**Deletion of GARP on Mouse Regulatory T Cells is not Sufficient to Inhibit the Growth of
Transplanted Tumors**

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Abstract

GARP is a transmembrane protein that presents latent TGF- β 1 on the surface of regulatory T cells (Tregs). Neutralizing anti-GARP monoclonal antibodies that prevent the release of active TGF- β 1 inhibit the immunosuppressive activity of human Tregs *in vivo*. In this study, we investigated the contribution of GARP on mouse Tregs to immunosuppression in experimental tumors. Unexpectedly, conditional knockout (KO) mice challenged orthotopically with GL261 tumor cells or subcutaneously with MC38 colon carcinoma cells did not show prolonged survival or delayed tumor growth. Also, the suppressive function of KO Tregs was similar to that of WT Tregs in the T cell transfer model in allogeneic, immunodeficient mice. In conclusion, *garp* deletion in mouse Tregs is not sufficient to impair their immunosuppressive activity *in vivo*.

Keywords: LRRC32, GARP, tumor models, immune response, regulatory T cells

1 Introduction

The immune system plays pivotal roles in the control of onset and development of cancer. Tumor cells use various strategies to escape immunosurveillance. An important mechanism adding to tumor progression is the inhibition of anti-tumor immune responses by regulatory T cells (Tregs). Tregs are a highly specialized subpopulation of CD4⁺ T cells that maintain self-tolerance and inhibit potential harmful autoimmune responses in physiological conditions. Tregs use multiple mechanisms to suppress immune responses (1). Hence, counteracting Tregs can be mediated in various ways that all represent highly interesting options to restore anti-tumor T cell immunity. Immune checkpoint therapy targeting CTLA-4, TIM-3, TIGIT, all expressed by Tregs, are approved or are proceeding to the clinic (2). However, even with several agents against different immune checkpoints on the market, the observed effects in many cancer patients are often limited. Therefore, the identification of new targets involved in controlling Treg activity is definitely far from complete.

Recently, the immunosuppressive properties of Glycoprotein A Repetitions Predominant (GARP), also known as leucine rich repeat containing 32 (LRRC32), were demonstrated (3,4). GARP binds and presents latent TGF- β 1 on the Treg surface and is upregulated upon activation. GARP/latent TGF- β 1 complexes can be released in cell supernatants as a result of shedding of the membrane complexes (4–6) and can prevent T cell-mediated destructive inflammation by enhancing Treg and inhibiting T effector cell activity (7). Importantly, integrin α V β 8 induces the release of active TGF- β 1 from GARP/latent TGF- β 1 on the Treg surface (8–10). Neutralizing monoclonal antibodies specific for GARP/latent TGF- β 1 complexes or downregulation of GARP expression with siRNA have been shown to block the release of active TGF- β 1 and inhibit the immunosuppressive activity of human Tregs *in vitro* (3,4). These findings were confirmed in a xenogeneic graft-versus-host-disease (xGVHD) model induced by transfer of human peripheral blood mononuclear cells in NSG mice. In this model, disease severity is reduced by co-transfer of autologous human Tregs. This protection was abrogated by a blocking anti-human GARP antibody, indicating that targeting GARP reduces human Treg function and thus indirectly promotes the function of activated effector T cells (Teff) *in vivo* (3). This is considered to be one of the ultimate goals in cancer-immunotherapy. The presence of GARP in the microenvironment of human tumors was previously described. High levels of GARP were measured on Tregs in ascites from ovarian cancer patients, in tumor tissues of lung cancer patients, and in peripheral blood of hepatocellular carcinoma patients (11–13). Furthermore, expression of GARP was reported on melanoma cells and in intratumoral tissues of patients with gastric cancer (14,15). Hence, GARP is currently being investigated as a promising immune-oncology target.

Whether GARP contributes to the suppressive function of Tregs in the context of malignancy has not yet been thoroughly elucidated. To address this question, we monitored tumor growth after transplantation of syngeneic tumor cells, or after tumor induction by treatment with a chemical carcinogen, in mice carrying a Treg specific deletion of *Garp*.

