with 1% BSA in PBS for 2 hours at room temperature. Samples were treated with HCl for 10 minutes and neutralized with Tris/NaOH to obtain total TGFβ. Both active and total TGFβ were detected using anti-mouse Biotin-TGFβ Antibody (BioLegend), Streptavidin-HRP (BioLegend), and TMB Substrate reagents (BD Biosciences).

Generation of human GARP-Jurkat cells
For bacteria-specific IgA, a fecal supernatant from μMT mice was used as shown in ref. 20 and the protein concentration was measured using Bradford assay (Bio-Rad). Each well of the ELISA plate was coated with 0.5 μg/ml μMT fecal supernatant. Serum was serially diluted and bacteria-specific IgA was detected with anti-mouse IgA-HRP antibody (Southern Biotech).

RNA isolation and quantitative RT-PCR
Total RNA was isolated using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and analyzed by quantitative PCR on StepOne Plus machine (Applied Biosystems) using SYBR Green reagent (Bio Rad) and analyzed by quantitative PCR on StepOne Plus machine (Applied Biosystems) using SYBR Green reagent (Bio Rad) and the following primers: human CD103 forward, 5’-TGAGCTGCGTTGACATGTC-3’; human CD103 reverse, 5’-GAGTGACAGCTGCGTTGACATGTC-3’; human β-actin forward, 5’-CCCTGGACATCTGCCAGAA-3’; human β-actin reverse, 5’-CCAGGAAAGGAGCGCTGGA-3’.

Detection of ANA
NOVA Lite HEp-2–coated slides (INOVA Diagnostics) were incubated with diluted sera (in PBS), followed by FITC-conjugated goat anti-mouse IgG or anti-mouse IgM antibody (Thermo Fisher Scientific), and examined by fluorescence microscopy. The fluorescence staining intensity was graded as 0 (negative), 1 (slight staining), 2 (moderate staining), and 3 (bright staining).

Histology
Mouse organs were fixed in 4% formalin, embedded in paraffin, sectioned, and stained following standard hematoxylin and eosin staining protocol.

Statistical analysis
Data analysis was performed using GraphPad Prism software (GraphPad Software, Inc.). Results are expressed as mean ± SEM. P values were determined with Student t test. Two-way ANOVA with Sidak multiple comparison test was used for multiple group comparisons in tumor curves and body weight graphs. P < 0.05 was considered statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.005).

Results
Treg-specific GARP deletion leads to chronic intestinal inflammation
To directly determine the role of GARP in Treg cell–mediated homeostasis, we generated mice with specific deletion of GARP on Treg cells by crossing mice with a conditional allele of Lmtc32 with mice expressing Fopp35W.Cre (Lmtc32 flox/flox if/ if x Fopp35W.Cre mice, herein called if/ if cre− mice). TCR activation of CD4+CD25+ Treg cells increased GARP levels (Supplementary Fig. S1A and S1B), in accordance with what has previously reported, that GARP selectively identifies activated Treg cells (16). Young if/ if cre− mice (4–6 weeks old) developed normally with no apparent changes in the systemic serum levels of active and total TGFβ or any key cytokines including IL2, IL6, TNF, and IFNγ (Supplementary Fig. S2A). The percentage of splenic CD4+ and CD8+ T cells was lower in if/ if cre− compared with WT mice. No changes in frequency were observed in the thymic single CD4+ or CD8+ T-cell populations (Fig. 1A). Notably, the frequencies of CD4+ Fopp35W+ T cells were elevated in both thymus and spleen of if/ if cre− mice (Fig. 1B and C). CD4+ and CD8+ T cells displayed a more activated effector-like phenotype with an increase in the CD44+CD62L− population in if/ if cre− mice (Fig. 1D). Histologically, slight increases in lymphocyte infiltration were detected in the liver, and the lung (Supplementary Fig. S2B). In addition, the villi in the small intestine were slightly shorter (Supplementary Fig. S2B). Moreover, IgG antinuclear antibodies (ANA) in the serum were also increased in the KO mice (Supplementary Fig. S2C).

