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Biochemical and Immunological Study of the Roles of GARP/TGF-β Axis in Cancer

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Preface

The work described in this dissertation was performed between April 2013 and July 2017. The focus of the work was to study the expression, activity and regulation of the novel latent-TGF-β1 receptor named Glycoprotein-A repetitions predominant protein (GARP). Since its discovery in 1992, the scientific literature regarding GARP can be divided into three consecutive time periods; each of them emphasizes research on a specific aspect of the protein (Figure 1).

Initially, GARP gene *Lrrc32* was isolated from human breast cancer. GARP gained attention between 1992 and 2006 because of its gene amplification in the aggressive forms of human carcinomas such as breast cancer.

In 2009 GARP was identified as a latent TGF-β1 receptor, expressed on immune cells, specifically on regulatory T cells and megakaryocytes/platelets. At this time, GARP is gaining much more attention from the scientific community as an activation marker of regulatory T cells and for its ability to regulate the bioavailability of TGF-β. More than 50 publications have been dedicated to understanding the importance of the GARP-Foxp3-TGF-β1 triad in tolerance from 2009 to present.

The last two years have marked the third period when GARP expression and the TGF-β related function have been implicated as direct promoters of oncogenesis.

Work of this dissertation started when the importance of GARP as a regulator of TGF-β bioavailability in Treg cells was discovered. Three specific aims have been proposed and subsequently addressed, centered on the roles of GARP in cancer, and together they constitute my PhD thesis:

- Aim 1. Investigate the formation and function of a novel soluble GARP/latent TGF-β complex
- Aim 2. Determine the expression and functions of GARP/TGF-β on cancer cells
- Aim 3. Study the role of GARP/TGF-β axis in platelets in the context of cancer

Of note, the work in Aims 2 and 3 have been summarized and published in the journals *Cancer Research* and *Science Immunology*, respectively. The manuscript related to Aim 1 is currently in preparation and will be submitted shortly for publication. Because the purpose is to have a better understanding of GARP/TGF-β axis through the aforementioned aims, the following introduction will cover several aspects of GARP protein structure and function. The importance of TGF-β regulation and the role played by platelets in cancer will be also discussed.

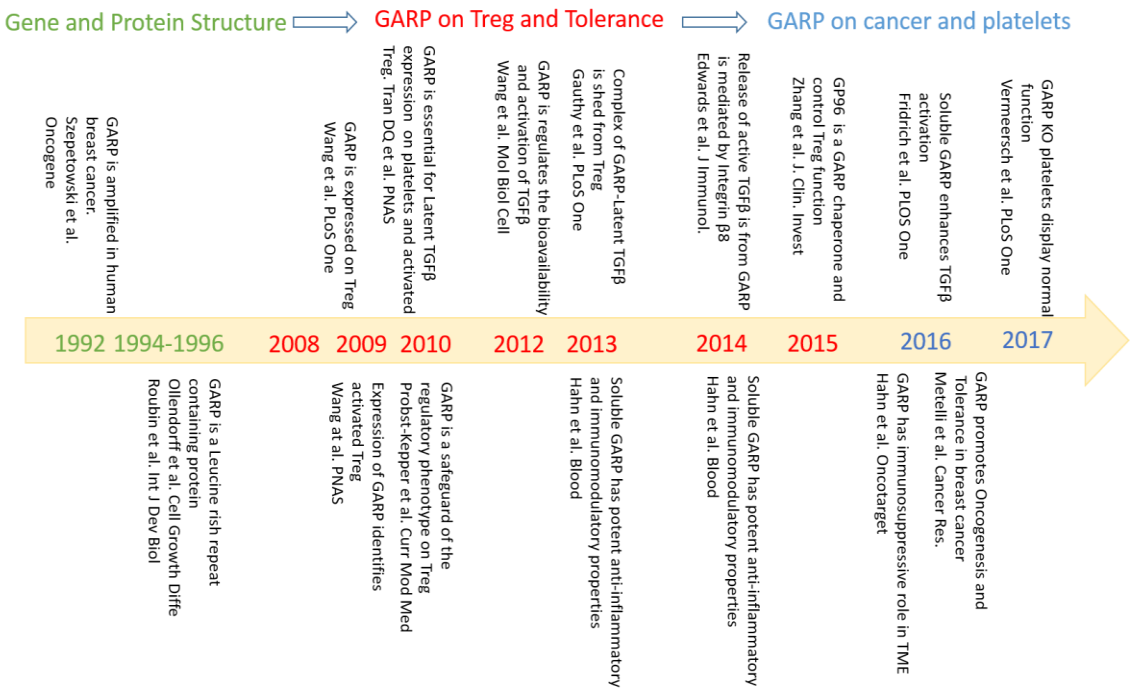

Figure Preface-1: Timeline of literature review about GARP

GARP literature can virtually be divided into 3 time windows; each emphasizes a specific aspect of GARP function: the first round of research focused on the characterization of gene and protein structure; the second round was dedicated to GARP function on Treg cells and tolerance; research during the third round analyzes GARP expression and function on platelets and cancer.

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List of Abbreviations

LRRC32: Leucine Rich Repeats Containing 32

TGF-β: Tumor Growth Factor β

LAP: Latency Associated Peptide

NK: Natural Killer

Treg: Regulatory T Cells

CTL: Cytotoxic T Lymphocytes

TCR: T Cell Receptor

Th: T helper lymphocytes

UTR: Untranslated Region

miRNA: micro RNA

mRNA: messenger RNA

TME: Tumor Micro-Environment

DC: Dendritic Cells

TIL: Tumor infiltrating lymphocytes

CY: Cyclophosphamide

TGF-βR: TGF-β receptor

ER: Estrogen receptor

PR: Progesterone receptor

HER2: Human epidermal growth factor receptor 2

EMT: Epithelial to mesenchymal transition

ADCC: Tumor-killing through antibody-dependent cell cytotoxicity

CDCC: Complement-dependent cell cytotoxicity

CTX: Cyclophosphamide

ATC: Adoptive cell transfer

TCIPA: Tumor Cell-Induced Platelet Aggregation

PR: Platelet Releasate

Abstract

GARP encoded by the *Lrrc32* gene is the cell surface docking receptor for latent TGF-β1 mostly expressed on regulatory T cells (Treg) and platelets. Although GARP has been extensively studied for the ability to enhance latent TGF-β1 activation in the context of Treg, the expression and relevant functions on cancer cells and platelets had not been explored when this work started 4 years ago. In addition, a soluble form of GARP has been described as shed from the Treg cell surface; however, the mechanism to explain the soluble molecule formation and how it becomes biologically active remains elusive.

The results contained in this dissertation cover several unknown aspects of GARP biology and shed light on GARP as a potential therapeutic target in cancer treatment and prognosis.

The first part of the results section focuses on the mechanism behind the formation and the biological activity of soluble GARP. Here we describe two putative mechanisms that explain the generation of the soluble protein. The first mechanism is mediated by thrombin that enzymatically cleaves surface GARP to generate two cleaved products. The second mechanism is mediated by extracellular vesicles (EVs) secreted by cells, specifically exosomes, which include GARP/latent TGF-β as a complex. Importantly, this part of the thesis dissertation demonstrated the importance of integrins belonging to the alpha V family that mediate the endocytosis of soluble GARP (sGARP) in epithelial cells.

In the second chapter of the results section, I report that GARP exerts oncogenic effects, promoting immune tolerance by enriching and activating latent TGF-β1 in the TME. In collaboration with other members in Dr. Zihai Li's laboratory, I found that human breast, lung, colon, and prostate cancers expressed GARP aberrantly. In genetic studies utilizing normal mammary gland epithelial and carcinoma cells, GARP expression increased TGF-β bioactivity and promoted malignant transformation in immunodeficient mice. In immunocompetent breast carcinoma–bearing mice, GARP overexpression promoted $Foxp3^+$ Treg cell activity, which in turn contributed in enhancing cancer progression and metastasis. Notably, administration of a GARP-specific monoclonal antibody, made by Dr. Zihai Li's laboratory, limited metastasis in an orthotopic model of human breast cancer. Overall, these results define the oncogenic effects of the GARP–TGFβ1 axis in the TME and suggest mechanisms that might be exploited for diagnostic and therapeutic purposes. These results have been summarized and published in the journal *Cancer Research* in a paper entitled "Expression of TGF-β Docking Receptor GARP Promotes Oncogenesis and Immune Tolerance in Breast Cancer"[1].

In the third part of the result section, I focused on the role of platelet GARP. Here, I hypothesized that constitutive GARP expression on platelets enhances the activation of latent TGF-β1 released by platelets. This phenomenon is critical in the cross-talk between platelets and cancer cells where GARP promotes malignancy and resistance to therapy. Indeed, platelet-specific deletion of GARP-encoding

gene *Lrrc32* blunted TGF-β1 activity at the tumor site and potentiated protective immunity against both melanoma and colon cancer. This work was recently published in *Science Immunology* in an article entitled: "Platelets Subvert T Cell Immunity Against Cancer via GARP/TGF-β Axis"[2].

Overall, the results included in this thesis demonstrate that 1) the formation of soluble GARP is mediated by two novel mechanisms that can be exploited for cancer immunotherapy; 2) GARP is expressed on cancer cells and has oncogenic properties; and 3) GARP/TGF-β axis on platelets reduces the efficacy of the anti-tumor immunity by blunting anti-tumor T cell activity.

Chapter 1
Introduction & Significance

I-A. GARP gene, protein and regulation

GARP Gene

Garp gene *Lrrc32* was first cloned in human breast cancer in 1992 [3]. In their studies on DNA amplification, Szepetowski and colleagues identified a new independent unit present in the telomeric region at 11q13-q14, that they named GARP. *In situ* hybridization studies revealed that murine *Lrrc32* gene is localized on chromosome 7 in a conserved region between human and murine genome[4]. Interestingly, *Lrrc32* gene locus is part of a 3-5 Mb chromosomic region frequently altered in human cancers. Specific *Lrrc32* gene amplification was observed in primary and metastatic neck lymph nodes in oral squamous cell carcinoma[5]; also in prostate cancer, *Lrrc32* amplification rate increases with the decrease of hormone sensitivity[6]. Conversely, deletion and rearrangement of *Lrrc32* locus was described in two cases of hibernoma, thus unveiling the ambiguous behavior of *Lrrc32* gene product in cancer[7]. Nucleotide blasting analysis showed that human and murine *Lrrc32* gene have a similar structure, i.e., they share the 81% of homology and both comprise of two coding exons; the first exon encodes for the signal peptide and 9 amino acids, and the second exon encodes for the majority of the coding region [8].

GARP Protein

After gene isolation, human and mouse protein putative sequences were deciphered. GARP protein structure can be divided into 3 domains: the extracellular domain, which constitutes about 70% of the protein, the hydrophobic transmembrane domain, and the 15-residue long cytoplasmic domain. As part of the Leucine Rich Repeats-Containing (LRR) proteins family, the extracellular domain of GARP contains 20 leucine rich motifs divided into two groups by a proline rich region[8]. Among the extracellular LRR proteins, GARP together with the Toll like receptors, GP1bα and GP1bβ, belongs to the LRR_Tollkin subfamily, a group of proteins important in inflammation[9]. As well as Toll like receptors, GARP requires the master chaperone gp96 for its folding and surface expression[10,11]. The proline rich region located between the LRR resembles the hinge domain of the latent TGF-β1 binding protein (LTBP-1). This domain confers flexibility to the protein and suggests that GARP might be involved in protein-protein interaction[12,13]. Additionally, as well as LTBP, GARP covalently disulfide links with Latency Associated Peptide (LAP): site specific mutagenesis from Cysteine to Alanine demonstrated that Cys-192 and Cys-133, located on the 7th and 12th LRR respectively, are responsible for the disulfide linkage between GARP and Cys-4 of LAP[14] (Figure 1-1). Despite the high homology in the extracellular domain, murine and human cytoplasmic tails show a 33% difference in the amino acid sequence, yet they both have a conserved tyrosine residue. Of interest, other members of LRR_Tollkin family, like TLRs, have a cytoplasmic phosphorylated tyrosine

involved in signal transduction, suggesting a possible tyrosine phosphorylation dependent function for GARP[15].

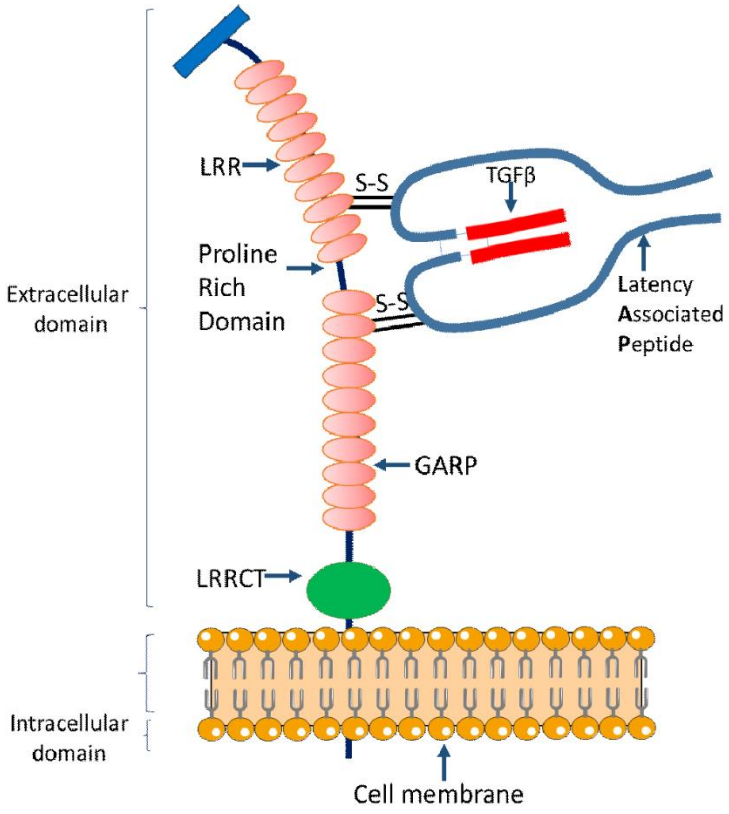


Figure 1-1: Structure of membrane bound GARP/latent TGF-β1 complex. GARP protein is structurally divided into 3 domains based on its primary sequence: the extracellular domain, the transmembrane domain, and the intracellular domain. The extracellular domain contains two sets of 10 LRRs divided by a Proline Rich Domain. Two conserved Cys residules (Cys-192 and Cys-133) are located on the 7th and 12th LRR, respectively, and are responsible for two disulfide linkages between GARP and Cys-4 of LAP of latent TGF-β.

Tissue Distribution and Cell Expression

In human tissues, GARP is expressed in peripheral blood, placenta,[16] and pancreas[17] in a concentration higher than 10 ppm. At the cellular level, GARP expression has been reported for mesenchymal stromal cells[18], Treg cells, megakaryocytes/platelets[19] [20], LAP^+ $\gamma\delta$T cells[21], hepatic stellate cells, [22] and activated human B cells[23]. GARP is widely expressed on mouse lymphoid organs: on resting Treg in the spleen, in mesenteric lymph nodes (mLNs) and in peripheral lymph nodes (pLNs), in thymus, and in payer's patches[24]. In accordance with gene expression, GARP protein is expressed on human breast cancer, lung, and colon cancer, where it correlates with tumor aggressiveness[1].

GARP protein has been described as a membrane bound soluble protein that is shed from the T cell membrane[25]. The possibility of a shedding process was first discussed by Roubin and colleagues in 1996 when, describing a GARP deduced amino acid sequence, they observed the presence of a hydrophobic leader sequence. They hypothesized that this domain might be the signal peptide for protein secretion[8]. Soluble GARP indeed is present in human plasma, yet the mechanism of the protein's shedding or secretion is not clear[26].

GARP Gene Regulation

Upon TCR engagement, GARP expression is only restricted to Treg cells; no significant surface expression of GARP has been described in human or mouse conventional T helper (Th) cells. [19,27]. Cell and context specific expression of GARP gene is the result of the interplay of two alternative promoters: upstream Promoter 1 (P1) and downstream Promoter 2 (P2). Both promoters drive GARP gene transcription; however, the variance in their methylation status in different cell populations dictates where, and under which conditions, GARP will be expressed. P2 is almost completely demethylated in both Treg and Th, yet only in Th is the transcription initiation from P2 blocked by several methylated CpG islands present in the downstream P1. Also, by inhibiting binding with any transcription factors, the methylated CpGs maintain P1 in a closed chromatin configuration. In contrast, the less pronounced methylation status of P1 in Treg allowed the binding of Foxp3 that remodels the promoter region towards an open configuration status. This allows the subsequent binding of NFAT and NF-kB for driving the transcription of the GARP gene[28]. A clear example of this Foxp3 mediated GARP expression is the conversion of tumor Th17 cells to ex-Th17 Foxp3^{+} cells that show upregulation of surface GARP as a transdifferentiation-associated marker[29]. Accordingly, Foxp3 shRNA in Treg reduced surface GARP, yet GARP shRNA did not affect Foxp3 expression[30]. Treg might not be the only cell population that experiences GARP-Foxp3 co-regulation; human and murine megakaryocytes and platelets constitutively express both Foxp3 and the surface GARP/LAP complex. Interestingly, platelets upon activation upregulate both GARP and Foxp3: PAR4-

AP increases surface GARP, while phorbol ester myristate acetate upregulates Foxp3 expression[19,31,32]. Although the simultaneous upregulation of GARP and Foxp3 needs to be demonstrated, these findings suggest that platelets are another subset where GARP and Foxp3 interdependence might play a role. Furthermore, human melanocytes simultaneously express Foxp3 and GARP[33].