2 Material and methods

2.1 Mice

NSG (NOD scid gamma) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Foxp3 promotor-driven Cre recombinase transgenic mice (*Foxp3*^{YFP-Cre}, courtesy A. Liston, VIB, KU Leuven) and mice carrying the *Garp*^{fl/fl} alleles (courtesy D. Unutmaz, The Jackson Laboratory for Genomic Medicine) were described previously (16–18). Exon 1 of *Garp*^{fl/fl} mice is flanked by *loxP* sites. Foxp3 conditional *Garp* knockout (KO) mice were generated by first mating *Foxp3*^{YFP-Cre} mice with the *Garp*^{fl/fl} mice. *Garp*^{fl/-}Cre^{+/-} mice were backcrossed with *Garp*^{fl/fl} mice to generate Foxp3 specific *Garp* KO mice (*Garp*^{fl/fl}Cre^{+/-}) and littermates (LM, *Garp*^{fl/fl} Cre^{-/-}).

2.2 Polymerase chain reaction (PCR)

Genotyping was performed by PCR amplification of total genomic DNA obtained after NaOH extraction of ear tissue samples, using two pairs of primers: 5'-GCAAAGCAGACGGTCATACA-3', 5'-TCTGGAAGTCAAGAGGCTGAG-3' for the floxed GARP allele and 5'-AGGATGTGAGGGACTACCTCCTGTA-3', 5'-TCCTTCACTCTGATTCTGGCAATTT-3' for the Foxp3 driven YFP-Cre allele.

2.3 Cell lines and short-term *in vitro* stimulation of T cells

The GL261 and MC38 cell lines were obtained from S. De Vleeschouwer (KU Leuven) (19) and B. van den Eynde (de Duve Institute). Tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin and 2 mM l-glutamine, 100 µg/mL streptomycin, 100 µg/mL gentamycin sulfate for GL261 (all from Lonza, Verviers, Belgium) and 10 mM Hepes, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids for MC38.

Splenocytes of KO and *Foxp3*^{YFP-Cre} mice were lysed using erylisis buffer (16 mM NH₄Cl and 10 mM NaHCO₃ in demiwater) and 1 x 10⁶ lymphocytes were stimulated for 24h in a 24 well plate using mouse T-activator CD3/CD28 Dynabeads (Invitrogen, Carlsbad, CA) at a 1:1 cell:bead ratio in RPMI 1640 Medium supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM l-glutamine. Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

2.4 Flow cytometry

GARP expression and number of cells was determined using fluorescently-labeled antibodies against murine GARP (YGIC86), Foxp3 (FJK-16s) (all from eBioscience), LAP (TW7-16B4), CD4 (RM4-5) (all from Biolegend, San Diego, CA) or CD4 (REA604, Miltenyi Biotec, Bergisch-Gladbach, Germany). Antibody dilutions were used according to the manufacturer's instructions. Intracellular staining of Foxp3 was performed using the Foxp3 intracellular staining kit (eBioscience) and samples were acquired on a FACS Verse flow cytometer (BD Biosciences).

2.5 Western Blot

Tregs were enriched from the splenocytes of LM and KO mice by magnetic cell sorting using anti-CD25 coated beads. The isolated cell populations, containing approximately 50% CD4+CD25+Foxp3+ cells as determined by FACS analysis, were incubated with or without anti-CD3/CD28 coated beads (1 bead : 2 cells) during 24 hours. Cells were collected and analyzed by Western Blot with antibodies against phosphorylated SMAD2 (pSMAD2) or β -actin. ECL signals were measured on a Fusion Solo 4S system (Vilber).

2.6 Tumor models

Syngeneic GL261 and MC38 tumor cells were implanted in male KO and LM mice. Each mouse, anesthetized with 5% isoflurane in O₂, was implanted with 5 x 10⁵ tumor cells in 200 μ L PBS by subcutaneous (sc) injection in the left flank (MC38) or in 10 μ L medium by orthotopic injection in the right striatum (GL261) using a stereotaxic frame as described (20). Alternatively, mice were challenged once subcutaneously in the left hind leg with 400 μ g 3-methylcholantrene (MCA, Sigma-Aldrich, St. Louis, MO) in 100 μ L of olive oil and in the right hind leg with vehicle. For GL261 challenged mice, overall survival was monitored. Tumor growth in sc challenged mice was monitored by measuring length (L) and width (W) in MC38 challenged mice and L, W and height (H) in MCA challenged mice, with a digital caliper. Rate of

follow up was three times a week for MC38-bearing mice and once a week for MCA-induced tumor-bearing mice. Tumor surface was calculated according $\frac{L \times W \times \pi}{4}$ and tumor volume according $\frac{L \times W \times H \times \pi}{6}$.