To further investigate whether these immune alterations predispose the mice to the development of a chronic autoimmune disease, the mice were aged to one year. We observed an increased frequency of CD4+ T cells in both colon (not significant) and mLN, and as well as an increased CD8+ T-cell percentage in the spleen (Fig. 1E). We also found that aged if/ if cre− mice had significantly higher levels of bacteria-specific IgA in the serum, suggesting increased gut permeability due to loss of gut tolerance (Fig. 1F). Histologically, we noted aberrant villi structure (thick, short, and fused) in small intestine of if/ if cre− mice, which reflects partial villous atrophy that is known to manifest in patients with celiac or other inflammatory bowel diseases (Fig. 1G; ref. 21). Thus, lack of GARP on Treg cells resulted in disruption of immune homeostasis and made them incapable of maintaining intestinal homeostasis with aging, suggesting that GARP might play a key role in maintaining Treg-mediated immune regulation.

GARP is important for optimal Treg differentiation and controlling lupus-mediated inflammation
Our data suggest that GARP deletion in Treg cells might affect their suppressive function. Using an in vitro suppression assay, we confirmed Edwards and colleagues data (15) that the lack of
Figure 1. Deletion of GARP in Foxp3⁺ T cells leads to systemic inflammation. A, Percentage of CD4⁺ and CD8⁺ T cells in thymus and spleen of 6- to 8-week-old WT and f/f cre mice. B, Representative flow cytometry plots of CD4⁺Foxp3⁺ T cells in thymus and spleen. C, Quantification of percentages of CD4⁺Foxp3⁺ T cells in B. D, Representative flow cytometric plots and quantification of splenic CD4⁺ and CD62L⁺ T cells based on CD44 and CD62L expression. E, Percentage of CD4⁺ and CD8⁺ T cells in spleen, colon, and mLNs from 1-year-old WT and f/f cre mice (n = 5) was determined by flow cytometry and quantified. F, Gut bacteria-specific IgA was measured in the sera of mice by ELISA (n = 5–6). G, The small intestine of WT and f/f cre mice were stained with hematoxylin and eosin. Arrows, stunted and fused villi. Statistical analyses were performed by unpaired Student t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Error bars, mean ± SEM.
GARP on Treg cells did not alter their suppressive capacity on CD4$^+$ T cells (Supplementary Fig. S3A). However, in vitro Treg suppression assays do not necessarily recapitulate in vivo processes. When we tested the expression of different Treg markers gated on CD4$^+$Foxp3$^+$ cells, we found that CD25 (Fig. 2A), ICOS, and KLRG1 (Supplementary Fig. S3B and S3C) expression were significantly lower in GARP$^{-/-}$ Treg cells compared with WT cells after 72-hour TCR stimulation. In fact, TGFβ signaling has been shown to induce the expression CD25 (22). KLRG1 defines terminally differentiated Treg cells and is linked to IL2ra (CD25) (23). On the other hand, the expression of GITR (24) and CTLA-4 was unchanged (Supplementary Fig. S3D and S3E). In addition, mice lacking GARP in Treg cells showed increased frequency of Foxp3$^+$Helios$^+$ T cells (Fig. 2B), typically defined as thymic Treg cells (25), indicating a possible dysregulation in the generation of peripheral Treg cells, which are TGFβ-dependent (10). Indeed, the frequency of differentiated iTreg cells from primary CD4$^+$CD25$^-$ T cells was significantly less in the absence of GARP (Supplementary Fig. S3F). These data suggest that GARP may affect the function of Treg cells.

To this end and based on data showing increased systemic ANA level in aged if/cre$^+$ mice (Supplementary Fig. S2C), we hypothesized that mice with GARP$^{-/-}$ Treg cells might be less suppressive under chronic inflammatory conditions such as systemic lupus erythematosus. Therefore, we employed a pristane-induced lupus model, which is a result of induction and proliferation of autoreactive lymphocytes that produce proinflammatory cytokines, pathogenic autoantibodies, and immunocomplexes (20, 26). We observed that deletion of GARP in Treg cells leads to decreased ability to control the disease. Indeed, several hallmarks of autoimmunity, such as lymphocytopenia, systemic IgG, and elevated GL7$^+$ germinal center B cells levels were more prominent in if/cre$^+$ than WT mice (Fig. 2C). In addition, immune cell infiltration into the lungs was more severe in if/cre$^+$ mice (Fig. 2D). These data imply that GARP is crucial for Treg cells to exert their regulatory function during inflammation.