Conversely, other reports indicate that Foxp3 is not required for GARP expression in Th cells upon TCR stimulation[24]. For example, the expression of GARP/LAP in $Foxp3^{-}$ $Helios^{+}$ Treg is additional confounding evidence. Thus, the interdependence of GARP and Foxp3 expression is an intriguing area that is far from being completely understood[34].

As discussed later, GARP is a latent TGF-β receptor that enhances furin-mediated pro-TGF-β cleavage, yet GARP expression is independent of both TGF-β and Furin[24].

Post-transcriptional regulation is another important checkpoint in GARP expression. The distal part of the 3' UTR region of GARP transcript is targeted by six miRNAs that decrease GARP protein expression. Among these six miRNAs, miR-142-3p is expressed 2.5 times more in Th cells than in Treg cells, and upon TCR stimulation, miR-142-3p expression decreases in both T cell populations[25]. MiR-142-3p facilitates the formation of a complex that together with Argonaute 2 and GARP-mRNA controls GARP expression via post-transcriptional regulation[35].

1-B. GARP in TGF-β1 regulation and tolerance

TGF-β1 in Cancer

The importance of TGF-β1 is demonstrated by the lethal phenotype of the global TGF-β1 knockout mice: TGF-β1 null mice die within 3-4 weeks after birth due to multiorgan inflammation[36]. On the other hand, TGF-β1 induces a variety of malignant cellular phenotypes like invasion, loss of cellular adhesion, epithelial to mesenchymal transition, and metastasis[37]. Retrospective studies highlight that in late stages of cancer, TGF-β1 levels positively associate with aggressiveness and poor prognosis. Consistent with this notion, intense TGF-β1 expression has been positively correlated with metastasis in breast carcinoma, prostate cancer, and colorectal cancer.[38,39] Additionally, aggressive forms of human cancers upregulate the production of TGF-β1, thus supporting their own growth in an autocrine and/or paracrine fashion.[40] Consequently, TGF-β expressing cancer cells are constantly supported by a positive feedback loop and have a selective advantage against the tumoricidal natural and lymphocyte-activated killer cells. TGF-β1 modulates the TME by favoring the evasion of cancer cells from immune-surveillance, by tempering both the anti-tumor innate and adaptive immunity[41]. With regard to innate immunity, TGF-β1 induces M2 macrophages polarization[42] and inhibits NK cells and DC maturation[43,44]. Similarly TGF-β1 impairs the adaptive anti-tumor immunity by directly inhibiting the clonal expansion and cytotoxicity of the $CD8^+$ T CTLs[45,46]. Furthermore, TGF-β1 indirectly attenuates CTLs by inducing the expression of Foxp3, which confers a regulatory and immune suppressive phenotype to $CD4^+$ T cells[47].

During tumorigenesis, TGF-β1 has a dual role: it can act both as a tumor suppressor and as a tumor promoter cytokine[48,49]. In breast cancer, for example, the dual role of TGF-β1 action appears to be cancer stage-specific: TGF-β1 functions as a powerful tumor suppressor in early stage breast cancer, while behaving as an oncogene in aggressive and advanced stage mammary tumors. In the early stages of breast cancer development, TGF-β1 controls cell proliferation mainly by inhibiting cell cycle progression through G1-arrest and by inducing or activating cyclin-dependent kinase (cdk) inhibitors. However, in advanced stage mammary tumors, cells acquire a TGFβ-1 dependent immunosuppressive ability and a mesenchymal phenotype[50]. In metastatic breast cancer, TGF-β is the major contributor to lung and bone metastasis through Smad-dependent signaling pathway[51]: human breast cancer bone metastasis shows a Smad4-dependent activation of pro-metastatic genes[52].

GARP Enhances TGF-β Formation and Activation

TGF-β is widely expressed in most tissues and exists in at least 4 different forms: 1) freely soluble TGF-β; 2) soluble TGF-β associated with LAP, known as latent TGF-β; 3) TGF-β-LAP-LTBP, latent TGF-β associated with large TGF-β binding protein (LTBP); and 4) membrane latent form of TGF-β[53]. GARP monomers are expressed on the cell surface where they act as a latent TGF-β1 receptor[54]. Three forms of TGF-β exist: TGF-β1, TGF-β2, and TGF-β3, encoded by 3 different genes; yet, TGF-β1 is the most studied among the three isoforms[55]. The close association with GARP seems to be almost uniquely limited to latent

TGF-β1, since latent TGF-β3 failed to bind to GARP, and latent TGF- β2 showed a much lower binding affinity[19].

As a very powerful cytokine, Robertson et al. refer to TGF-β as the “beast”[56]; its production and secretion consist of multiple tightly regulated steps, and interestingly, GARP plays a role in each of them. First, TGF-β is synthesized and secreted by the Golgi as inactive homodimeric pro-proteins that are cleaved by furin-type proteases to generate a mature TGF-β. At this stage, the newly synthetized molecule is non-covalently associated with the LAP, referred to as latent TGF-β. A study from Sophie Lucas’ laboratory clearly demonstrated that GARP increases the rate of pro-TGF-β cleavage in a furin independent manner[25].

Subsequently, latent TGF-β associates with the latent TGF-β binding protein (LTBP), creating the large latent complex (LLC)[57]. GARP can interfere with this association due to its higher affinity to latent TGF-β: when both GARP and LTBP are co-expressed in 293T cells, GARP outcompetes with LTBP for latent TGF-β disulfide binding at the Cys4. Interestingly, electron microscopy analysis showed that GARP and latent TGF-β association also can be mediated by non-covalent binding[14]. The nature and function of this weakly associated complex might mediate TGF-β activation upon surface shedding[58], as discussed below.

Finally, the release of the biologically active peptide requires the proteolytic separation of the mature form of TGF-β from the LAP. Multiple mechanisms have been evoked to describe this critical step, where cell surface integrins are the main orchestrators. αVβ6 and αVβ8 can activate latent TGF-β through proteases-dependent and protease-independent mechanisms. In the proteases, independent

mechanism integrins αVβ6 and αVβ8 bind to the latent TGF-β and, deforming the surface LAP, they mediate the releases of the mature form of TGF-β. In the protease-dependent mechanisms, integrins recruit metalloproteinases or serine proteases that cleave LAP and, subsequently, free TGF-β[59,60]. For example, thrombin mediates the activation of latent TGF-β bound to αVβ6 in a mouse pulmonary edema model[61]. Novel findings demonstrated that membrane bound GARP facilitates the protease independent TGF-β activation facilitating the formation of a complex together with αVβ6 integrins and latent TGF-β. Intriguingly, the association of the GARP/latent TGF-β complex with integrins does not disrupt the ring like structure of the pro-TGF-β, suggesting that the integrins interaction is essential, yet it is not sufficient for secretion of mature TGF-β[14]. This may indicate that, similarly to latent TGF-β alone, integrin binding to LAP predisposes the complex for the release of the active peptide; however, extra tensile force is required for the removal of the "straightjacket" elements of LAP[62]. This might explain why GARP/latent TGF-β complex does not release active TGF-β, yet is still able to activate TGF-β signal transduction, as shown in Treg[63], B cells[63] and TGF-β reporter cell lines[14].

Integrins contribution also has been described in activated Treg, where αVβ8 integrins are responsible for the release of latent TGF-β from the cell surface and for the formation of biologically active TGF-β[64]. Also in this case, the biological activity of TGF-β was not directly measured in culture medium, but it was indirectly measured by a Th17 induction co-culture system. Intriguingly, integrins and membrane tensile forces do not explain the release of mature TGF-β from soluble

GARP (sGARP). This conundrum was partially unveiled by Fridrich and colleagues when they observed that mature TGF-β can be released from sGARP only when GARP and latent TGF-β are non-covalently associated[58]. However, the intimate mechanism is still obscure.

GARP and Peripheral Tolerance

As previously discussed, GARP promoter has a binding region for Foxp3, indicating that Treg-specific transcription factor is required for GARP expression. Accordingly, silencing Foxp3 in human Treg reduces surface GARP upon TCR stimulation. On the other hand, enforced expression of GARP in human Th endows cells with suppressive capability by upregulating several Treg signature genes like Foxp3, CD25, and CTLA4[27,65]. These findings suggest that the tolerogenic Treg phenotype might be enforced by a positive feedback loop between GARP and Foxp3. Accordingly, in Treg cells which are differentiated *in vitro*, GARP sh-RNA partially impairs normal suppressive ability[30]. The critical role of GARP in tolerance is demonstrated by fatal inflammatory disease developed by mice with a Treg-specific *gp96* deletion. As mentioned above, gp96 serves as chaperone for GARP folding: its deletion prevents GARP/latent TGF-β expression on activated Treg and consequently the acquisition of a suppressive phenotype. Accordingly, the fatal phenotype is partially rescued by exogenous active TGF-β administration[10]. This work might give a mechanistic explanation for the atopic dermatitis manifested by patients with gene mutations in the *Lrrc32* gene locus that prevents GARP surface expression[66].

In allergen-specific gut inflammation, injection of anti-GARP blocking antibody was sufficient to reduce the therapeutic effect of activated Treg cells[67], while during oral tolerance, blocking IL-6 signaling in presence of TGF-β polarized CD4+ T cells versus a $GARP^{+}LAP^{+}$ Treg that was critical to maintain oral tolerance in a DHT (delayed type hypersensitivity) reaction model[68]. Furthermore, monoclonal GARP/latent TGF-β antibody blocks the autocrine production of active TGF-β in Treg restraining their immunosuppressive activity in xenogeneic GVHD[69]. In line with these results, sGARP has been proven useful as an anti-inflammatory therapeutic agent by sustaining regulatory T cells differentiation in two different models of xenogeneic GVHD[67,70]. In addition, allergic airway inflammation is mitigated by sGARP injections in a TGF-β dependent way[71]. Conversely, other reports indicate that Foxp3 is not required for GARP expression on Th cells upon TCR stimulation and that $Foxp3^{+}$ Treg maintains the same suppressive phenotype even in absence of GARP[24].

GARP and Tumor Tolerance

Although in inflammation-driven phenotypes GARP offers several therapeutic benefits, in tumor settings the tolerogenic Foxp3/GARP/TGF-β axis is a mediator of the immunosuppressive microenvironment that enhances tumor growth. Human ovarian cancer ascites are infiltrated with $Foxp3^{+}GARP^{+}$Treg[29]. Higher frequency of $GARP^{+}Foxp3^{+}$ Treg expression positively correlates with an elevated immunosuppressive and more aggressive phenotype in advanced hepatocellular carcinoma[72]. Human melanocytes express and secrete membrane

bound GARP and sGARP, respectively, that skew M2 macrophages toward a polarized phenotype and constrain the proliferation of CTLs and the production of cytokines [33]. GARP is highly expressed in human breast, colon, and lung cancer where GARP/TGF-β axis sustains primary tumor growth and distal metastasis formation. Antibody mediated blocking of GARP and latent TGF-β interaction is a therapeutic strategy in a syngeneic mammary carcinoma model[1]. Likewise, LAP blocking antibody, that prevents secretion of active TGF-β, reduces tumor growth in several animal models by decreasing the number of LAP^+ Treg[73].

1-C. Platelet GARP

Platelets and Cancer Bidirectional Activation

When circulating through blood vessels with an intact and healthy endothelium, platelets remain in an inactivated state. However, any injury to the endothelium will expose platelets to molecules such as collagen, thromboxane A2, ADP, and thrombin that are present in the subendothelial matrices which will trigger the activation of platelets. Activated platelets express receptors like glycoprotein (GP) Ib IX-V, GP Ia/IIa, GP VI, and p-Selectin, which mediate the aggregation of platelets and clot formation. Furthermore, upon activation platelets secrete a “releasate” rich in α granules that contain over 300 proteins[74]. Alan T. Nurden classified the proteins in the platelets releasate (PR) based on their biological activity in: metabolites like adhesive proteins, clotting factors, fibrinolytic factors, proteases and anti-proteases, growth and mitogenic factors, chemokines, cytokines, antimicrobial proteins, and membrane glycoproteins[75]. In addition to tissue homeostasis, these proteins are involved in the modulation of innate and adaptive immunity, in inflammation, and in major diseases like atherosclerosis, multiple sclerosis, and cancer.[76]

The leaking tumor vasculature (the tumor is a wound that does not heal)[77] causes adhesion and subsequent activation of platelets that, in response, secrete several proteins that fuel tumor growth. This platelets-cancer loop is a vicious circle where activated platelets and growing tumor reciprocally feed and support each other. [78], In addition to the leaky vasculature, tumors have the ability to produce tissue

factors, ADP, and thrombin to activate the surrounding platelets through the peroxisome activation receptors (PARs) expressed by platelets. On the other hand, each activated platelet releases up to 80 α granules that secrete platelet derived growth factors (PDGFs) in tumor proximity. One of the most abundant and critical PDGFs is TGF-β.[79-81] In cancer-driven platelets activation, TGF-β released by platelets supports primary tumor growth by mitigating anti-tumor immunity and promotes intravasation to neighboring blood vessels, thus facilitating the metastatic process[82]. Venous thromboembolism (VTE) is a direct consequence of the massive platelet activation that occurs in cancer patients[83]. This phenomenon, better known as, Cancer-Associated thrombosis, is the second most common cause of mortality in patients with cancer[84]. Epidemiological estimates show that the annual incidence of venous thromboembolism (VTE) in cancer patients may be as much as 500/100,000/year compared to ≈ 70–113 cases/100,000/year in the general population[79].

GARP on Platelets

As mentioned before, GARP was first identified on activated regulatory T cells and platelets[19]. Despite the increasing knowledge about the role of GARP in regulatory T cells, little attention has been given until now to the role of GARP on platelets. It is not totally clear whether GARP plays a role in platelet activation and function, since two studies in two different animal models seem to contradict each other. A first study performed on *Danio rerio* (zebrafish) demonstrated that GARP

is important for thrombus initiation and tissue homeostasis: knockdown of the *Lrrc32* gene resulted in spontaneous bleeding events.[85] A second study, performed on a genetic mouse model where *Lrrc32* is specifically knocked out from platelets and megakaryocytes, shows that GARP is not necessary for thrombus formation and clot retraction. Interestingly this last work shows that *ex-vivo* platelets activation triggers increase in GARP surface expression, indicating that GARP might play a role in active platelets beside thrombus formation[31].

1-D. Thesis Significance

GARP's ability to maintain the peripheral tolerance in the steady state is now accepted, delineating a fascinating scenario where GARP imposes itself like a new marker of activated and fully suppressive Treg in a TGF-β dependent manner[67].

Conversely, several other aspects of GARP biology still need to be studied to have a complete understating of GARP function.

First, this thesis addresses the mechanistic aspect of soluble GARP (sGARP) generation and function. sGARP is present in plasma[26] and has anti-inflammatory functions in the xenograft model of GVHD[70] and in allergic airway inflammation[71], yet how sGARP is generated and how the biologically active TGF-β is released remain unknown. This thesis describes two possible scenarios in which sGARP/latent TGF-β complex can be released. sGARP is a vehicle of latent TGF-β that eventually will become activated; thus, understanding the mechanism behind the generation of this soluble complex is a necessary step to control its presence and spread in diseases where TGF-β plays a major role.

Second, it is known that the *Lrrc32* gene is amplified in human aggressive carcinomas; however, no evidence of GARP expression had been reported when this thesis started. Many tumors are TGF-β dependent environments, and GARP concomitant expression is a newly described phenomenon that gives more insights into TGF-β regulation on cancer cells. Also, this thesis explores the possibility that

GARP is a novel tumor biomarker as well as a target for anti-GARP antibody based therapy.

Finally, regardless of the extensive study of GARP function on Treg, only one study about GARP on megakaryocytes and platelets occurred prior to the current thesis[85]. This first study showed that GARP on platelets is important for coagulation; however, other major platelet functions were not explored. The third part of this thesis investigates the function of GARP-specific platelets in physiological conditions and in the presence of tumor. The current study examines platelet GARP as a potential mediator and enhancer of the cross-talk between tumor cells and platelets. Given that platelets are the major TGF-β reservoir and GARP enhances TGF-β activation, we aim to extend the knowledge on the role of GARP in the TGF-β-mediated bidirectional talk between platelets and cancer.

Chapter 2

Materials and Methods

Cell Lines

Pre-B cell line (70Z/3) was a gift from Dr. Brian Seed (Harvard University) [86]. 4T1, NMuMG, and NMuMG* were obtained from Dr. Philip Howe (Medical University of South Carolina). NMuMG* subline with silencing of hnRNP E1 were described previously[87]. 70Z/3 was validated by flow cytometry using B cell lineage markers. MC38 tumor cells were obtained from Dr. Mark Rubinstein (Medical University of South Carolina). 293FT and other cell lines were purchased from ATCC. 4T1, NMuMG, HEK-293FT, B16-F1 and MC38 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. PreB cells were cultured in RPMI medium with 10% fetal bovine serum, 100 units/ml penicillin,100 µg/ml streptomycin, and 1% β-Mercapto Ethanol in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cancer cells were authenticated by gene expression analysis, *in vivo* growth, and histology. All the lines were monitored for pathogens as per MUSC regulations, and we routinely perform mycoplasma analysis on the lines.

Mice

BALB/c and NOD–*Rag-1*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Platelets-specific GARP KO were obtained by crossing *Lrrc32flox/flox*[24] mice with *Pf4-Cre* mice[88]. C57BL/6 WT mice were purched from the Jackson Laboratory. All animal experiments were conducted under approved protocols by the Institutional Animal Care and Use Committee at MUSC.