2.7 Treg in vivo suppression assay

Phycoerythrin-conjugated anti-CD4 antibodies (GK1.5, eBioscience, San Diego, CA) and CreYFP fusion protein expression was used to purify CD4⁺Foxp3⁻ effector T cells (Teff) from CD4⁺Foxp3⁺ regulatory T cells (Treg) with a FACS Aria fluorescent cell sorter (Becton Dickinson, Franklin Lakes, NJ) out of splenocytes from male KO and Foxp3^{YFP-Cre} mice. Sorted Teff were injected either alone or in combination with Treg via the tail vein into adult NSG mice at a dose of 1 x 10⁶ Teff and 2.5 x 10⁵ Treg in 200 µL PBS. The mice were sacrificed 7 days later and the number of Teff in the spleen were measured using flow cytometry (21).

2.8 Ethics Statement

Animal experiments were approved by the Institutional Animal Care and Use Committee of KU Leuven, Leuven, Belgium (Permit Number: P263-2014).

2.9 Statistics

Data are represented as mean ± standard error of the mean. One-way and two-way ANOVA with Bonferroni post test was performed to compare CD4 cell numbers and differences in tumor volumes in function of time respectively. Survival analysis and onset of the MCA tumor analysis was performed using the Logrank test.

3 Results

3.1 Generation of Treg-specific *garp* KO mice

Cre/LoxP recombination technology was used to ablate *Garp* in Foxp3 expressing cells. Parental mice, either transgenic for Cre recombinase driven by the *Foxp3* promotor (*Foxp3*^{CreYFP} allele), or harboring LoxP sites flanking the *Garp* gene, were used to generate hemizygous male Treg specific *Garp* KO mice (*GARP*^{fl/fl} *Foxp3*^{CreYFP}) and LM controls (*GARP*^{fl/fl} *Foxp3*⁺). Only hemizygous male KO and LM controls were used to avoid the problem of deletion of *Garp* in only a subset of Tregs from heterozygous *Foxp3*^{CreYFP/+} females,

due to random X chromosome inactivation. Genotyping confirmed the presence of *Garp*^{fl} alleles (181 bp) and the *Foxp3*^{CreYFP} allele (346 bp) in genomic DNA of KO mice (**Fig 1A**). GARP expression was measured on stimulated CD4⁺Foxp3⁺ Tregs from *littermate* mice, but not from *KO* mice, confirming the absence of GARP on Tregs of KO mice (**Fig 1B**). To investigate the effect of the absence of GARP from Treg on TGF- β , surface LAP staining was measured on stimulated Treg. LM Treg demonstrated co-expression of GARP and LAP on the surface of stimulated CD4⁺ Foxp3⁺ cells. KO mice did not express LAP on the surface of the Treg (**Fig 1B**). Finally, production of active TGF- β was determined by measuring Smad 2 phosphorylation, a downstream marker for TGF- β receptor activation. Upon stimulation, LM Treg demonstrated a high increase in Smad 2 phosphorylation. This was less pronounced in KO Tregs and demonstrates that the release of active TGF- β is decreased in absence of GARP, although not completely abrogated (**Fig 1C**).

3.2 Similar growth of transplantable tumors in KO and LM mice

To address whether *Garp* expressed on Tregs contributes to suppression of anti-tumor responses in the context of malignancy, we performed three independent tumor models. We hypothesized that Tregs in KO mice would display a decreased immunosuppressive capacity, hence resulting in a slower tumor growth. In a first model, we injected syngeneic MC38 tumor cells subcutaneously (*s.c.*) and tumor surfaces were monitored thrice-weekly. Unexpectedly, the tumor surfaces in Treg-specific *Garp* KO mice (78.6 ± 19.6 mm² on day 17 post injection (*p.i.*)) were similar to those in LM controls (86.1 ± 29.9 mm²) (**Fig 2A**). Second, we injected syngeneic GL261 glioma tumor cells in the striatum of KO mice (orthotopic injections). The median overall survival of KO mice was similar to that of LM mice (31 *versus* 32 days *p.i.*). Logrank survival analysis did not show a statistical difference between the two groups. (**Fig 2B**). Third, we induced fibrosarcoma tumors by injecting MCA in the hind leg after which tumor development was followed. After 41 weeks *p.i.*, 9 of the 10 LM mice had developed fibrosarcoma, *versus* only 4 of the 8 KO mice. Along the same line, the median time during which the KO mice were tumor free was 33 weeks *versus* 20 weeks in LM (**Fig 2C**). However, in mice that did develop a tumor, the kinetics of tumor development were similar

between KO and LM controls (**Fig 2D**). Our data indicate that in transplantable tumor models, absence of GARP on Tregs is not sufficient to reduce tumor growth. It is noteworthy however that in a carcinogen-inducible tumor model, the proportion of KO mice developing tumors was lower than that of LM. Although this trend did not reach statistical significance, it may still suggest that on the long term, reduced immunosuppression by GARP-deficient Tregs may improve immune surveillance of developing tumors.