GARP deletion on Treg cells abrogates their ability to suppress T-cell responses during inflammation

Given that the intestinal compartment was clearly affected in mice lacking GARP on Treg cells, we hypothesized that Treg-GARP...
might play an important role in managing immunopathology during ongoing intestinal inflammation. To address this possibility, we tested their ability to suppress inflammatory responses in the intestine using a T-cell transfer model of colitis. CD4+CD45RBhiCD25- T cells from WT mice were injected intraperitoneally together with either WT or GARP-/- Treg cells into RAG2-/- mice. Functional Treg cells have been shown to reverse the disease in this mouse model (27). As anticipated, the control group of RAG2-/- mice injected only with CD4+CD45RBhiCD25- T cells without Tregs (Fig. 3A) showed severe body weight loss (Fig. 3B). Interestingly, GARP-/- Treg cell cotransfer completely failed to control the disease in the recipient mice, which showed significant weight loss (Fig. 3B). The histologic score was also indistinguishable from control mice that did not receive Treg cells (Fig. 3C). This was associated with increased CD4+ T cells in the colon (Fig. 3D). Notably, the frequency of GARP-/- Treg cells in the colon was significantly lower compared with WT Treg cells (Fig. 3E). As expected, this was associated with increased TNFα, IFNγ, CD4+ T cells (Fig. 3F), tissue inflammatory monocytes/macrophages (CD11b+Gr1hi), and neutrophils (CD11b+Gr1hi) (Fig. 3G; ref. 28). These data clearly demonstrate that GARP is important for the suppressive function of Tregs and may additionally affect their colonic accumulation.

GARP+/+ Treg cells fail to accumulate in the colon

To further assess whether GARP expression could modulate CD103 expression, mRNA level of CD103 was determined in Jurkat human T cells with GARP overexpression (OE). We found that CD103 mRNA levels were higher in GARP OE compared with WT Jurkat cells (Fig. 5A). Next, we tested whether in vitro GARP OE can also modify CD103 expression. To this end, we employed a doxycycline-inducible GARP OE mouse model where a Tet-on element is knocked into the GARP promoter (19, 20). After 6 weeks of doxycycline treatment, the mice were analyzed and GARP expression was confirmed to be upregulated in the thymic and splenic CD4+ and CD8+ T cells (Fig. 5B). No differences were observed in the proportion of thymic Treg cells or their CD103 expression (Fig. 5C). Notably, the frequencies of CD4+Foxp3+ and CD103+Foxp3+ T cells were more abundant in the periphery of the GARP OE mice, especially in the spleen and the colon (Fig. 5D–F). To confirm the modulatory role of GARP on CD103, we used another mouse model for inducible global GARP deletion (ROSA26-creERT2-GARP). Splenocytes were isolated from ERT2-cre+ and GARP deletion was induced by 4-HT treatment in vitro (Supplementary Fig. S4A). The percentage of CD103+ cells was reduced in ERT2-cre+ Tregs after 6 days of 4-HT treatment, following GARP deletion. (Supplementary Fig. S4B). As expected, no modulation of either GARP or CD103 was observed in control cells after 4-HT treatment.

To validate that in human setting, in vitro–induced human Treg cells were generated from peripheral blood CD4+CD25- cells from four healthy subjects and treated with a pool of polyclonal mouse anti-human GARP antibody made in our own laboratory (at 10 μg/mL each) or their isotype control for up to 10 days. As an additional control, cells were also treated with 10 μg/mL of neutralizing anti-TGFβ antibody. The media were replenished with fresh antibody-containing media every 2 days. Both the percentage of CD103+ Treg cells and their expression level of CD103 were significantly decreased after 8 and 10 days of either GARP or anti-TGFβ antibody treatment compared with treatment with the isotype antibody for the same period of time (Fig. 5G). These data further support the modulatory function of GARP on CD103 expression.