Generation of GARP-expression vectors

GARP was amplified by PCR and subcloned between the BglII and HpaI sites in a MigR1 retroviral vector[11]. A cDNA construct for expression of the GARP-Fc fusion protein (sGARP) was generated by joining the extracellular domain of GARP to the sequence encoding the Fc portion of murine IgG2a by PCR using the following primers:

GARP-FC fusion forward:
AAGGGAGGGCTGAAGAATGTCAACGGCGGAGGTGGGTCGGGTGGCGGC

GARP-FC fusion reverse:
GCCGCCACCCGACCCACCTCCGCCGTTGACATTCTTCAGCCCTCCCTT

Plasmid containing mouse Fc domain of IgG1 were donated by Stephen Tomlinson (Medical University of South Carolina)

Retrovirus production and cells spin infection

Ecotropic GARP and sGARP retroviral particles were packaged into the Phoenix-Ecotropic cells in presence of Lipofectamine 2000 (Fisher)[10,11]. After 48 h, virus containing medium was collected, centrifuged at 3000 rpm for 10 minutes, filtered with 0.25 µm filter, and stored at -80°C. For the spin infection, 2×10^5 cells were suspended in 1 ml along with 8ug/ml of polybrene and centrifuged for 90 minutes at 32°C and 3,200 rpm. 1 ml of fresh medium was then added to the cells. Cells were stably selected by culturing in the presence of Blasticidin 48 h post transduction for at least 72 h.

Lentivirus production and transduction

For mouse GARP and thrombin knockdown and control, scrambled lentivirus vector-expressing short hairpin RNA (shRNA) transcripts were purchased from Sigma-Aldrich (St. Louis, MO), and viral particles were produced in HEK293FT cells as described previously[11,89]. Briefly, 239 HEK cells were transiently transfected with recombinant DNA vector in the presence of Delta 8.9 and VSV-G plasmids. Subsequently, the cells were spin infected with lentiviral supernatants. The knockdown efficiency was assessed by RT-PCR (Applied Biosystems Step-One Plus), flow cytometry (BD Verse), and western blot using an anti-mouse GARP antibody (eBioscience).

Purification of soluble GARP

sGARP protein was purified in the Dr. Stephen Tomlinson laboratory. For purification of sGARP, MigR1 vector was transfected into Chinese hamster ovary (CHO) cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stably transfected clones were selected by blasticidin (5 µg/ml), and protein expression was quantified by SDS-PAGE and western blot under reducing conditions using anti–mouse GARP and anti–mouse Fc antibody. Recombinant sGARP was purified from cell culture supernatants by protein A affinity chromatography (GE Health).

Surface and intracellular Flow Cytometry

All staining protocol, flow cytometry instrumentation, and data analysis were performed in single cell suspension. Cells were washed in FACS buffer twice, FcR blocking was performed 10 minutes at room temperature, and surface antibodies were applied for 30 minutes at 4°C in FACS buffer. For transcription factors and cytokine analysis, cells were fixed and permeabilized using Foxp3/Transcription factor Set (eBioscience cat # 00-5523-00) according to the manufacturer's protocol.

Tumor infiltrating Lymphocytes (TIL) isolation

For TIL isolation, tumors were resected, chopped in small parts, and enzymatically digested in 1% Collagenase at 37°C for 1 hour shaking at 250 RPM. Tumors were filtered to have a single cell suspension, and lymphocytes were separated by density gradient centrifugation using Histopaque 1083 (Sigma cat #

10831). Lymphocytes were then analyzed by surface and intracellular flow cytometry.

Human tumor microarrays

All human tumor microarrays (TMAs) were derived from formalin-fixed, paraffin embedded tissues collected at the Medical University of South Carolina (Charleston, SC). Each patient specimen in these TMAs was represented in two cores on the slide, and each core measured 1 mm in diameter. These patient specimens were available in a single core of 2 mm in diameter. Clinical and demographic information were obtained from the Cancer Registry of the Hollings Cancer Center at MUSC or provided by the commercial source. This study was approved by the Institutional Review Board (IRB) at MUSC.

Immunohistochemistry (IHC)

The mouse anti-human GARP (hGARP) antibody (ALX-804-867-C100, Enzo Life Sciences) was first verified by western blot using hGARP-transfected HEK-293 cells and by IHC with hGARP-transfected 70Z/3 cells. Both analyses demonstrated specificity of the antibody at 1:100 dilution. TMA slides were processed, and antigen retrieved as described previously[90]. For mouse IHC, tissue was either placed into OCT media for fresh frozen sections or fixed in 4% paraformaldehyde overnight. Fixed tissue was incubated in 70% ethanol overnight prior to paraffin embedding, then cut for hematoxylin and eosin (H&E) staining. For p-Smad-2/3 on fresh frozen tumor sections, 5 µm sections were fixed with 4% paraformaldehyde followed by incubation with 3% H_2O_2. To minimize nonspecific

staining, sections were incubated with the appropriate animal serum for 20 min at room temperature, followed by incubation with primary anti-p-Smad-2/3 antibody (EP823Y; Abcam) overnight at 4°C. Staining with secondary antibodies (Vectastain ABC Kit) was then performed before development using DAB substrate (Vector Labs SK-4100). The staining intensity of GARP and pSmad-2/3 was graded as follows with the sample identity blinded (0: negative; 1: faint; 2: moderate; 3: strong but less intense than 4; and 4: intense).

Generation and characterization of anti-GARP antibody

Four BALB/c mice were immunized with recombinant human GARP (R&D Systems, Minneapolis, MN) with Freund's complete adjuvant, followed by boosting with SP2/0 cells stably expressing human GARP for 2-3 times. Splenic B cells from mice with high anti-GARP antibody titers were fused to SP2/0 cells in the presence of polyethylene glycol. Hybridomas were selected in HAT medium and cloned by limiting dilution assay. The specificity of antibody was screened and determined by ELISA and flow cytometry using 70Z/3 cells stably transduced with empty vector (70Z/3-EV) and overexpression of human GARP (70Z/3-GARP).

Protein extraction, immunoprecipitation, and western blot analysis

Cells were harvested by trypsin-EDTA when necessary, washed in PBS, and lysed on ice in radio-immunoprecipitation assay (RIPA) lysis buffer in the presence of a protease inhibitor cocktail (Sigma-Aldrich). Nuclear-free protein lysate was quantified by Bradford assay (Bio-Rad), and an equal amount of lysate

was analyzed by SDS-PAGE and western blot under reducing conditions using anti–mouse GARP (AF6229; R&D system), anti-mouse Vimentin (D21H3; Cell signaling), anti-mouse E-Cadherin (24E10; Cell Signaling), and anti-mouse p-Smad-2/3 (EP823Y; Abcam) antibodies.

Cell proliferation and in vitro wound healing assay

To measure cell proliferation, 2.5 x10^4 NMuMG* cells were seeded in a 96-well plate in complete medium (DMEM, 10% FCS, 1% penicillin-streptomycin) and incubated overnight. Proliferation was determined with 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which was added to the cells at the indicated times and incubated for an additional 3 h at 37°C. The medium was then removed and mixed with 100 µl of DMSO for 15 minutes by shaking. Absorbance at 570 nm was then measured using a plate reader. The cell migration was measured by the wound-healing assay: at 100% confluence, two parallel wounds were made using a 1 ml pipette tip. Migration was assessed after 24, 48, and 72 hours, and quantification of wound closure was measured using the ImageJ software (NIH).

4T1 Tumor model, $CD25^+$ cell depletion and GARP antibody therapy

Female 6-8-week old BALB/c mice were inoculated in the fourth mammary fat pad subcutaneously (s.q.) with 5 x 10^5 cells (4T1-EV, 4T1-GARP, or 4T1-sGARP). Tumor growth was monitored three times per week with a digital vernier caliper, and tumor volume was calculated using the following formula: tumor

volume (mm^3) = [$(width)^2$×length]/2. In GARP antibody therapy experiments, beginning at 3 days post-tumor inoculation anti-GARP antibody or isotype-controlled antibody (0.1 mg/mouse in 0.1 mL PBS; three times per week) were administered intraperitoneally (i.p.) into mice. For combination therapy with cyclophosphamide (CY) and antibody, mice were treated with one injection of CY (4 mg/mouse) 3 days post-tumor inoculation in addition to the antibody treatment. For $CD25^+$ cell depletion, mice received 500 μg PC61 antibody via i.p. administration every 4 days, beginning 2 days before 4T1 injection. At end-point, mice were sacrificed, and the primary tumor, spleen, and lungs were isolated. Primary tumors were weighted, and IHC was performed. Lung macro- and micro-metastases were determined by visual inspection and microscopic analysis respectively. Tumor infiltrated lymphocytes were isolated by Collagenase D (Sigma) digestion followed by Histopaque-1083 (Sigma) mediated density separation.

NMuMG tumor model

Female NOD-*Rag-1*$^{-/-}$ (n=5 each group; 6-8 week old) mice were inoculated in the fourth and left mammary fat pad using 5 x 10^5 cells (NMuMG*-EV, GARP knockdown NMuMG*). Each week the animals were weighed, and tumors were measured. At endpoint, primary tumors, lungs, and livers were harvested. In another experiment: female NOD-*Rag-1*$^{-/-}$ mice (n=4-5 each group; 6-8 week old) were inoculated in the fourth left mammary fat pad with 5 x 10^5 cells (NMuMG-GARP-Luc, NMuMG-sGARP-Luc or NMuMG-Luc cells). *In vivo* luciferase imaging

was conducted weekly as follows: mice were intraperitoneally injected with D-luciferin (Perkin Elmer) at a dose of 150 mg/kg per mouse and anesthetized. Bioluminescence images were then acquired using the Xenogen IVIS imaging system. Bioluminescence signal was quantified as photon flux (photons/s/cm^2) in defined regions of interest using Living Image software (Xenogen).

GARP ELISA

Plasma from prostate cancer patients and healthy controls were kindly donated by Dr. Michael Lilly and Dr. Jennifer Wu (Medical University of South Carolina). Samples were analyzed in duplicate according to the manufacturer's protocols (BioLegend, San Diego, CA).

GARP/TGF-β1 sandwich ELISA

To measure GARP/TGF-β1 complex by ELISA, 96 well plates were coated with TGFβ1 capture antibody according to the manufacturer's instructions (BioLegend, San Diego, CA). Samples were incubated for 2 h at room temperature followed by incubation with the anti-hGARP detection antibody developed by our lab for two hours.

Thrombin time

Mice were anesthetized. A superficial incision was made approximately 1 inch from the base of the tail. The incision was then blotted with Whatman paper every 10 seconds until bleeding ceased. The time from the incision to bleeding cessation was recorded.

Actiavtion of platelets and Preparation of releasate from activated platelets

Platelets-specific GARP KO and control mice were anesthetized, and blood was withdrawn to a 5 mL tube containing another 0.5 mL of acid citrate dextrose (ACD) buffer (39 mM citric acid, 75 mM sodium citrate, 135 mM dextrose, 1 μg/mL prostaglandin E1, pH 7.4). Samples were centrifuged for 10 min at 100 g, with no brake, and the upper layer of platelet-rich plasma was collected. Platelets were washed 2x with citrate washing buffer (128 mM NaCl, 11 mM glucose, 7.5 mM Na2HPO4, 4.8 mM sodium citrate, 4.3 mM NaH2PO4, 2.4 mM citric acid, 0.35% BSA and 50 ng/mL prostaglandin E1, pH 6.5), then resuspended in RPMI or PBS, enumerated by a blood cell counter, and diluted to a final concentration of 1x108/mL. Purified platelets were incubated with or without mouse thrombin (Enzyme Research Laboratories, South Bend, IN) for 1 hour at 37°C. Stimulated platelets were sedimented by centrifugation for 15 min at 3,200 g, and supernatant was collected and stored at -80°C.

Platelets Releasate western blot

Stimulated platelets with and without 1U/ml of thrombin were sedimented by centrifugation for 15 min at 3,200 g, and supernatant was collected. For reducing conditions, western blot PR was boiled for 5 minutes and loaded in the presence of dithiothreitol (DTT) and β-Mercaptoethanol. For non reducing and non denaturating western blot, no DTT and no β-Mercaptoethanol were used. Also samples were not boiled.

Soluble TGF-β1 ELISA from Serum and Platelet Releasate

Mouse serum samples were obtained from the lateral tail vein. Capture ELISA for TGF-β1 was performed according to manufacturer instructions (BioLegend, San Diego, CA). Blood was collected in Eppendorf tubes, allowed to coagulate for 30 minutes at room temperature, and centrifuged at 5,000 rpm for 15 minutes. Active TGF-β was measured with no additional manipulation. Total TGF-β1 was measured following acidic activation using 1 M HCl for 10 min at room temperature, and neutralization occurred with 1.2 NaOH, 1 M Hepes. Active TGF-β1 and total TGF-β1 levels were measured using TGF-β1 ELISA kits according to the manufacturer's protocols. Similar procedures were adopted for platelets releasate.

MC-38 model

WT or Plt-GARPKO mice were injected in the right flank with $1x10^6$ MC38 colon cancer cells. Tumor area was measured every 3 days with digital vernier caliper kinetically. Tumor infiltrating lymphocytes were isolated from fresh primary tumors by density gradient, after single cell suspensions were made with mechanics and enzymatic digestions as previuously described.

B16-F1 model and Adoptive T cell therapy

Treatment of B16-F1 melanoma by adoptive transfer of ex vivo activated Pmel T cells occurred as described previously[91]. 6-8-week old female C57BL/6J mice were inoculated subcutaneously (s.q.) in the right flank using 2.5×10^5 cells and, where specified, treated with one intra-peritoneal injection of cyclophosphamide (CY, 4 mg/mouse) one day prior to adoptive T cell therapy (ACT). To obtain gp100-specific T cells, the splenocytes from Pmel1 TCR transgenic female mouse were stimulated with hgp100 (25-33 epitope, 1 µg/ml, American peptide Company) and mouse IL-12 (10 ng/ml, Shenandoa) for 3 days. ACT occurred through tail vein injection of $2x10^6$ activated Pmel T cells per recipient mouse one day after injection of CY. Growth of primary tumors was monitored three times per week with vernier calipers. Peripheral adoptively transferred Pmel cells were monitored at 2, 3, 4, and 5 weeks after ACT. Ex-vivo Pmel IFN-γ production was assessed by stimulating Pmel cells for 3 hours in the presence of hgp100 and brefeldin A BFA at 37°C and analyzed by flow cytometry.

T-250

Thrombin cleavage bloking experiment were performed using the following peptide:

DLRENKLLHFPDLAVFPRLLIYLNVSNNLIQLPAGLPRGSEDLHAPSEGWSA

generated in Hangzhou Dangang biological technology (HangZhou, China).

Dabigatran and Dabigatran Etexilate

For *in vivo* experiments, mice daily received gavage 3mg/mouse of Dabigatran Etexilate (Boehriger Ingelheim) dissolved in water. For *in vitro* experiments, platelets were treated with 4 µg Dabigatran (Cayman)

PD1 bloackade therapy

200 µg of anti mouse PD-1 blockade antibody (Bioxcell) were intraperitoneally administrated every 3 days.

Mass Spectrometry

Cell lysate was loaded, and SDS-PAGE was run. Gel was stained with Coomassie blue, and the fragment of interest was analyzed by mass spectrometry.

Thrombin digestion

10^6 Pre-B cells were centrifuged and resuspended in thrombin buffer: 50 mM Tris HCl pH 8.0, 100mM NaCl, 2.5 mM $CaCl_2$, 0.1% β Mercapto Ethanol. Cells were digested for 2 h at room temperature with 4 µg of thrombin in 100 µl volume. Cells were then vortexed briefly, centrifuged, and lysed in RIPA buffer.

Site direct mutagenesis

Proline-Arginine at 267-268 and 286-287 amino acidic positions were both mutated to Alanine-Alanine using the QuikChange II XL Site-directed Mutagenesis kit (Stratagene). The following primers we used:

267-268 point mutation forward: GGCCGTGTTCGCGGCACTCATCTACC

267-268 point mutation reverse: GGTAGATGAGTGCCGCGAACACGGCC

286-287 point mutation forward: GCGGGGCTGGCCGCAGGCAGTGAGG

286-287 point mutation reverse: CTCACTGCCTGCGGCCAGCCCCGCA

All constructs were subcloned into MigR1 retroviral vector for retrovirus production as previously reported[11]. The efficiency of mutagenesis was assessed by DNA sequencing and Western blot analysis.

RNA isolation and quantitative RT-PCR.

RNA was isolated using Trizol reagent and analyzed by quantitative RT-PCR with the following primers using SYBR Green reagent:

Mouse Alpha V Forward: AGGTTGACCTCGCCGAAAAG

Mouse Alpha V reverse: GCTGAACAATAGGCCCAACG

Mouse beta 3 forward: TTACCACGGATGCCAAGACC

Mouse beta 3 reverse: CCCCAGAGATGGGTAGTCCA

Mouse beta 5 forward: CCGCTTAGGTTTCGGGTCTT

Mouse beta 5 reverse: GGGACGCAGTTGGGGAATAA

Mouse beta 6 forward: TCATAAAGCCAGTGGGGCTG

Mouse beta 6 reverse: CCACACTGGAAGGAGCCATT

Mouse beta 8 forward: CAACTGCATCCAGGAGCTGA

Mouse beta 8 reverse: CAAGACGGAAGTCACGGGAA

Mouse Thrombin forward: CCGTGTGTTTGCCAGACAAG

Mouse Thrombin reverse: TTGGTTGTCCATGTCTCCCG

Mouse GARP forward: CGCTTCGTCACCTGGATTTA

Mouse GARP reverse: ATTGTGGGCCAGGTTAAGG

NMuMG stimulation with soluble GARP and RGD peptide: confocal analysis and flow cytometry

Cells were stimulated with 5µg/ml of soluble GARP or multiple mouse IgG in serum free medium for 1 hour at 37° C. Cells were then washed and fixed only, or fixed and permeabilized using Image-iT Fix-Perm kit (Molecular probes). Anti-

mouse Alexa Fluor 594 conjugated antibody was used. For the RGD blocking experiment, 5ug/ml of RGD peptide was added to the cells 30 minutes before adding soluble GARP. For GFP- p-SMAD3 reporter vector[92], transformed cells were stimulated with the indicated concentration of soluble GARP and analyzed by flow cytometry for GFP expression.