3.3 Comparable suppression of KO Tregs and WT Treg in an in vivo suppression assay

To measure the immunosuppressive function of *Garp* KO Tregs *in vivo*, but this time in non-tumor bearing animals, we co-transferred WT effector CD4⁺ T cells and Tregs isolated from *Garp^{fl/fl} Foxp3^{CreYFP}* or *Garp^{wt/wt} Foxp3^{CreYFP}* control mice into immunodeficient NSG mice. The model measures the ability of Tregs to suppress the proliferation of CD4⁺ T cells transferred into allogeneic recipients. One week after T cell transfer, numbers of WT Teff cells, recovered from mice that had not received Tregs (transfer of Teff alone), were increased 27.6-fold by comparison to numbers of Teff cells transferred on day 0, indicating strong proliferation of WT Teff cells *in vivo*. As expected, co-transfer of WT Tregs resulted in a significant inhibition of WT Teff cell proliferation, resulting in 5 times less CD4⁺ T cells in recipient spleens by comparison to transfer of Teff alone. Strikingly, KO Tregs showed a similar ability to suppress the proliferation of CD4⁺ T cells as WT Tregs (**Fig 2E**).

4 Discussion

We investigated the contribution of *Garp* to Treg-mediated immunosuppression in tumor-bearing mice. To do so, we generated Treg-specific *Garp* KO mice. Three different syngeneic cancer models were performed in these mice. The well-established MC38 and GL261 tumor models are documented to be immunogenic and significantly influenced by Tregs (22–24). Prophylactic elimination of Treg through anti-CD25 antibody treatment completely protected mice against orthotopic GL261 tumor challenge (23,25,26). Similarly, MC38 tumor cell growth was delayed upon anti-CD25 antibody treatment in B6 mice and upon treating DEREK mice with diphtheria toxin (24,27). Although the orthotopic GL261 model might mimic human carcinogenesis because tumor cells are introduced at the site of the primary tumor origin compared

to the sc MC38 model, the disadvantage of these models is that the tumor cells are artificially introduced in the mouse tissues as bulk mass. Consequently, the tumor cells and the immune system probably do not interact in the same way during early disease onset in situations where tumor cells are injected or tumor growth is induced. To take these early interactions into account, we also performed the MCA induced fibrosarcoma tumor model in which it is known that depleting Tregs reduced tumor development (27,28). Here, we demonstrate that genetic deletion of *Garp* in Tregs is not sufficient to cause a delay in tumor growth in transplanted tumor models or to completely protect mice from tumor development in the MCA model. However, there is a trend for the KO mice to develop fewer fibrosarcoma tumors. This could indicate that the GARP/latent TGF- β complex and the release of active TGF- β may be important in the early interactions in the tumor microenvironment or may be involved in the formation of the tumor stroma (29), which may be less likely in transplanted tumors.

The above results were rather unexpected, as a role for GARP in immunosuppression by human Tregs was clearly established *in vivo*, using blocking anti-GARP antibodies in a xGVHD model induced by human T cells in NSG mice (3). Our data are consistent with a previous report by the group of Li, showing that deletion of *Garp* in mouse Tregs does not improve the efficacy of adoptive T cell therapy in melanoma (30). This warranted further assessment of the immunosuppressive function of *Garp* KO Tregs *in vivo*, in non-tumor bearing animals. To this end, we used the less complex immunological setting of CD4⁺ Teff cell transfers in allogeneic, immunodeficient NSG mice. In this model, co-transfer of WT Tregs inhibits the proliferation of Teff cells, allowing to measure immunosuppression by Tregs *in vivo*. We observed that *garp*-KO and WT Treg were equally capable of suppressing the proliferation of Teff cells.

Our data revealed residual active TGF- β from stimulated Treg from KO mice and, originating thus from another source than the GARP/latent TGF- β complex. We hypothesize that the remaining level of active TGF- β may be sufficient to maintain both the immunosuppressive tumor microenvironment in transplanted tumors and the capacity to suppress alloreactive effector T cells.