Deletion of GARP in Treg cells leads to reduced colitis-associated colon cancer development

We next investigate the impact of GARP on the colonic accumulation of Treg cells in the context of colorectal tumor. WT and if/cre+ littermates were subjected to colitis-associated colon cancer (CAC) based on a combination of the mutagenic agent AOM and DSS (32). CAC was induced by injecting a single dose of AOM, followed by three cycles of DSS administration (Fig. 6A). The mice were sacrificed at week 12 after initial AOM injection. No weight difference was observed between WT and if/cre+ mice (Fig. 6B). While no changes were observed in splenocyte cell counts (Fig. 6C), the cell numbers were significantly increased in the mLNs of if/cre+ mice (Fig. 6D). Interestingly, mice with GARP deletion in Treg cells developed fewer tumors and the tumor load in these mice was significantly reduced compared with the WT mice (Fig. 6E). Notably, this was associated with a significant increase in the percentage of CD4+ T cells, but not CD8+ T cells, within the tumor-infiltrating lymphocytes (TIL) population (Fig. 6F). Indeed, TILs from if/cre+ mice had a significant decrease in the percentage of Treg cells compared with control littermates (Fig. 6G). Functional analysis of TILs also showed that both CD4+ and CD8+ T cells had higher TNFα expression (Fig. 6H). Altogether, these data are in accordance with the aforementioned results that GARP improves the accumulation of Tregs in the gut.

To further confirm the association of GARP/CD103 and colonic Treg cells, MC38 colon cancer cells were implanted subcutaneously into the flanks of WT and if/cre+ mice. The
Figure 3.
GARP Expression is essential for Treg cell–suppressive function during intestinal inflammation. 

A, Experimental outline. Rag2−/− mice received 3.5 × 10⁵ WT or GARP−/− Treg cells (CD4⁺CD25⁺) T cells). B, Body weight trend of the RAG2−/− recipient mice upon T-cell transfer of WT or GARP−/− T reg cells (5–6 mice in each group are displayed; mean ± SEM; *, P < 0.05, two-way ANOVA with multiple comparison). C, Representative hematoxylin and eosin staining of colon tissue section and their histology score based on acute inflammation as absent (0), minimal (1), mild (2), moderate (3), or severe (4). D, Total cell number of colonic CD4⁺ T cells. E, Representative dot plots and cumulative frequency data of WT and GARP−/− colonic CD4⁺Foxp3⁺ T cells. F, Representative dot plots and bar graphs showing percentage and number of TNFα⁺ and IFNγ⁺ CD4⁺ T cells in the colon. G, Number of monocytes/macrophages (CD11b⁺Gr1int) and neutrophils (CD11b⁺Gr1hi). Each point represents an individual mouse. The results are representative of two independent experiments with 5–6 mice in each group. Statistical analysis performed by unpaired Student t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Error bars, mean ± SEM.
tumor growth was similar (Supplementary Fig. S5A) with no changes in tumor-infiltrating CD4⁺, CD8⁺, or CD4⁺Foxp3⁺ T cells (Supplementary Fig. S5B and S5C). The percentage of Foxp3⁺CD103⁺ Treg cells was also comparable (Supplementary Fig. S5D), consistent with the previous data that GARP is more important for the accumulation of Treg cells specifically in the gut.