Exosomes isolation

HEK 293 cells were grown to confluence in T75 cm flasks, and complete medium was changed to serum-free medium for 24 h. Medium was collected and centrifuged at 2,000x*g* for 10 min, then supernatant was collected and centrifuged at 10,000x*g* for 30 min. Supernatant was further collected and centrifuged at 100,000x*g* for 90 min. The pellet containing exosomes was suspended in RIPA buffer to solubilize proteins. Solubilized exosome proteins were quantified and subjected to SDS-PAGE and Western blotting.

L-lactate measurement

Serum was deproteinizated with perchloric acid and subsequently neutralized with KOH. L-lactate concentration was then measured according to the manufactures instructions (Eton Bioscience).

Statistical Analysis

In TMAs where specimens were spotted in duplicate, the average of both cores was used as the representative value. The Student's *t*-test was implemented to compare categorical variables such as normal versus cancer or different disease stages or categories. Kaplan-Meier analysis for correlation of GARP with survival was performed using X-tile software[93]. Population characteristics were tested for statistically significant differences between low and high GARP expressers using the Chi-square test. Tumor curve analysis was performed using 2-way analysis of variance (ANOVA) or a Wad test. For bioluminescence imaging study, random effects linear regression was used to model tumor volume over time. To adhere to model assumptions, a square-root transform of tumor size was calculated before model estimation. Bayesian information criteria were used to determine the best fitting model. The final model included three main effects of time and interactions between group and time. Wald tests were used to compare coefficients and significance across groups; all other experiments were analyzed using the Two-tailed Student's *t*-test with GraphPad Prism. All data are presented as mean ± SEM. *P*-values less than 0.05 were considered statistically significant. The Chi-square test was used to analyze the correlation between high and low serum GARP, with the percentage of prostate cancer patients showing high PSA1 (higher than 10) and secondary tumors.

Chapter 3

Thrombin cleaves GARP and enables latent TGF-β release

Rationale

GARP is a latent-TGF-β receptor expressed abundantly on Treg, platelets, and cancer cells. We and others have demonstrated that GARP can be shed form the cell surface and released extracellularly: plasma from prostate cancer patients contains GARP in association with TGF-β (See figure 4-8), platelets resealed GARP in platelets releasate (PR) (See figure 5-6), and GARP/latent TGF-β complex shed from T cell membrane[25]. The possibility of a shedding process was first discussed by Roubin and colleagues in 1996 when, describing GARP deduced amino acid sequence, the researchers observed the presence of a hydrophobic leader sequence. They hypothesized that this domain might be the signal peptide for protein secretion[8], yet the mechanism for the protein's shedding or secretion is not clear[26]. Although the mechanism of GARP secretion remains evasive, its immunosuppressive abilities have been already investigated. Soluble GARP, indeed, is an anti-inflammatory therapeutic agent that sustains regulatory T cells differentiation in two different models of xenogeneic GVHD[67,70]. In addition, allergic airway inflammation is mitigated by soluble GARP injections in a TGF-β dependent way[71]. In tumor settings, soluble GARP derived by cancer cells exerts its pro-tumorigenic potentials by inducing a tolerogenic TME rich in TGF-β induced

Tregs[1,33]. Soluble GARP therapeutic potentials in autoimmune diseases as well as its pro-tumorigenic activity in malignancies, prompted us to investigate how its formation occurs and how it is regulated.

There are several mechanisms for the formation of a soluble protein, like alternative splicing that removes the transmembrane domain[156] and surface shedding exerted by proteases[157]. Our first observations led us to focus on a potential surface shedding of membrane bound GARP and to investigate the role of thrombin as enzyme involved in the protein's cleavage.

Thrombin is a trypsin like serine protease involved in the conversion of fibrinogen to fibrin. Phage display technology has shown that thrombin active site binds and cleaves a consensus recognition sequence containing Proline and Arginine[158].

As described herein, we performed site specific mutagenesis of putative thrombin binding sites on GARP to investigate the role of thrombin as potential enzyme involved in soluble GARP formation.

Another elusive aspect is the mechanism that soluble GARP uses to elicit TGF-β signaling. Hahn and colleagues clearly showed that soluble GARP drives Treg induction; however, this effect was inhibited in presence of TGF-β receptor II blocking antibody[70]. These results indicate that mature TGF-β is released from soluble GARP/latent TGF-β complex in order to bind to its own receptor on the cell surface.

Fridrich and colleagues observed that there are two forms of soluble GARP, one covalently and one non-covalently associated with latent TGF-β. The researchers

also observed that mature TGF-β can be released from soluble GARP only when GARP and latent TGF-β are non-covalently associated;[58] however, how the release of active TGF-β occurs is not known.

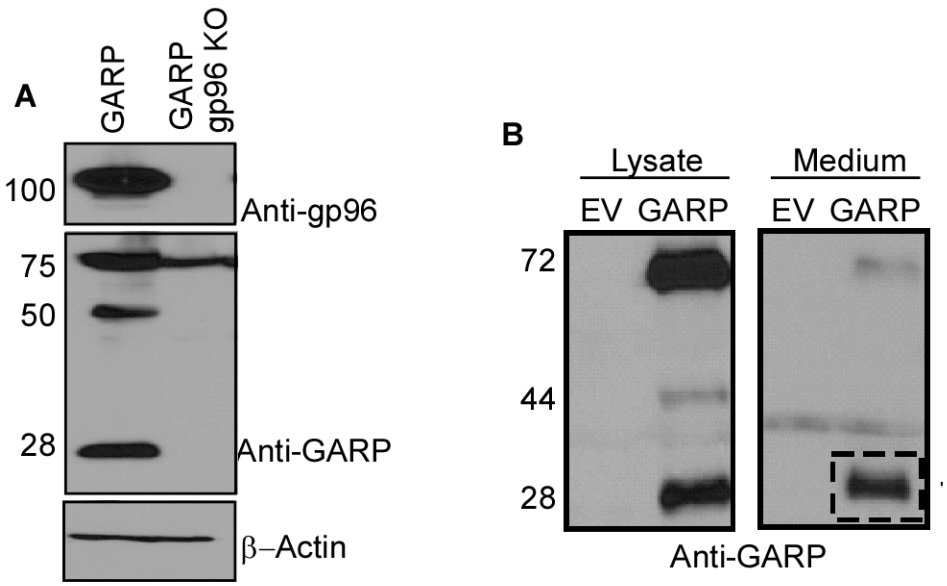
Lastly, another critical mechanism that cells utilize to secrete proteins is through the exosome machinery. These nanoscale vesicles of endocytic origins are secreted by most cells in the extracellular milieu where they travel until they fuse to the membrane of other cells. Exosomes mediate the inter-cellular communication carrying RNA, proteins, and DNA that can affect target cells[159,160]. Interestingly, tumor cells secrete exosomes containing oncoproteins that can mediate distant metastasis formation[161].

Herein we show evidence to support two mechanisms that involve soluble GARP formation: thrombin mediated shedding and exosome mediated GARP shedding. Additionally, we investigate the role of integrins in soluble GARP signal transduction.

Results

GARP is cleaved on the cell surface releasing a 29 KDa fragment in the extracellular environment.

We demonstrated that the molecular chaperone gp96 is critical for cell surface expression of GARP and membrane latent TGF-β[10]. To investigate the role of gp96 in the formation of the soluble GARP, we expressed GARP in WT and gp96 KO Pre-B cells. Cell lysates analysis revealed the presence of three forms of GARP protein: full length protein (72 KD) expressed in both WT and gp96 KD cells, and 2 smaller forms of GARP protein (44 KD and 29 KD) not present in the gp96 KD cells (Figure 3-1A). The formation of smaller fragments of GARP only in presence of gp96 supported the idea that GARP might be shed at the cell surface and be released in the extracellular environment. To address this possibility, we analyzed the presence of GARP in cell lysate and conditioned medium from GARP expressing cells. We observed that the 29 KD fragment was abundantly present in the conditioned medium, and only a small fraction of the protein was present as full length in the extracellular environment (Figure 3-1B). Mass spectrometry analysis showed that the 29 KD GARP fragment belongs to part of the N^+ terminal domain (Figure 3-1C).



C

MCG51019, isoform CRA_b OS=Mus musculus GN=Lrrc32 PE=4 SV=1 - [G3XA59_MOUSE]	44.22	1.96
A3	Sequence	# PSMs
High	LRTLSLAENSLTR	4
High	TLSLAENSLTR	10

Figure 3-1. GARP is cleaved on the cell surface.
(A) Western blot analysis of GARP expressing Pre-B cells WT or gp96 KD. Cell lysates were analyzed by using antibodies against mouse GARP and mouse gp96 (B) Parallel western Blot analysis of cells lysates and conditioned media of Pre-B cells expressing GARP or control vector. (C) Mass spectrometry analysis of the fragment present in the conditioned medium indicated in the box.

Surface GARP is cleaved by thrombin.

The next logical question that we asked aimed to define the mechanism of GARP cleavage. First, we tested the roles of furin, several matrix metalloproteinases, and serine protease inhibitors. We found that the lower GARP fragment decreased only when the serine proteases were inhibited (data not shown). In parallel, GARP amino acidic sequence was analyzed using the resource portal ExPASy to predict the potential proteases cleavage sites. Among the list of enzymes that were suggested to interact with GARP, thrombin attracted our attention for two reasons: first, thrombin is a surface serine protease; second, based on the ExPASy prediction, thrombin mediated cleavage generates two GARP fragments of the same molecular weight of the fragment that we already observed, 44 and 29 KD. (Figure 3-2A). For these reasons, we decided to continue studying the potential role of thrombin in GARP cleavage. We found that upon treatment with increasing concentration of thrombin, both 44 and 29 KD fragments increased in a dose dependent fashion (Figure 3-2B). Because GARP cleavage occurred in the presence and absence of serum in the conditioned medium (data not shown), we reasoned that thrombin was produced by the tumor cells. To address this point further, we knocked down thrombin in GARP expressing cells, and we noticed that the 29 KD GARP fragment was almost completely abrogated, while full-length protein staining intensity was increased (Figure 3-2C). In chapter 5, GARP expression and function of platelets will be analyzed. Just like preB cells, platelets GARP is cleaved by thrombin, revealing the generality of our findings (Figure 3-2D).

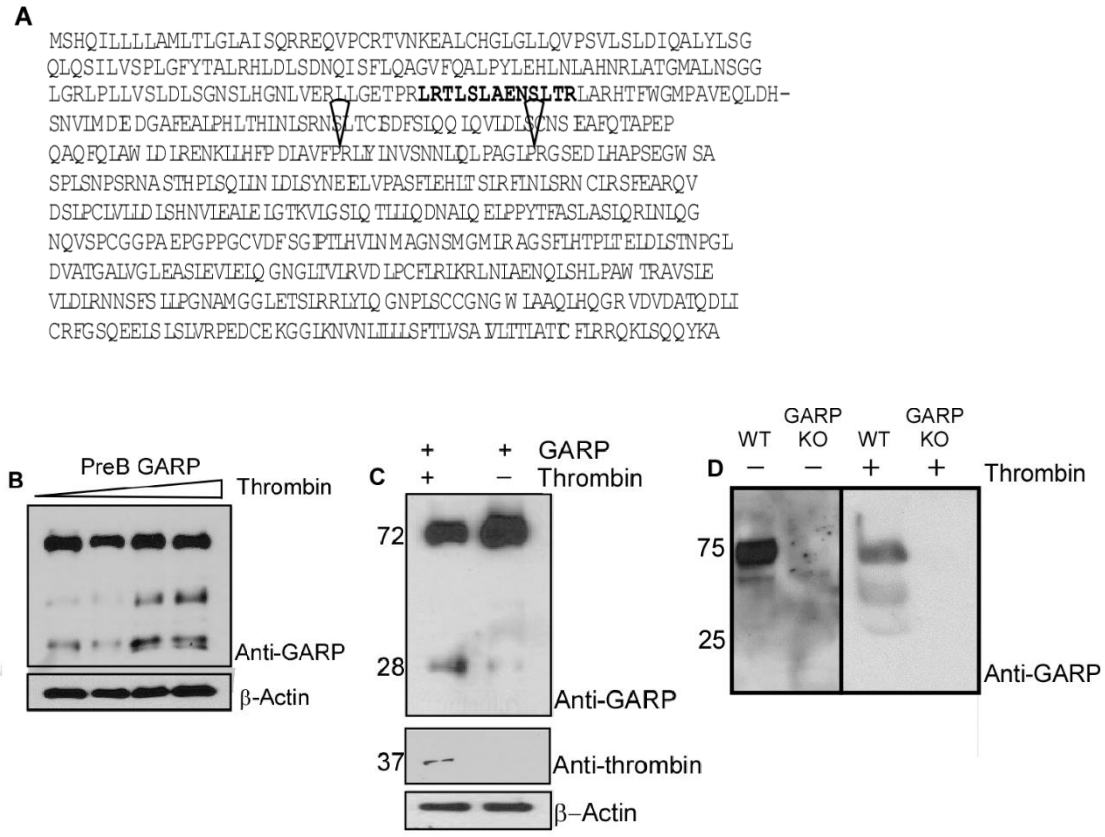


Figure 3.2 Surface GARP is cleaved by thrombin.
(A) GARP amino acidic sequence: The part in bold indicates the sequence found in the conditioned medium; the arrows indicate the predicted thrombin cutting sites based on ExPASy analysis. (B) GARP expressing Pre-B cells were digested with increasing concentration of thrombin (0, 1, 2, and 4 µg in 25 µl), followed by western blot analysis. (C) shRNA mediated KD of thrombin in Pre-B cells expressing GARP followed by western blot analysis. (D) Western blot analysis of WT and GARP KO platelets treated with thrombin.

GARP upregulates thrombin gene expression.

Several cancers upregulate thrombin expression as a survival factor to amplify TCIPA as described in Chapter 5[162,163]. Moreover, as described in Chapter 1, GARP exerts an oncogenic function in epithelial cells[1]. For these reasons, we next asked whether GARP expression could upregulate thrombin expression. Interestingly, enforced GARP correlated with enhanced expression of thrombin mRNA (Figure 3-3A), suggesting a positive correlation between GARP and thrombin gene transcription. In support of a GARP-thrombin correlation, we found a positive association (R=0.127) between GARP and thrombin mRNA in a cohort of 59 breast cancer patients (Figure 3-3B).

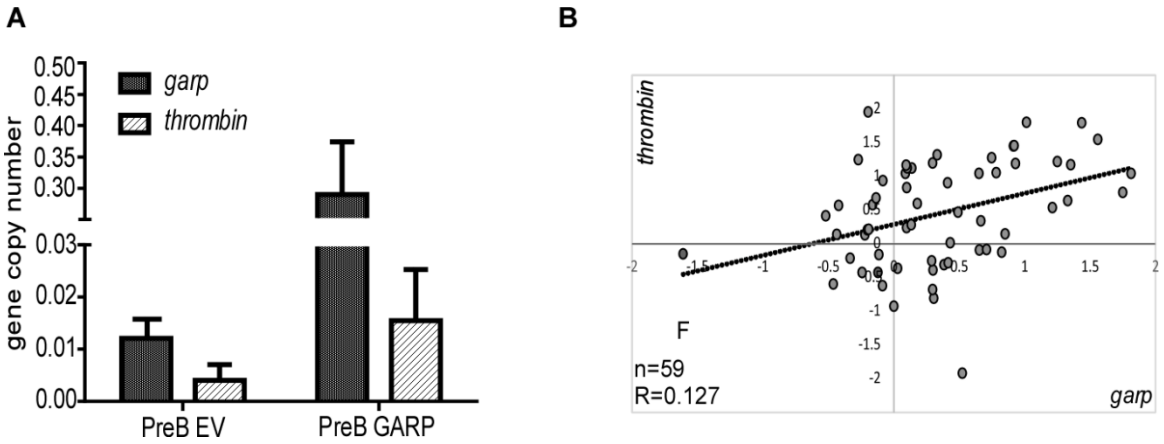


Figure 3.3 GARP upregulates thrombin gene expression.
(A) RT-PCR analysis of *Lrrc32* and *thrombin* gene expression in Pre-B cells expressing GARP or control vector. (B) Regression analysis of GARP and thrombin expression. Data obtained from TCGA, Breast Cancer proteomic database.

Thrombin cleaves GARP at the amino acid position 267 and 286 between proline and arginine.