Additionally, it was recently shown that integrin $\alpha V\beta 8$, which interacts with GARP/latent TGF- $\beta 1$ complexes and is involved in the release of active TGF- $\beta 1$ from human and mouse Tregs, is not required for the Treg-mediated suppression of colitis induced by the transfer of naïve T cells (8,9,31). It must be noted that in this model, whereas *Itgb8* deficient Tregs were as suppressive as WT Tregs when they were transferred to recipient mice at the same time as naïve T cells, they were incapable of suppressing ongoing colitis when transferred at later time points (31). This inability of *Itgb8* deficient Tregs to suppress ongoing colitis contrasted with that of WT Tregs, indicating that active TGF- $\beta 1$ released by integrin $\alpha V\beta 8$ from GARP/latent TGF- $\beta 1$ complexes on the surface of Tregs may be required for their immunosuppressive function *in vivo*, in conditions of ongoing, active inflammation.

Notwithstanding the above, we cannot exclude that mouse Tregs may cause immunosuppression through mechanisms different from that of human Tregs, which were shown to depend on GARP to suppress immune responses *in vivo*.

5 Conclusion

Our aim was to provide a better insight as to whether GARP influences Treg immunosuppression *in vivo*. Deletion of GARP from mouse Tregs was not sufficient to impair immune suppression in transplanted tumor models and in an *in vivo* suppression assay in non-tumor bearing mice. Of course, synergies between GARP deletion on Tregs and treatment with checkpoint inhibitors would be worth to explore. Recent evidence has arisen that GARP expressed on platelets is involved in protective immunity against malignancy (30). Therefore, the question remains whether deletion of GARP on non-Treg cell types could affect tumor associated immunosuppression in a more profound way.

6 Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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9 Author contribution statement

EV, S.Liénart, AC and WM performed the experiments and analyzed the data. EV and WM wrote the manuscript. KV, SFDM, HD, SLiénart, S.Lucas, AG and DU reviewed the manuscript. CG and DU provided mice. AG gave technical support about the MCA model.

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11 Legends

Fig. 1. Generation of Treg-specific Garp KO mice. (A) Genotypic analysis of genomic DNA from Treg-specific *Garp* KO mice (*Garp*^{fl/fl} *Foxp3*^{CreYFP}), LM controls (*Garp*^{fl/fl} *Foxp3*⁺), *Garp*^{fl/wt} and *Foxp3*^{CreYFP} mice. Band size of the targeted loxP allele (*Garp*^{fl}) is 181 bp; wild type allele (*Garp*^{wt}) is 111 bp, *Foxp3*^{CreYFP} allele is 346 bp. (B) GARP and LAP staining on CD4⁺Foxp3⁺ cells from LM and KO mice using flow cytometry. Splenocytes were stimulated with anti-CD3/CD28 beads for 24h. (D) Production of active TGF- β 1 by Tregs is decreased in the absence of GARP. Tregs were enriched from the splenocytes of mice with the indicated genotypes by magnetic cell sorting, using anti-CD25 coated beads. The isolated cell populations were incubated in the presence (stim) or absence (no stim) of anti-CD3/CD28 coated beads. Cells were collected and analyzed by Western Blot with antibodies against phosphorylated SMAD2 (pSMAD2) or β -actin.

Figure 2: Tumor models in Foxp3 specific *garp* KO and Treg homeostasis assay in NSG mice. **(A)** Subcutaneous MC38 tumor surfaces over time. Data are pooled from 2 independent experiments (LM n=9; KO=9). The data are represented as mean $\pm$ SEM, statistically analyzed using two-way ANOVA. **(B)** Kaplan Meier analysis of mice orthotopically grafted with 5×10^5 GL261 tumor cells. Data are pooled from 2 independent experiments (LM n=16; KO n=11). **(C)** Incidence of MCA-induced tumors during 41 weeks after 400 μ g MCA induction (LM n=10; KO n=8). **(D)** Tumor volumes over time in the hind leg injected with MCA. Solid, black line represents LM mice; dashed grey lines represents KO mice. Statistical difference was determined by a Log rank (Mantel-Cox) test. **(E)** CD4 cell numbers in the spleen one week after splenocyte injection. Statistical difference was determined by one-way ANOVA with Bonferroni post test (***, $p < 0,001$).

A

B

C

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