Discussion

The role of TGFβ in the development of Treg cells and suppression of various immune cell subsets including T cells, dendritic cells, B cells, NK cells, and myeloid cells is well established (33–35). The presence of GARP specifically on Treg cells underlines its importance for these cells. However, it is not clear how the absence of GARP on Treg cells modulates their function and ability to operate under inflammatory conditions. Our study is the first comprehensive research done on the role of GARP in the generation, maintenance, suppressive function, and migration of Treg cells in various inflammatory and cancer settings. We found that GARP is required to maintain systemic immune homeostasis, particularly in the intestine. GARP is also important for the optimal generation of Treg cells from naïve CD4⁺ T cells. We observed that even though GARP deletion affected the expression level of Foxp3 after activation, GARP⁻/⁻ Treg cells were able to control the proliferation of CD4⁺ T cells in vitro. However, they were unable to control chronic inflammatory diseases such as inducible lupus. GARP⁻/⁻ Treg cells were also unable to suppress T-cell–mediated colitis or antitumor responses in the colon. This was not only due to the defect in the suppressive function, but also...
Figure 5.
GARP modulates CD103 expression. A, Expression of CD103 was determined in WT and GARP OE Jurkat cells by qPCR. B, The expression levels of GARP in thymic and splenic CD4^+ and CD8^+ T cells in WT and Tet-inducible GARP-overexpressing (OE) mice were determined by flow cytometry. Representative mean fluorescence intensity (MFI) plots are shown. C–F, Percentage of Treg cells and CD103^+ Treg cells in the thymus, spleen, pLN, and colon was determined by flow cytometry and quantified. G, Graph bars show the change in the percentage of CD103^+ and CD103 MFI in human Tregs treated with GARP antibodies. Human Treg cells were generated in vitro from CD4^+ CD25^- T cells of four different healthy individuals in the presence of anti-CD28/CD3, IL2, TGFβ, anti-IL4, and anti-IFNγ. After 72 hours, the media were removed and replenished with fresh T-cell media containing IL2 and either anti-GARP antibody mix or isotype antibody or anti-TGFβ neutralizing antibody. The cells were harvested after 8 and 10 days of antibody treatment, stained, and analyzed by flow cytometry. Statistical analysis was performed by unpaired Student t test (A–F) or paired t test (G, * P < 0.05; ** P < 0.01; *** P < 0.001). Error bars, mean ± SEM.
Figure 6.
Mice with GARP deletion in Treg cells have better protective tumor immunity in a mouse colon cancer model. A, Experimental outline. Eight- to 12-week-old WT and f/f cre+ mice were intraperitoneally injected with 12.5 mg/kg AOM, followed by three cycles of DSS administration. Mice were sacrificed at week 12 after AOM injection. The results are representative of two independent experiments. B, Body weight change during the course of the experiment. C, Total cells count in spleen. D, Total cells count in mLNs. E, Total tumor count in each individual mouse and tumor load defined by the total tumor size in each mouse. F, CD4+ and CD8+ T-cell percentage in TILs gated on CD45+ cells. G, CD4+Foxp3+ T cells percentage in TILs gated on CD45+ cells. H, Bar graphs and representative flow cytometry plots show IFNγ and TNFα expression in CD4+ and CD8+ T cells in TILs gated on CD45+ cells. Statistical analysis was performed by unpaired Student t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Error bars, mean ± SEM.
because of their defective accumulation in the colon, which might be CD103-mediated.

GARP was previously shown to be expressed on activated Treg cells (16). The main function of GARP is to bind latent TGFβ, indicating that GARP might play a role in tuning the function of Treg cells. In our work, we used a transgenic mouse model in which GARP was specifically deleted on Foxp3 Treg cells, which allowed us to elucidate important and previously unknown functions of this molecule on Treg cells. In particular, Foxp3 expression in GARP−/− Treg cells was not affected under homeostatic conditions. When the cells were activated by TCR stimulation, we noticed that the expression levels of key markers such as CD25, ICOS, and KLRG1 were reduced, which might be associated with the compromised function and stability of Treg cells. Accordingly, in chronic inflammatory disease models such as the pristine-induced lupus model, GARP−/− Treg cells were not able to control the disease. In fact, previous studies have shown that Treg cells from active lupus patients exhibited functional and stability abnormalities (36, 37). In parallel, the TGFβ-mediated signaling in the cells defined by SMAD activation was consequently weaker. This signaling pathway is critically involved in the induction of Foxp3 because deletion of both SMAD2 and SMAD3 in CD4+ T cells leads to their failure to upregulate Foxp3 in response to TGFβ (11). Another evidence of the abnormal TGFβ signaling as a consequence of GARP deletion is that in vitro generation of Treg cells, which is largely TGFβ-dependent differentiation mechanism, was significantly reduced. In other words, by regulating of TGFβ bioavailability, GARP might modulate the function of Treg cells. Although, TGFβ is important for the generation, maintenance, and function of Treg cells, it has been reported that cell-specific deletion of TGFβ did not result in any immune abnormalities and the mice developed normally (38). Thus, more work is needed to clarify these controversial findings.