To prove that thrombin cleaves GARP at the predicted cleavage sites, we mutated the two proline-arginine binding sites at 267-268 and 286-287 amino acidic positions to alanine-alanine. We thus generated 3 types of mutants: PR 267-268AA (GARP 267), PR 286-287AA (GARP 286), and double mutant (DM) PR 267-268AA PR 286-287AA (GARP 267-286, -DM). Previously, we showed that GARP cleavage occurs on the cell surface, so we decided to confirm that the mutations did not affect surface GARP expression. Flow cytometry analysis showed that GARP harboring 267, 286, and both 267+286 (GARP-DM) mutations are normally expressed on the cell surface (Figure 3-4A). Strikingly, parallel western blot analysis of cell lysates and conditioned media revealed that thrombin indeed cleaves GARP at the predicted cleavage sites (Figure 3-4B). Notably, the smaller cleaved GARP fragment from GARP 267 and GARP 286 are reduced in molecular weight and intensity, yet are still present. The 29KDa fragment from GARP harboring the two mutations 267-286 (GARP-DM) is completely abrogated, indicating that thrombin cleaves GARP at both binding sites (Figure 3-4B). Next, we tested whether GARP-DM was resistant to the cleavage of exogenous thrombin. To this end, we treated PreB GARP-WT and PreB GARP-DM with increasing concentration of thrombin. As expected, both 44 and 29 KD fragments increased in a dose dependent fashion in GARP-WT, and no cleaved products were observed in GARP-DM (Figure 3-4C). To further confirm thrombin dependent GARP cleavage, we performed a competition assay using a recombinant fragment of GARP containing the two Proline Arginine thrombin binding sites, named T250

(Figure 3-4D). In accordance with the previous results, T250 competed with surface GARP in binding with thrombin and was able to reduce GARP cleavage even in the presence of exogenous thrombin (Figure 3-4E).

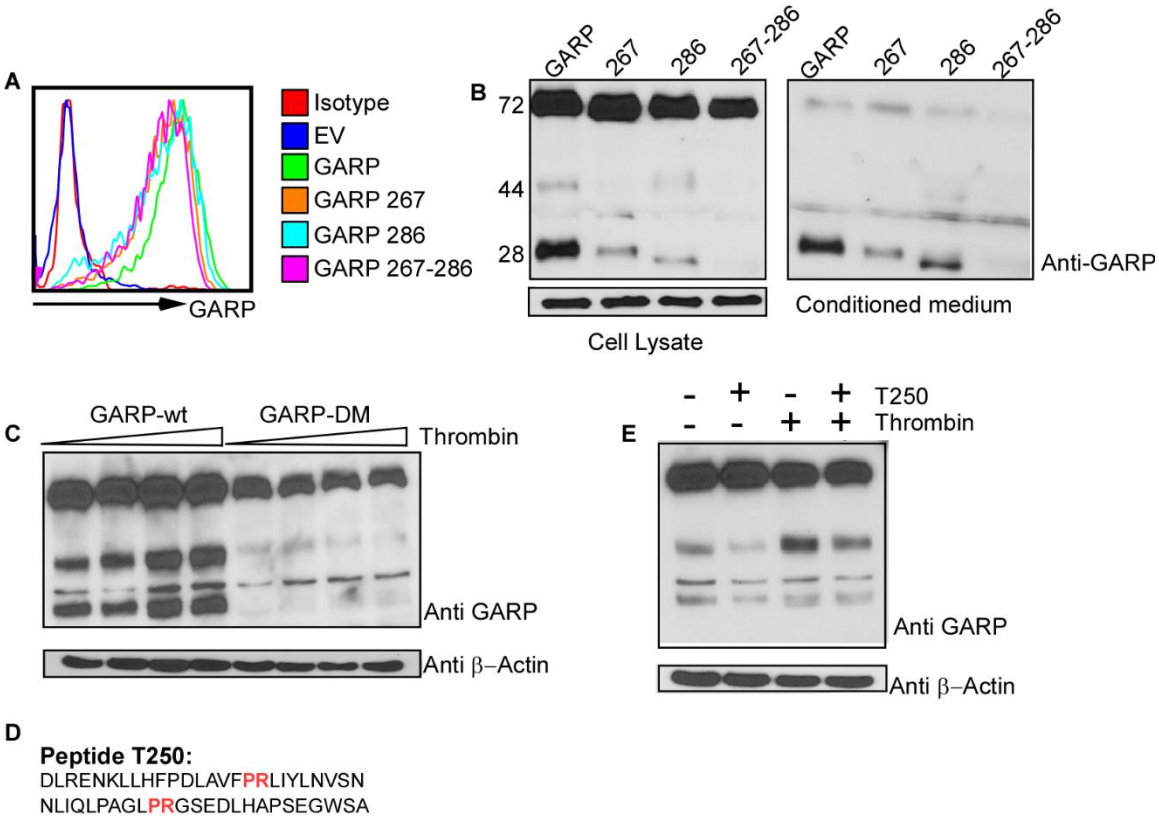


Figure 3-4 Thrombin cleaves GARP at the amino acid position 267 and 286 between proline and arginine.

(A) Surface flow cytometry analysis for GARP of WT PreB cells and GARP expression in PreB cells expressing WT GARP, GARP with single point mutation 267 aa, GARP with single point mutation 286 aa, GARP with double point mutation 267/286 aa (DM), and relative control vector. (B) Western blot analysis of cells lysates and conditioned media of PreB cells expressing WT GARP, GARP with single point mutation 267 aa, GARP with single point mutation 286 aa, or GARP with double point mutation 267/286 aa using antibody against GARP. (C) PreB cells expressing WT or DM GARP were digested with increasing concentration of thrombin (0, 1, 2, and 4 µg in 25 µl), followed by western blot analysis. (D) Recombinant fragment of GARP containing the two Proline Arginine thrombin binding sites (T250). (E) Western blotting analysis of GARP expressing PreB cells digested with 4 µg of thrombin in presence and absence of 4 µg of T250.

Thrombin-mediated cleavage facilitates latent TGF-β release from cell surface, yet does not affect mature TGF-β formation.

GARP expression facilitates cleavage of pro-TGF-β1 in mature TGF-β1 and secretion of latent TGF-β [25]. We hypothesized that thrombin mediated cleavage might be a mechanism to regulate the activation of latent TGF-β bound to GARP. A fundamental prerequisite was the binding of latent TGF-β to mutated GARP; thus, we checked if the mutations affected the ability of GARP to bind latent TGF-β. Surface flow cytometry analyses showed that the 3 GARP mutants still retained the ability to bind to LAP, and interestingly, LAP binding increased in GARP-DM (Figure 3-5A). GARP has been shown to enhance pro-TGF-β maturation and to mediate latent–TGF-β secretion from Tregs[25]. Thus, we decided to test whether thrombin plays a role in these two GARP functions. Surprisingly, the inhibition of thrombin-mediated cleavage did not affect the ability of GARP to facilitate pro-TGF-β1 maturation (Figure 3-5B); however, such inhibition affected the secretion of latent TGF-β1 in the cell supernatant (Figure 3-5C). This result is consistent with the previous finding that thrombin cleavage occurs only on the cell surface and sheds light on the importance of thrombin in releasing latent TGF-β. Accordingly, low total TGF-β in conditioned medium of GARP DM correlated with the higher expression of LAP on the surface as shown by surface flow cytometry analysis (Figure 3-5A).

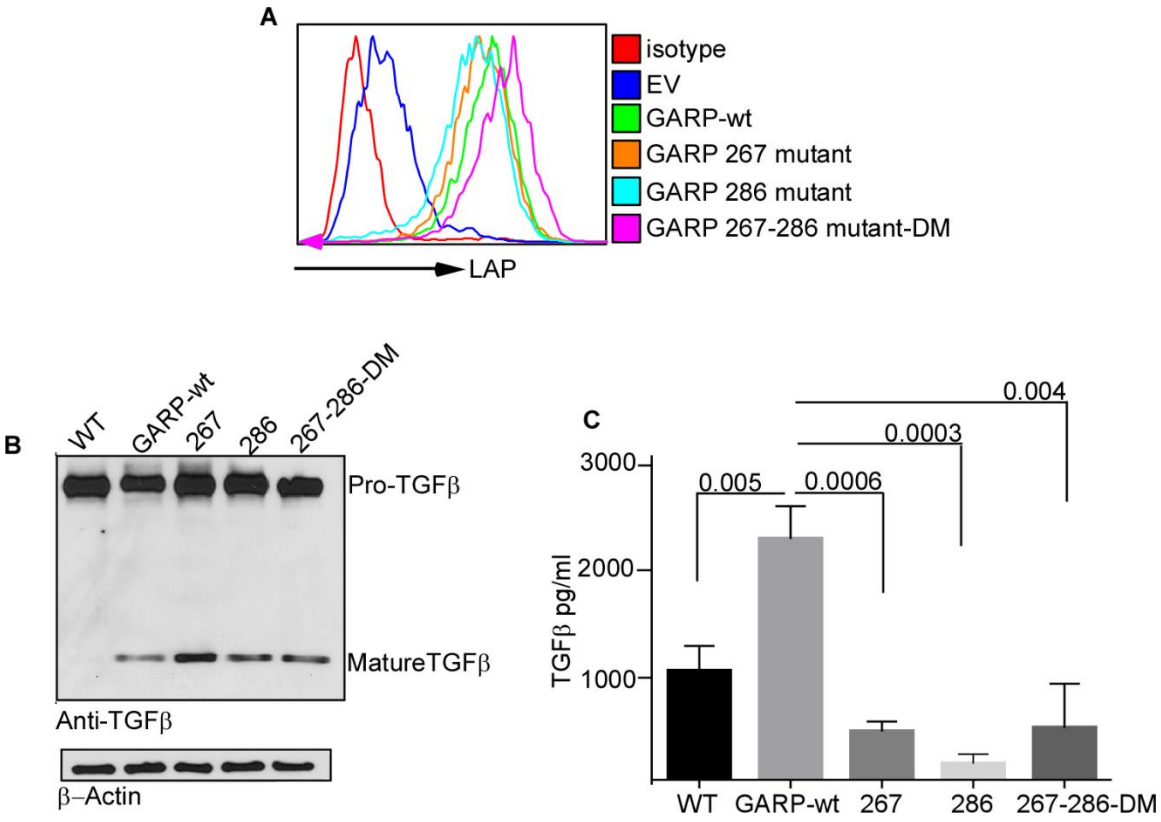


Figure 3-5. Thrombin-mediated cleavage facilitates latent TGF-β release from cell surface yet does not affect mature TGF-β formation.
(A) Surface flow cytometry analysis for LAP of WT PreB cells and GARP expression in PreB cells expressing WT GARP, GARP with single point mutation 267 aa, GARP with single point mutation 286 aa, GARP with double point mutation 267/286 aa (DM), and relative control vector. (B) Parallel western blot analysis of cells lysates and conditioned media of PreB cells expressing WT GARP, GARP with single point mutation 267 aa, GARP with single point mutation 286 aa, or GARP with double point mutation 267/286 aa using antibody against TGF-β. (C) Total TGF-β ELISA of conditioned medium of PreB cells expressing WT GARP, GARP with single point mutation 267 aa, GARP with single point mutation 286 aa, or GARP with double point mutation 267/286. Statistical significance was analyzed by two-tailed T-test in C.

Recombinant GARP protein is bound to latent TGF-β and is cleaved by thrombin.

To further study the function of GARP cleavage, we employed a recombinant form of soluble GARP (sGARP) where the transmembrane domain was replaced by IgG1 Fc fragment. The function of the Fc domain was to facilitate the protein purification using a Protein A column system. This recombinant protein mimics the soluble cleaved GARP product. Western blot analysis of the purified sGARP revealed that the recombinant protein was isolated in complex with latent TGF-β. Indeed, non-reducing and non-denaturating SDS-PAGE (ND) showed a large complex that was recognized at the same molecular weight by both anti-GARP and anti-TGF-β antibody. When denatured and reduced (D), the complex dissociated in sGARP and TGF-β (Figure 3-6A). TGF-β ELISA of sGARP indeed showed that, at serial dilutions, sGARP corresponded with a parallel dilution of total TGF-β (Figure 3-6B). Soluble GARP/latent TGF-β complex was analyzed by western blot analysis upon dose dependent treatment with thrombin. As expected, digestion of soluble GARP with thrombin gave rise to 3 fragments: GARP full length (72 KD), and the 2 smaller fragments of 44 and 29 KD (Figure 3-6C). More interestingly, in parallel with the formation of cleaved GARP products, latent TGF-β was released as represented by non-reducing conditions western blot analysis (Figure 3-6D). No active TGF-β dimer (25KDa) was detected by western blot analysis. Interestingly, GARP cleavage and latent TGF-β occurred in parallel, suggesting that GARP might be released in association with its own ligand. This disulfide-linked GARP-latent TGF-β complex has been already described as secreted from Treg surface[25]. The high amount of TGF-β bound to sGARP was

furthermore demonstrated in the reducing and denaturing SDS-PAGE (Figure 3-6E).

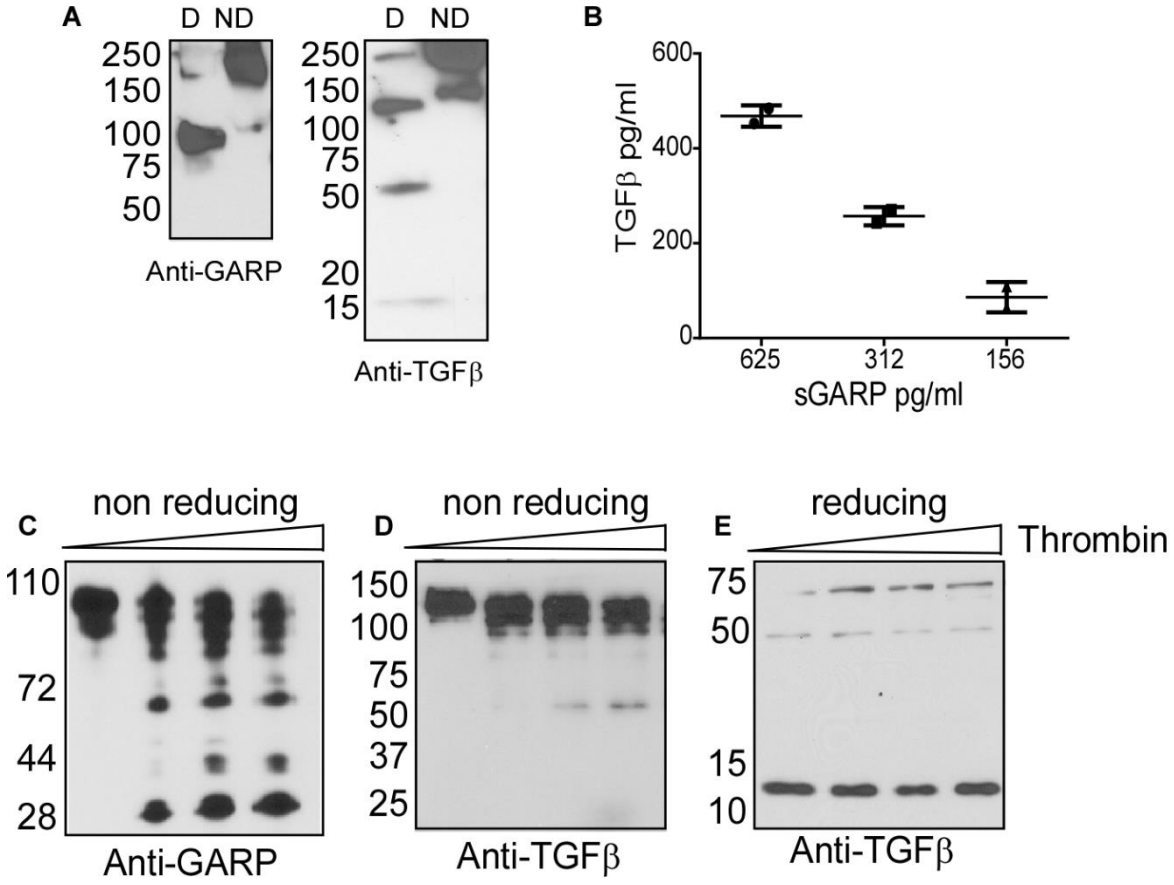


Figure 3-6. Recombinant GARP protein is bound to latent TGF-β and is cleaved by thrombin. (A) Western blot analysis of sGARP in reducing denaturating (D) and non-reducing non-denaturating (ND) conditions. Antibody against GARP and TGF-β were used. (B)Total TGF-β ELISA of 3 serial dilutions of soluble GARP-Fc. (C) Western blot analysis of 1µg soluble GARP digested with increasing concentrations of thrombin (0, 1, 2, and 4 µg in 25 µl) in reducing and non-reducing conditions. Antibody versus GARP and TGF-β were used.

Soluble GARP enhances TGF-β through αV integrins.

We next studied how cleaved GARP/latent TGF-β binds to cell surface and enhances TGF-β activation. NMuMG SMAD3-GFP reporter cell line was stimulated with increasing sGARP concentrations. Remarkably, p-SMAD3 signaling increased in response to sGARP in a dose-dependent fashion, as indicated by GFP signal intensity (Figure 3-7A). We then asked how latent TGF-β in complex with GARP was able to elicit TGF-β signal transduction. Integrins have been extensively studied for their ability to bind and to activate TGF-β[59,164]. In particular, αVβ6 and αVβ8 integrins have been shown to mediate TGF-β activation from membrane bound GARP in HEK293 and Treg cells, respectively. In 293 HEK, indeed, integrin αVβ6 binds to LAP's RGD sequence of the membrane bound GARP/latent TGF-β and helps to release mature TGF-β[14]. Thus, it is plausible that integrins could mediate the binding and activation of latent TGF-β bound to sGARP. By flow cytometry and RT-PCR analysis, we observed that αV integrins are abundantly expressed on NMuMG cells (Figure 3-7B and C). We next checked the expression of the β chains of integrins that have been reported to activate TGF-β in vitro: αVβ6[165], αVβ8[60], αVβ5[166], and αVβ3[167]. Among the β integrins, β6 was the most expressed when compared to β3, β8, and β5 gene expression (Figure 3-7C). Strikingly, the increasing concentration of RGD peptide was sufficient to decrease sGARP-dependent p-SMAD3 signaling in NMuMG, indicating that latent TGF-β mediates the binding between sGARP and integrins (Figure 3-7D).

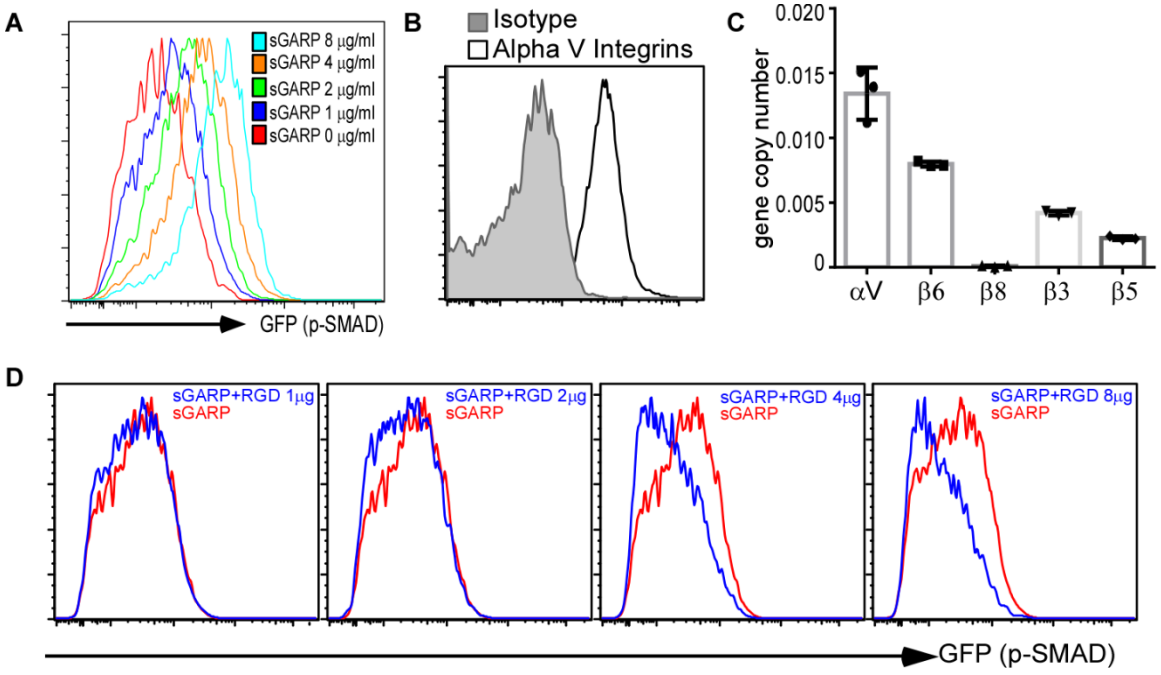


Figure 3-7. Soluble GARP enhances TGF-β through αV integrins. (A) Flow cytometry analysis of GFP p-SMAD NMuMG cells stimulated with soluble GARP. (B) Flow cytometry analysis of surface αV integrins in NMuMG cells. (C) RT-PCR analysis of αV and β integrins expressed on NMuMG cells. (D) Flow cytometry analysis of GFP expression in NMuMG cells stimulated with 8 μg of soluble GARP in presence of increasing concentration of RGD peptide.

Soluble GARP/latent TGF-β complex is internalized by cells through integrins.

Integrins bind to latent TGF-β on the cell surface where they predispose the complex for the release of the active peptide. We therefore attempted to detect soluble GARP complex on NMuMG cell surface. However, flow cytometry analysis and confocal pictures of NMuMG cells treated with soluble GARP or control IgG showed that after 1 hour of incubation, soluble GARP was not present as bound to the cell membrane (Figure 3-8A upper panel). Strikingly, we observed that sGARP was internalized as represented by flow cytometry analysis and confocal pictures of permeabilized NMuMG cells (Figure 3-8A lower panel). Integrins internalize ligands via receptor-mediated endocytosis. This is an interesting process that could give an alternative explanation to the integrin mediated surface release of active TGF-β[168]. Integrins' contribution to both binding and internalization of soluble GARP was investigated by confocal analysis, which confirmed first that soluble GARP is internalized, and second that this process is abrogated by RGD peptide, suggesting that the interaction between LAP and integrins mediates the endocytosis (Figure 3-8B).

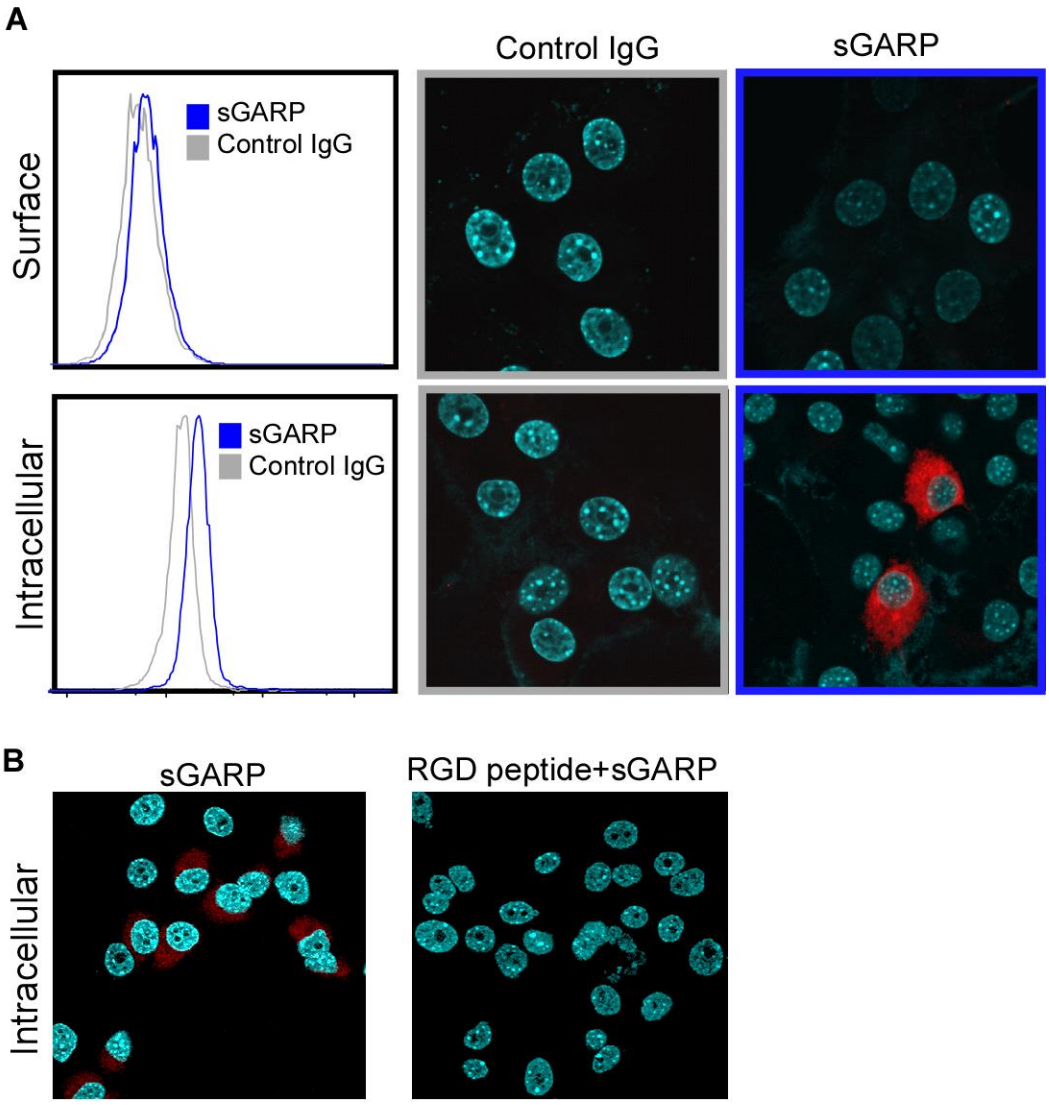


Figure 3-8. Soluble GARP/latent TGF-β complex is internalized by cells through integrins.

(A) Upper panel: surface flow cytometry analysis and confocal pictures of NMuMG cells stimulated with 1 µg of sGARP. Lower panel: intracellular flow cytometry analysis and confocal pictures of NMuMG cells stimulated with 1 µg of sGARP. (B) Confocal pictures of fixed and permeabilized NMuMG cells stimulated with 1µg of sGARP in presence or absence of RGD peptide.

GARP/latent TGF-β complex is released in exosomes.

Western blot analysis of conditioned medium from GARP expressing cells indicated the presence of two cleaved products that, as shown earlier, resulted from thrombin mediated cleavage. Interestingly, a faint signal reflecting a full length of GARP was always detected, indicating that either the conditioned medium was contaminated by residues of whole cells, or GARP was released as a full-length protein. The consistent detection of these full-length proteins prompted us to investigate if exosomes release constitutes an alternative mechanism of GARP secretion. We hypothesized that GARP is an oncogenic protein that might be liberated via exosomes to increase the metastatic potentials of the cancer cells. In support of this hypothesis, plasma collected from metastatic prostate patients revealed higher GARP concentration than non-metastatic patients. Additionally, following androgen deprivation therapy (ADT), we observed an important increase of soluble GARP concentration in the plasma of prostate cancer patients in parallel with a PSA1 decrease, suggesting GARP-rich exosomes are released upon cancer cell death (Supplemental Figure 2). Exosomes from 293 cells expressing TGF-β1 alone or in combination with GARP were isolated from serum free conditioned medium with serial ultracentrifugation and analyzed by western blot. Strikingly, a consistent concentration of GARP was detected (Figure 3-9A), and more importantly, immunoblot for GARP and TGF-β in non-reducing and non-denaturating conditions revealed that exosomes contained GARP bound to TGF-β (Figure 3-9B). The successful exosome isolations was demonstrated by detection of the known exosome marker CD63 (Figure 3-9C)

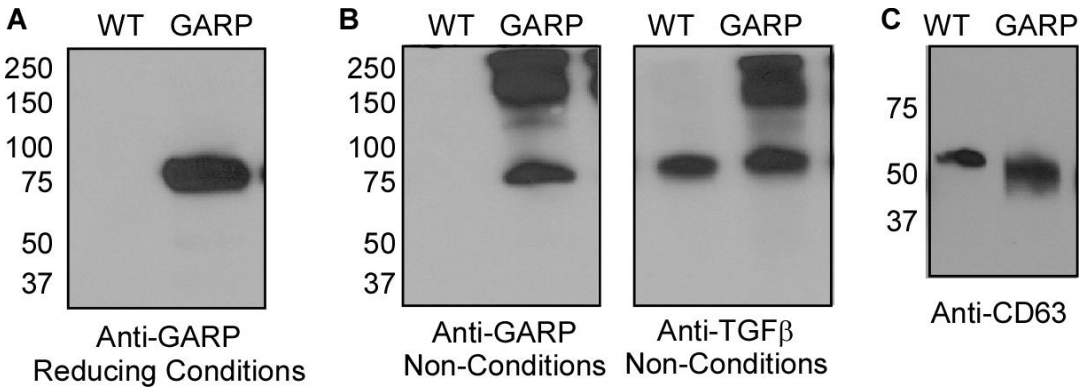


Figure 3-9. GARP/latent TGF-β complex is released in exosomes.
(A) Western blot analysis in reducing and denaturing conditions of exosomes isolated from conditioned medium of 293 HEK TGF-β with and without GARP expression. Anti-GARP antibody was used. (B) Western blot analysis in non-reducing and non-denaturating condition of exosomes isolated from conditioned medium of 293 HEK TGF-β with and without GARP expression. Anti-GARP and anti-TGF-β antibodies were used. (C) Western blot analysis of CD63 in reducing and denaturing conditions of exosomes isolated from conditioned medium of 293 HEK TGF-β with and without GARP expression.

Discussion

GARP as a pro-oncogenic receptor for latent-TGF-β gene in epithelial cells has been described[1]. Numerous evidence suggests that GARP is secreted in combination with TGF-β; however, the generation of this soluble complex as well as its signal transduction pathway are completely unresolved. Herein, we provide evidence of two mechanisms that explain soluble GARP formation. The first involves the serine protease thrombin as the enzyme responsible for cleaving surface GARP to generate two soluble products: a C-terminal bigger fragment and an N+ terminal smaller one. We found that this latter fragment is abundantly released in the extracellular milieu. Then, we generated a recombinant GARP protein (sGARP) lacking the transmembrane domain to mimic the N+ terminal fragment that is released. We observed that sGARP is complexed with latent TGF-β and drives Smad3 phosphorylation by binding to surface integrins through LAP. Additionally, our data showed that integrins are responsible for sGARP cell internalization. These observations shed light on a novel possible mechanism for active TGF-β release from GARP/latent TGF-β complex: our data propose the endocytosis-mediated internalization of sGARP bound to integrins and subsequent intracellular signaling. Integrins indeed can be endocytosed to release their cargo in the endosomes where they can be either recycled or degraded[169]. The endocytosis-mediated mechanism of TGF-β activation might explain the absence of active TGF-β release upon thrombin cleavage (See Figure 3-6D), yet the presence of Smad phosphorylation signaling is elicited by soluble GARP on NMuMG cells (See Figure 3-7A). GARP dependent TGF-β activation indeed, has

been mostly demonstrated by TGF-β reporter cell lines (mink lung epithelial reporter cells) and Smad 2/3 phosphorylation[14,170].

It is interesting that thrombin mediated activation of platelets leads to the secretion of active TGF-β through GARP (See chapter 5-6C and D); however, in cells with enforced GARP expression or in recombinant soluble GARP, thrombin cleavage causes release of latent TGF-β with no mature formed detected. The apparent discrepancy might be explained by three hypotheses:

1. Platelets express much more TGF-β than other cells. Thus, in cells other than platelets, the amount of active TGF-β produced is simply too low to be detected.

2. Upon activation, platelets release lactic acid in their supernatant that might facilitate the disruption of the non-covalent bond between LAP and TGF-β1. The acid mediated activation of latent TGF-β has been described for osteoblasts during bone resorption[171]. Of note, serum from *Pf4*creGARPf/f mice contains elevated lactic acid levels (Supplemental Figure 2). This phenomenon might be interpreted as a consequence of sustained platelet activation to compensate for the low circulating active TGF β.

3. Finally, there is a possibility that thrombin mediated cleavage requires thrombin binding to its receptor, largely expressed on platelets. Previous evidence, indeed, showed that PAR1 ligands can enhance activation of TGF-β bound to integrins through signals via RhoA and Rho Kinase[61], suggesting that thrombin needs to bind to its receptor to activate TGF-β.

Another observation that deserves attention is the thrombin-mediated cleavage of platelet GARP. Thrombin directly elicits platelet activation by binding to thrombin G protein couple receptors PAR1 and PAR4, and additionally our data demonstrate that thrombin upregulates GARP expression and activation of TGF-β. We also observed that thrombin releases soluble full length and cleaved GARP products in PR. These data strongly support the idea that thrombin-dependent GARP cleavage might be part of a novel mechanism to activate platelet membrane bound TGF-β.

In parallel, the evidence that full-length GARP is present in the exosomes starts to unveil another important chapter of GARP biology. In this context, GARP/latent TGF-β complex in exosomes might be a system adopted by GARP expressing cancer cells and Treg to fill the extracellular environment with TGF-β and promote more tumor growth and more Treg conversion in a positive feedback loop. Interestingly, plasma of AML patients is rich in exosomes that carry pro-TGF-β and inhibit NKG2D expression on NK cells[172]. Additionally, cancer cells produce the so-called TuEx (Tumor derived Exosomes) that express membrane bound TGF-β and can induce Treg[173]. It is reasonable to think that these exosomes contain and/or express GARP/latent TGF-β and are used by cancer cells to guarantee an immunosuppressed environment.

Of interest, Treg are among the lymphocytes that mediate many immunosuppressive effects by delivering miRNA and many other elements through exosomes[174]. Although this hypothesis has not been explored, it is

reasonable to assume that Treg derived exosomes contain GARP/latent TGF-β used to establish a paracrine feedback loop.

GARP and TGF-β rich exosomes become attractive therapeutic agents. Exosomes have been employed as a drug delivery system in several diseases to transport siRNA[175], chemotherapies[176] and enzymes[177]. Cai and colleagues showed the efficacy of TGF-β1 rich exosomes produced by bone marrow derived dendritic cells (BMDC) in attenuating Th17 driven IBD (Inflammatory Bowel Disease) by inducing Treg[178]. Thus, it is reasonable to think that exosomes rich in GARP and TGF-β might be a useful tool in inflammatory diseases.

Finally, we cannot exclude the possibility that thrombin cleavage and exosome release are two steps in the same pathway. It is possible, indeed, that soluble GARP/latent TGF-β complex is released upon thrombin cleavage and binds to the cell surface through integrins. After that binding, integrin mediated endocytosis could allow the intracellular sequestration of GARP and TGF-β that would be finally released in the exosomes.

This possibility is currently under investigation.

Chapter 4

Expression of TGF-β Docking Receptor GARP Promotes Oncogenesis and Immune Tolerance in Breast Cancer

Rationale

GARP gene *Lrrc32* was initially described as an amplified unit present in metastatic breast cancer. Subsequent studies showed that *Lrrc32* amplification is not only restricted to advance breast cancer, but it is an hallmark in hormone-resistant prostate cancer and metastatic oral squamous cells carcinoma as well[3,5,6]. Despite the multiple evidence of *Lrrc32* amplification in cancer, GARP protein expression in breast cancer or in any other cancer was not investigated yet.