Another key observation from our study is that the expression of GARP on Treg cells is a crucial factor to maintain the intestinal homeostasis. We observed that young mice with GARP−/− Treg cells had shorter villi and increased lymphocytes. Upon aging, the mice developed an intestinal inflammation mimicking celiac disease in humans with thick, shortened, and fused villi. We also detected significantly increased bacterial-specific IgA in the serum of aged mice, indicating increased intestinal barrier permeability compared with WT mice. This chronic disease development is induced by TGFβ signaling as also was shown in a recent study in mice lacking Tgfr1 on Treg cells (12). In addition, aging itself has been reported to decrease the function of Treg cells (39, 40). Therefore, further studies are needed to determine how the aging can affect the expression profile for TGFβ-associated mediators including GARP on Treg cells.

A prior study (41) demonstrated that deletion of GARP on CD4+ T cells leads to a reduction Treg cells in the laminae and Peyer patches during an oral tolerance disease model. It suggests that the generation of Treg cells in these tissues is affected due to GARP deletion. Our study brings to light unknown effects of GARP expression on Treg cells. In fact, not only it is necessary for the generation of new Treg cells, but it is also important for the optimal expression of CD103, which is an important factor in cell recruitment to the intestine. Indeed, GARP−/− Treg cells were not able to suppress T-cell transfer colitis because of the failure of GARP−/− Treg cells to accumulate in the colon. Moreover, mice with specific GARP deletion in Treg cells exhibit better antitumor responses in the AOM/DSS colon tumor model. Our finding is well in line with previous murine and human studies showing that depletion of Treg cells or a low density of Foxp3+ cells in the colon is associated with better colorectal antitumor immunity (42, 43). Furthermore, the role of TGFβ in the induction of CD103 has been previously reported in a variety of immune cells (44, 45). CD103 is not just functioning as a homing marker. Recent findings show that the Foxp3 expression level in Treg cells from CD103−/− mice was significantly reduced and these Treg cells were not able to suppress murine contact hypersensitivity reactions during the elicitation phase (46). Moreover, in vitro–generated alloantigen-primed CD4+CD103+ cells coexpressed CD25. These cells suppressed T-cell activation, and contained more Foxp3 mRNA than the CD103−/− cells isolated from the same cultures (47). The link between GARP and CD103 is therefore remarkable. Our findings have a translational relevance to imply GARP as a potential target for combination therapy against cancer to improve the current immunotherapy outcomes, especially because Treg cells constitute a major challenge in antitumor immunity. To this end, our laboratory has previously reported that targeting GARP potentiates protective immunity against both melanoma and colon cancer (48). In addition, it also limited metastasis in an orthotopic model of human breast cancer (49). Therefore, we believe that our study uncovers several novel aspects of GARP as a functionality tuning and trafficking enhancer by empowering TGFβ signaling. It is an interesting feature that can be utilized as a treatment strategy in immunotherapy combinations for autoimmune diseases and cancer especially after the recent findings that give clear structural and mechanistic insights on how GARP binds to TGFβ on human Treg cells (50).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Z. Li
Development of methodology: E. Ansa-Addo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Wallace, E. Ansa-Addo, H. Kwon, B. Riesenberg, B. Wu, S. Guglietta
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Wallace, B. Riesenberg, S. Sun, Z. Li
Writing, review, and/or revision of the manuscript: C. Wallace, B. Riesenberg, S. Guglietta, S. Sun, Z. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Salem
Other (initiated, performed, and designed the experiments, analyzed the data, and wrote the manuscript): M. Salem
Other (designed and performed experiments): M. Velegraki, A. Li, A. Metelli, Y. Zhang
Other (provided critical materials and scientific input): B. Liu

Acknowledgments
We thank the FACS core of Hollings Cancer Center, MUSC for their technical assistance. The research was funded by multiple grants from the NIH: R01AI077283, R01CA213290, R01CA188419, and P01CA186866 (all to Z. Li).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 22, 2018; revised September 21, 2018; accepted January 17, 2019; published first January 23, 2019.
References


GARP Dampens Cancer Immunity by Sustaining Function and Accumulation of Regulatory T Cells in the Colon

Mohammad Salem, Caroline Wallace, Maria Velegraki, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-18-2623

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2019/01/23/0008-5472.CAN-18-2623.DC1

Cited articles
This article cites 49 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/79/6/1178.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/79/6/1178.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/79/6/1178.
Click on “Request Permissions” which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.