Interestingly, the entire 11q13 region reveals the presence of a pool of 23 known genes frequently amplified in several human carcinomas[94], however *Lrrc32* is an independent unit since it does not require the amplification of the rest of the region to be extensively expressed. This aspect stimulated our interest and for this reason we decided to explore the oncogenic ability of GARP protein by enforcing its expression on mammary epithelial cells. We hypothesized that the single amplification of GARP gene was sufficient to initiate the mesenchymal transition on epithelial cells due to the subsequent accumulation and activation of surface TGF-β.

This hypothesis is also reinforced by the existence of several cases of single gene amplifications which confer oncogenic properties to epithelial cells like for example Myc and HER2/neu[95,96] in breast cancer.

As a receptor involved in the maturation of TGF-β, the presence of GARP on cancer cells acquires an interesting meaning. Of note, *Lrrc32* amplification was described in advanced stage cancers, when TGF-β acquires a pro-metastatic function. Indeed, histological analysis of primary breast[97], lung (NSCLC)[98], prostate[99] and colon[100] demonstrates that high TGF-β expression correlates with lymph node and distant metastasis. In this context, GARP might support cancer cells dissemination by providing an excellent reservoir of tumor TGF-β. Importantly, TGF-β produced by cancer cells exerts a paracrine function on the tumor microenvironment by regulating the innate and adaptive immune components and favoring tumor immune evasion. One of the most important mediators of tumor immune-tolerance are Treg, a cell population strongly induced in presence of mature TGF-β. It is not surprising that the accumulation of this cell type in TME inversely correlates with overall survival in several human solid tumors[101].

In the light of the pro-tumorigenic role of active TGF-β in late stage cancers, several clinical trials have been conducted to assess the efficacy of anti-TGF-β treatments[102]. Fresolimumab, for example, is a monoclonal TGF-β1, 2, and 3 antibody used in phase 1 clinical trial in 28 advanced melanoma patients. The results show that 7 patients experienced stable disease and one achieved a partial

response[103]. The results from this and many other clinical trials show that better biomarkers are needed in order to select patients whose tumors express TGF-β. In this context GARP might be considered an excellent biomarker that identifies tumors that express membrane bound TGF-β. Secondly, because of its anti- or pro-tumorigenic nature, blocking TGF-β might stimulate more cancer cell proliferation if the treatment is performed in early tumors. As previously mentioned, *Lrrc32* amplification has been described only in advance stage cancers where TGF-β acts as pro-metastatic cytokine, in this context GARP expression might be considered as a good candidate to define those cancers in which anti TGF-β treatment would be efficacious.

Other than tumor biomarker, GARP on aggressive cancers becomes also a valuable target to add to the list of those used in antibody based therapy. Pertuzumab for example is a monoclonal antibody used in HER2 positive breast cancer[104]. Targeting GARP is a fascinating treatment option because it consents to attack cancer from two angles: first, the antibody would exert its tumoricidal effect directly on the cancer cells; second, immunosuppressive Treg expressing surface GARP would be specifically depleted, restoring an effective anti-tumor immunity. Several clinical trials have already demonstrating that targeting Treg using CD25 or CTLA4 monoclonal antibody enhances anti-tumor cytotoxic T cells response. However both CTLA4 and CD25 are not uniquely expressed only on activated Treg, upon activation CTLs express these two proteins on their surface as well and this might restrain the efficacy of the monoclonal antibody therapy[105].

Among lymphocytes GARP expression has not been detected on any T effector cells, allowing the investigator to specifically aim on Treg.

Results

GARP expression and significance in mammary carcinoma

Considering that TGF-β is highly expressed in infiltrating ductal carcinomas[38] we first asked whether GARP might serve as a surface latent TGF-β receptor in breast cancer. To examine GARP protein expression in archived formalin-fixed cancer specimens, we developed an immunohistochemistry (IHC) assay. The specificity of the anti-human GARP antibody was ascertained by its staining of a Pre-B leukemia cell line stably transfected with human GARP (Figure 4-1A). Analysis of patient-matched uninvolved breast tissue versus primary breast cancer (n=16) indicated a significant increase of GARP expression on cancer tissues in 9 out of the 16 patients (Figure 4-1B and 4-1C). Consistent with previous findings, *Lrrc32* mRNA expression was increased by ≥ 2-fold in 28.5% of patients with breast cancer (n=42) compared with normal breast tissues (data not shown), suggesting an amplification of *Lrrc32* gene. In contrast with normal colon, lung and prostate epithelium, normal breast cancer shows a baseline GARP expression (Figure 4-1 B, 4-6 and 4-7) that might correlate with the baseline expression of TGF-β observed in normal mammary gland tissue[106]. Mammary gland development indeed requires a normal TGF-β signaling as shown by the lactation-deficient phenotype of mice expressing a constantly activated TGF-βRI[107]. A growing body of evidences suggests a reciprocal inhibitory effects between Estrogen Receptor Alpha (ERα) and TGF-β in breast cancer: while TGF-β restricts ERα mediated cell proliferation[108], ERα suppresses TGF-β signaling cascade via

G protein couple receptor 30 (GPR30)[109]. Nevertheless, no association between GARP and ER, PR, HER2 expression was found (data not shown).

As a receptor involved in the activation of its ligand, the presence of GARP on breast cancer acquires an interesting meaning. To understand the functional significance of GARP expression in mammary carcinoma, we screened murine mammary carcinoma cell lines for GARP expression by flow cytometry. A variant of the normal murine mammary gland epithelial cell line (NMuMG*), has been recently described as being capable of forming tumors in nude mice. In NMuMG* the RNA-binding protein hnRNPE1 is knocked down by RNA interference, inducing the constitutive translation of proteins involved in the epithelial to mesenchymal transition (EMT)[110]. Intriguingly, we found these cells expressed a significantly high level of endogenous GARP (Figure 4-1D-F), raising the possibility that heightened TGF-β biogenesis, in addition to the silencing of the TGF-β-mediated translation repression complex, drives mammary cancer in this model. To test this hypothesis, we performed short hairpin RNA (shRNA) knock down (KD) of GARP in the NMuMG* cells (Figure 4-1D-F). GARP silencing did not affect the *in vitro* proliferation of NMuMG* cells as determined by MTT assay (Figure 4-1G). Remarkably, silencing of GARP alone in the NMuMG* cells significantly attenuated their growth *in vivo* (Figure 4-1H). Further, the ability of these GARP KD cells to metastasize to the lungs was compromised (Figure 4-1I).

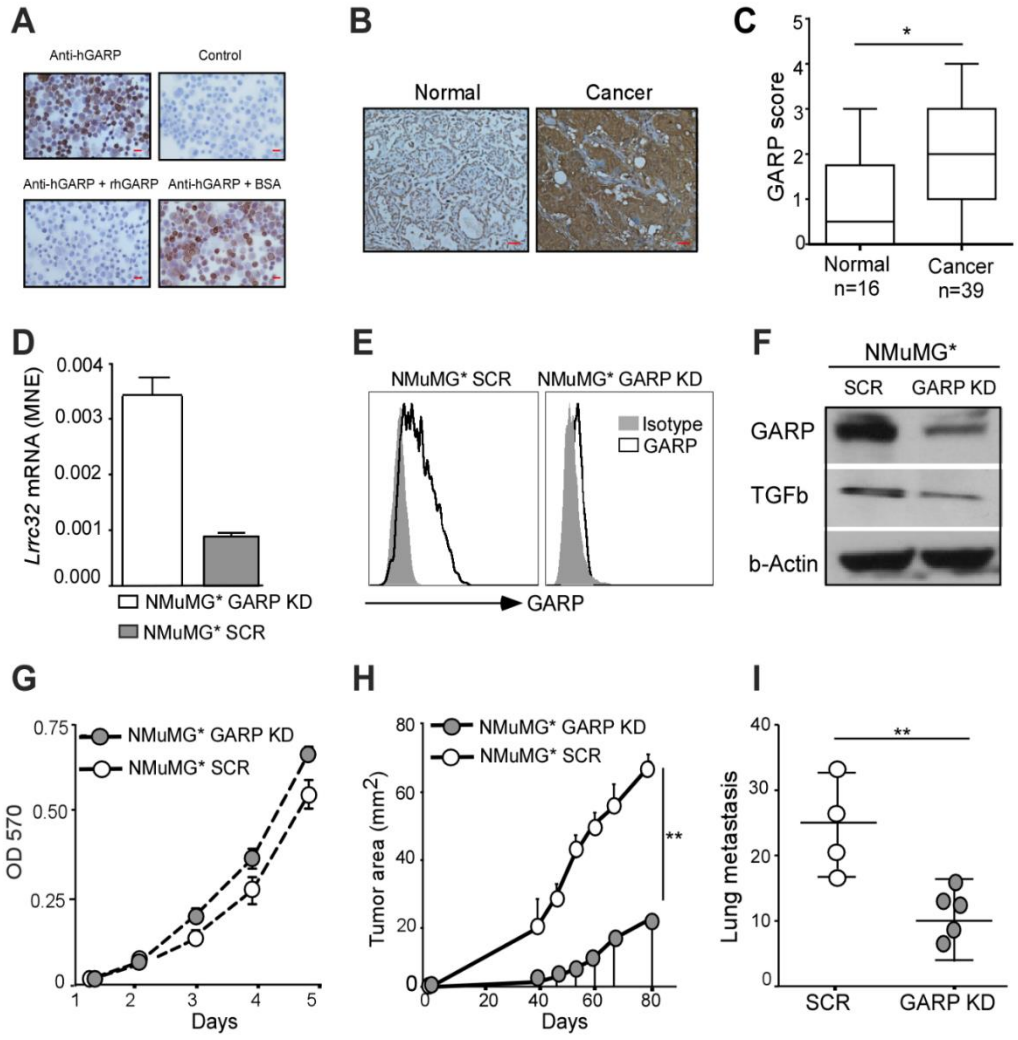

A
Anti-hGARP
Control
Anti-hGARP + rhGARP
Anti-hGARP + BSA
B
Normal
Cancer
C
GARP score
Normal n=16
Cancer n=39
D
Lrrc32 mRNA (MNE)
NMuMG* GARP KD
NMuMG* SCR
E
NMuMG* SCR
NMuMG* GARP KD
Isotype
GARP
F
NMuMG*
SCR
GARP KD
GARP
TGFb
b-Actin
G
OD 570
NMuMG* GARP KD
NMuMG* SCR
Days
H
Tumor area (mm2)
NMuMG* GARP KD
NMuMG* SCR
Days
I
Lung metastasis
SCR
GARP KD

Figure 4-1. GARP expression in human breast cancer and its oncogenic roles in murine mammary gland epithelial cells.

(A) IHC validation of the human GARP antibody using Pre-B cells expressing human GARP or empty vector control. (B) Representative images of GARP IHC (brown color) of breast cancer with their respective patient matched normal tissue. Each patient specimen in these TMAs was represented in two cores on the slide and each core measured 1 mm in diameter. (C) Expression intensity of GARP-positive cells in breast cancer specimens and patient matched adjacent normal tissue. (D) shRNA knockdown of GARP mRNA in NMuMG* cells. Cells treated with scrambled shRNA (SCR) were used as control. (E) Flow cytometry analysis of cell surface GARP expression on GARP KD and SCR NMuMG* cells. (F) Immunoblot of GARP and TGF-β level in GARP KD and SCR NMuMG* cells. (G) In vitro cell proliferation assay for GARP KD and SCR NMuMG* cells. (H) NMuMG* SCR and NMuMG*-GARP KD cells were injected into NOD-Rag1-/- mice, followed by weekly monitoring of the tumor growth kinetics (E). 120 days after tumor injection, lungs gross metastatic nodules were counted visually on the surface of the organ. $^{**}P<0.01$. Statistical analysis was determined by 2-way ANOVA or two-tailed *t*-test, where appropriate. Data is representative of two independent experiments.

Enforced GARP expression in normal murine mammary epithelial cells upregulates TGF-β bioavailability and drives oncogenesis

In the parental NMuMG cells, TGF-β exerts both a growth inhibitory response and an EMT response[111]. As such, NMuMG cells have been extensively utilized to study TGF-β signaling and biology [112]. We found that stable GARP expression induced Smad-2/3 phosphorylation and expression of vimentin, but downregulated E-cadherin, consistent with increased canonical TGF-β signaling (Figure 4-2A). As previously mentioned, GARP can also be secreted as a soluble form and that soluble GARP can increase the bioavailability of TGF-β[25,58,70]. To further address this point, we generated a soluble GARP (sGARP) by fusing the entire ectodomain of GARP with the Fc portion of IgG (Figure 4-2B). We found indeed that sGARP can induce EMT by inducing time- and dose-dependent upregulation of vimentin (Figure 4-2C). We next performed an *in vitro* "scratch" assay to gauge the migratory properties of GARP-expressing cells. The closure rate of the gap (created by scratching the culture plate) was significantly increased with GARP-expressing cells, indicating increased acquired migratory ability (Figure 4-2D and 4-2E). More importantly, we examined whether enforced GARP expression enabled NMuMG cells to establish tumors *in vivo*. To this end, female immunodeficient NOD-*Rag1*$^{-/-}$ mice were injected in the fourth mammary fat pad with GARP-expressing NMuMG cells or with EV control cells all of which were also engineered to co-express luciferase. By *in vivo* imaging of the bioluminescence, we found that the bioactive mass formed only in mice that received GARP^{+} or sGARP^{+} NMuMG, but not in mice receiving E- transduced cells (Figure 4-2F). This tumor formation by GARP-expressing cells was confirmed by histology (Figure 4-

2G). Collectively, we demonstrate that GARP has a transforming property via upregulation of TGF-β, identifying GARP as a potential novel oncogene.

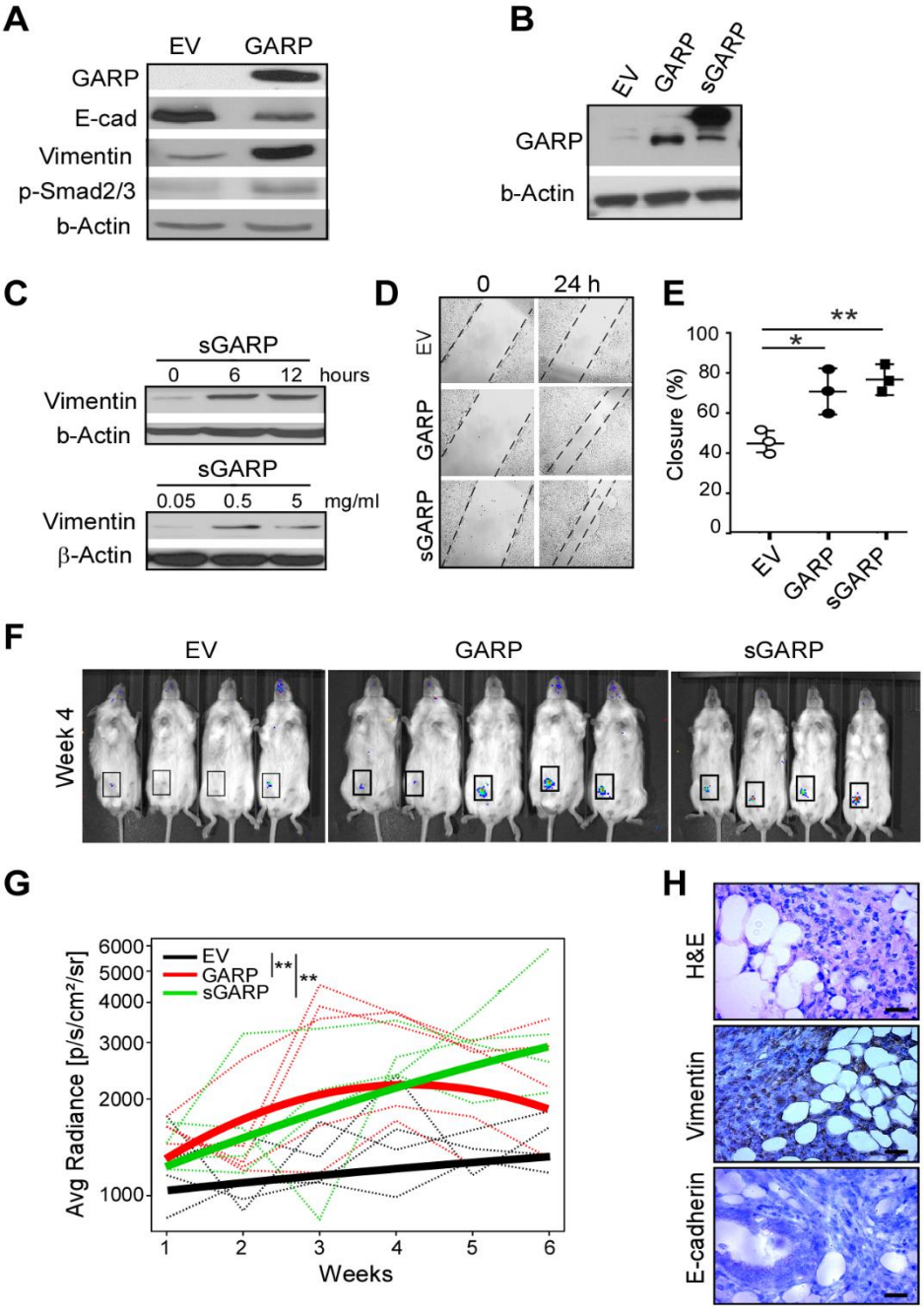

A
EV
GARP
GARP
E-cad
Vimentin
p-Smad2/3
b-Actin
B
EV
GARP
sGARP
GARP
b-Actin
C
sGARP
0
6
12
hours
Vimentin
b-Actin
sGARP
0.05
0.5
5
mg/ml
Vimentin
β-Actin
D
0
24 h
EV
GARP
sGARP
E
**
*
Closure (%)
100
80
60
40
20
0
EV
GARP
sGARP
F
EV
GARP
sGARP
Week 4
G
EV
GARP
sGARP
**
**
Avg Radiance [p/s/cm²/sr]
6000
5000
4000
3000
2000
1000
1
2
3
4
5
6
Weeks
H
H&E
Vimentin
E-cadherin

Figure 4-2: Recombinant soluble GARP and enforced soluble GARP expression drives EMT and invasion on normal mammary gland epithelial cells. (A) Immunoblot analysis for GARP, E-cadherin, Vimentin and Smad2,3 phosphorylation in NMuMG cells stably transfected with GARP vector or empty vector. (B) Immunoblot analysis for GARP in NMuMG cells stably transduced with GARP vector, sGARP vector or control vector. (C) NMuMG cells were treated for the indicated time points and with increasing doses of soluble GARP in serum-free culture medium, followed by immunoblot for vimentin. (D) *In vitro* scratch assay to indicate the difference in the gap closure at 24 hours. (E) Summary statistics of three independent scratch assays. (F) *In vivo* imaging of the luciferin-enhanced bioluminescence in mice after injection of GARP, sGARP and control NMuMG cells, with representative images from Week 4. (G) Quantification of luciferase signal through the use of ROIs; data were weekly quantified by average radiance (photons/s/cm2/steradian). (H) Week 4 H&E and IHC analysis of Vimentin and E-cadherin expression for membrane GARP expressing NMuMG tumors. **P*<0.05; ***P*<0.01. Statistical significance determined by two-tailed *t*-test. Western blot images are representative of 2 independent experiments and scratch assay are representative of 3 independent experiments.

GARP upregulation in murine mammary cancer cells promotes TGF-β activation, tumor growth, metastasis and immune tolerance

Cancer-intrinsic TGF-β signaling has been shown to promote breast cancer invasion and metastasis[48,107,113]. The other aspect of TGF-β biology in cancer is its cancer-extrinsic role via modulating the host immune response [114], which is under-studied. We thus turned our attention next to an examination of how GARP impacts cancer growth and metastasis in a syngeneic immune-sufficient setting. We chose the highly aggressive and metastatic 4T1 mammary carcinoma model in BALB/c mice [115]. Similar to the NMuMG system, over-expression of GARP or sGARP in 4T1 cells led to increased production of active TGF-β (Figure 4-3A). One of the key mechanisms by which TGF-β inhibits tumor-specific immunity is via the induction of Foxp3$^+$ Tregs. To this end, purified naïve CD4$^+$ T cells were cultured *in vitro* with conditioned media from 4T1-GARP, 4T1-sGARP and empty vector (EV) control cells in the presence of polyclonal T cell activators for 3 days. The conditioned media from GARP-expressing cells was 2-3 fold more efficient at inducing Treg differentiation compared to medium from control cells (Figure 4-3B). We next injected 4T1-EV, 4T1-GARP and 4T1-sGARP cells orthotopically in the fourth right mammary fat pad of 6-8 weeks old female BALB/c mice. We found that GARP-expressing cells were more aggressive, as indicated by both increased growth kinetics of the primary tumor (Figure 4-3C and 4-3D) and increased lung metastasis (Figure 4-3E). We found that this aggressiveness correlated with enhanced TGF-β signaling in the tumor microenvironment as determined by increased p-Smad-2/3 in cancer cells (Fig 4-3F and 4-3G), as well as by expansion

of tolerogenic Treg cells (Figure 4-3H and 4-3I).

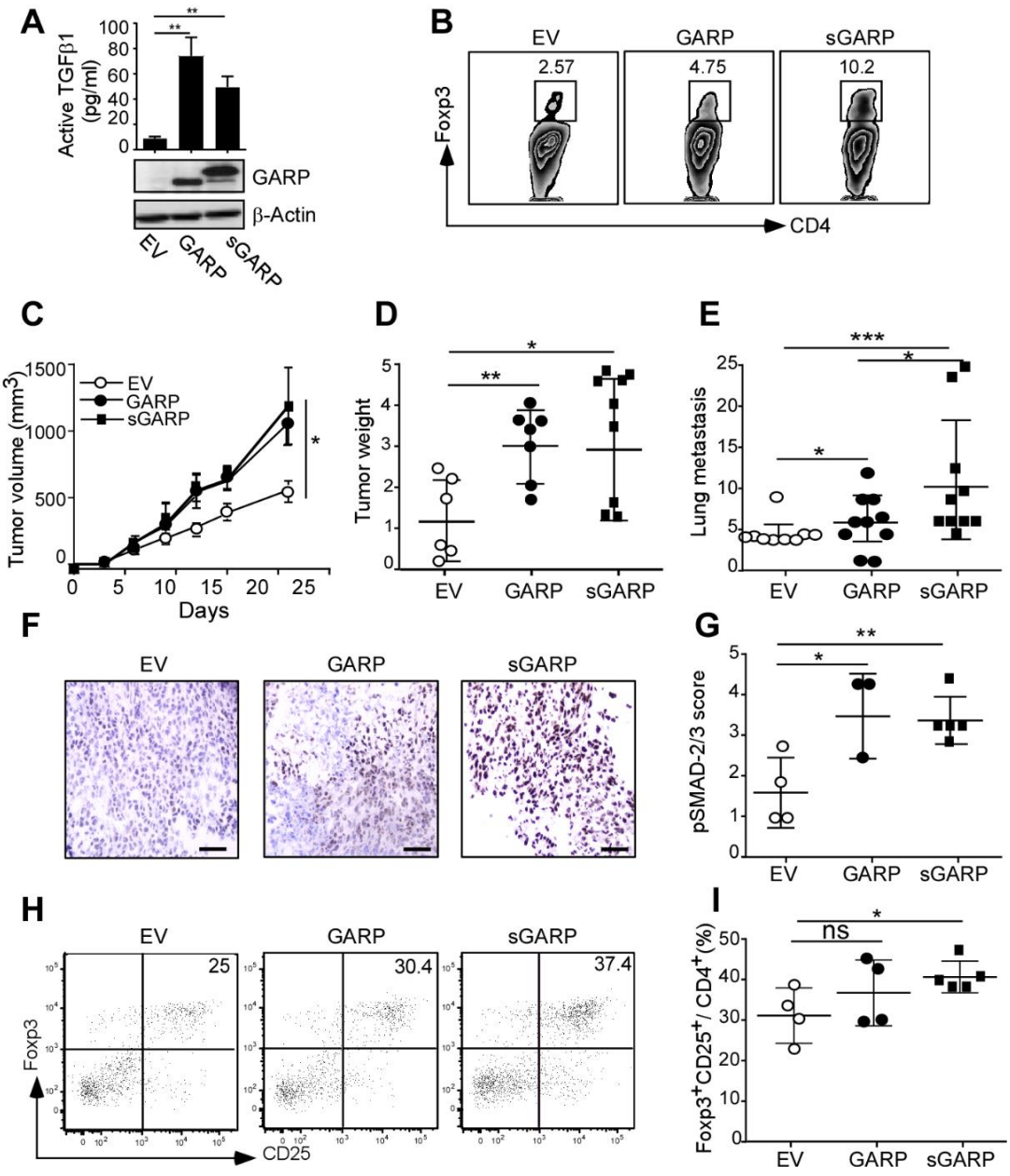

A
Active TGFβ1 (pg/ml)
GARP
β-Actin
EV
GARP
sGARP
B
EV
GARP
sGARP
2.57
4.75
10.2
Foxp3
CD4
C
Tumor volume (mm3)
EV
GARP
sGARP
Days
D
Tumor weight
E
Lung metastasis
F
G
pSMAD-2/3 score
H
25
30.4
37.4
Foxp3
CD25
I
ns
Foxp3+CD25+/ CD4+(%)

Figure 4-3. GARP upregulation in murine mammary cancer cells promotes TGF-β activation, tumor growth, metastasis and immune tolerance. (A) Concentration of active TGF-β in 3 days conditioned medium measured by ELISA and immunoblot for GARP in 4T1 cells stably expressing GARP, sGARP, or EV. (B) Regulatory T cells differentiation assay using $CD4^{+}CD25^{-}$ cells in the presence of 4T1-GARP, 4T1-sGARP, and 4T1-EV. (C) Female BALB/c mice were injected in the 4^{th} mammary fat pad with $5x10^{5}$ tumor cells. Tumor volume was measured every 3 days. (D) Three weeks after tumor injection, mice were sacrificed and the primary tumors were resected and weighted. (E) Lungs were isolated, paraffin embedded, and H&E stained for histological analysis. The number of micro-metastatic tumor nodules in the lungs were enumerated by a pathologist. (F) Portions of the tumors were isolated and embedded in OCT and snap frozen. The fresh frozen sections were stained for the presence of p-SMAD-2/3; representative images are shown. (G) Summary statistics for p-SMAD-2/3 staining intensity, defined independently by a pathologist (S.S). (H) Tumor-infiltrating lymphocytes were isolated and the number of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs were enumerated by flow cytometry. (I) Representative flow plots. (J) Summary of the percentage of Tregs in the tumor microenvironment. *P<0.05; **P<0.01; ***P<0.001. Statistical significance was determined by two-tailed *t*-test or Two-way ANOVA, where appropriate. Data is representative of at least two independent experiments.

Depletion of $CD25^+$ Treg cells abolished the aggressiveness of soluble GARP expressing mammary tumors

To determine if the increased aggressiveness of 4T1-sGARP cells was mediated by Tregs, we next depleted $CD25^+$ Treg cells with CD25-specific antibody PC61, followed by implementing either 4T1-EV or 4T1-sGARP (Figure 4-4A). Indeed, Treg depletion abolished the aggressiveness of 4T1-sGARP measured by tumor growth kinetics (Figure 4-4B**)**, as well as tumor volume (Figure 4-4C). We also examined the tumor-infiltrating T cells, and confirmed that PC61 injection resulted in reduction of $CD4^+CD25^+$ (Figure 4-4D) and $CD4^+CD25^+Foxp3^+$ Tregs (Figure 4-4E), and increased percentage of IFNγ-producing $CD8^+$ T cells (Figure 4-4F).

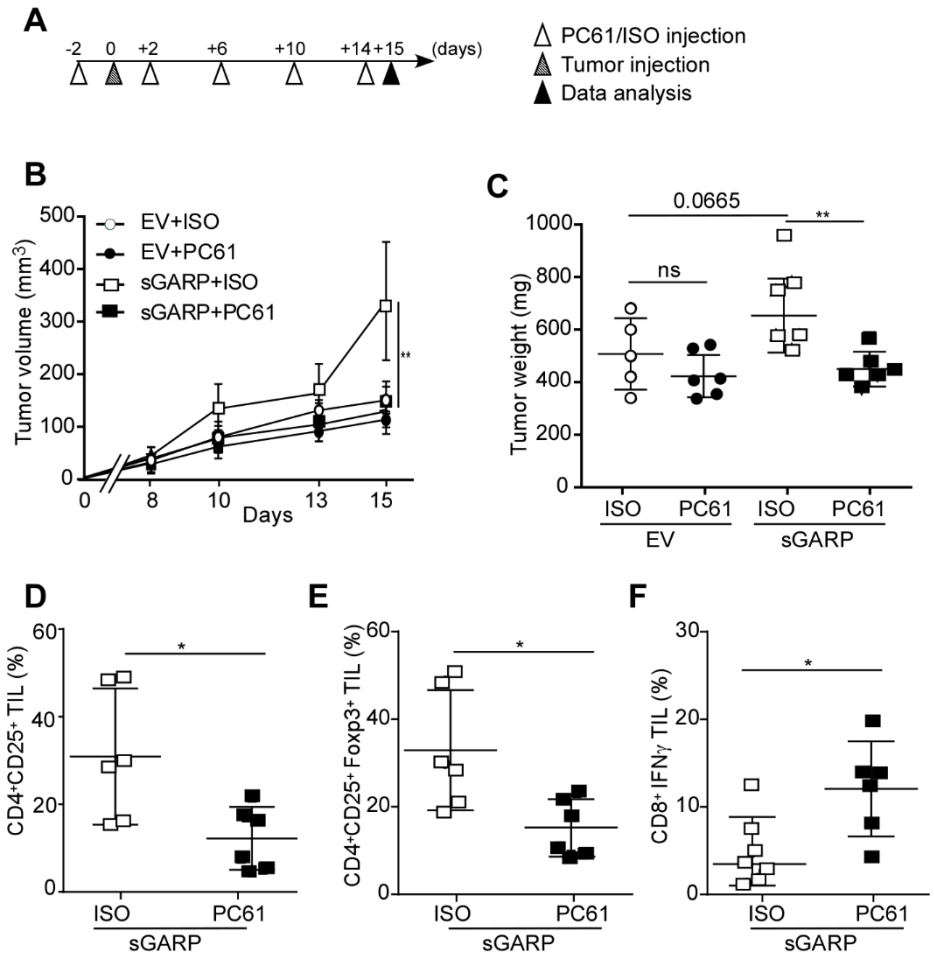
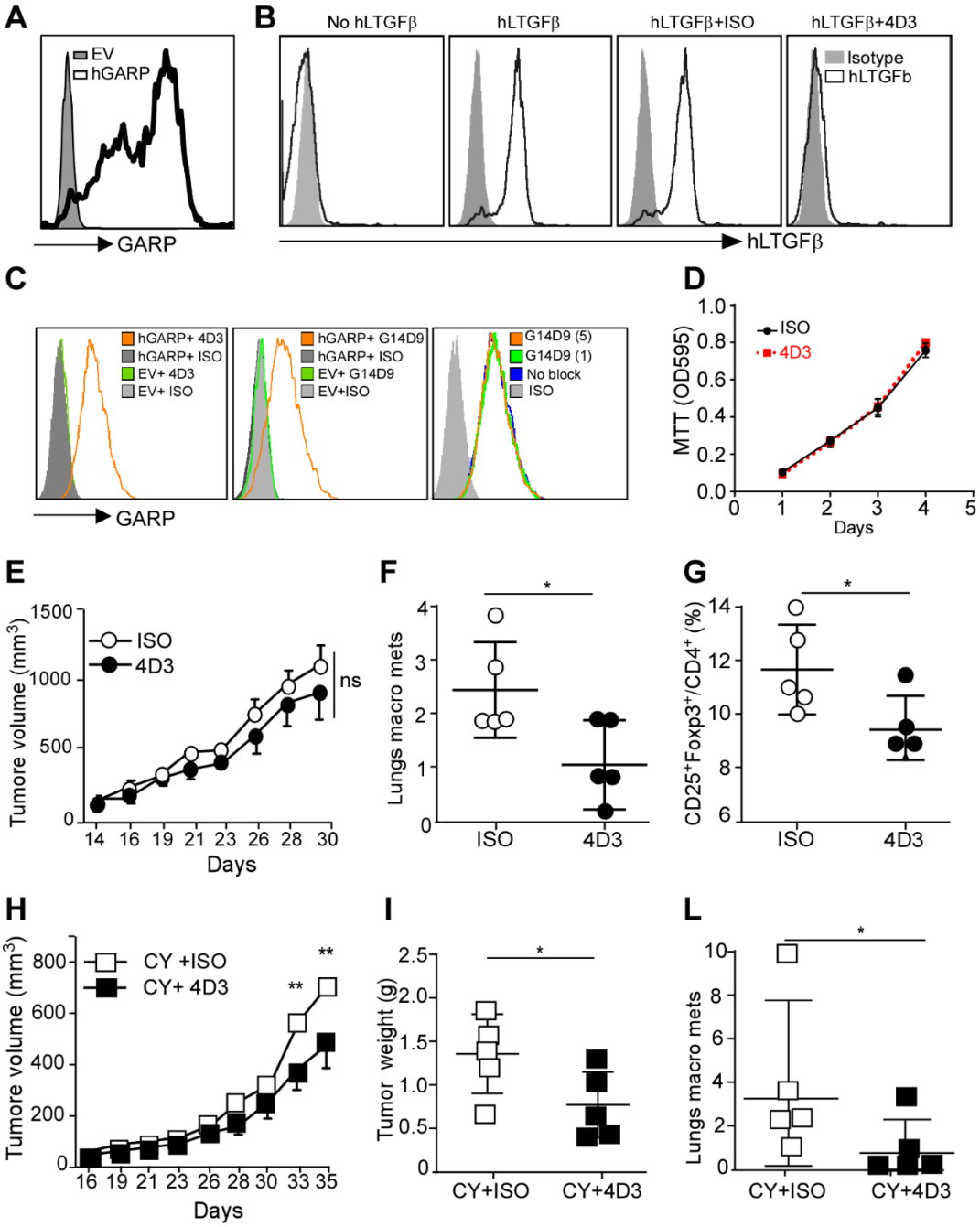


Figure 4-4. Depletion of CD25+ T cells abolished the aggressiveness of soluble GARP expressing mammary tumors. Experimental design. Female BALB/c mice received intra peritoneal injection of PC61 anti-CD25 monoclonal antibody (100 μl ascites) every 4 days, starting 2 days before tumor injection. On Day 0 mice were injected in the 4th mammary fat pad with 5x10^5 tumor cells. Mice were sacrificed on day 15. (B) Tumor volume measured every 3 days. (C) Mice were sacrificed at day 15 after tumor injection and the primary tumors were resected and weighted. (D-E) Tumor-infiltrating lymphocytes were isolated and the number of CD4$^+$CD25$^+$ T cells, CD4$^+$CD25$^+$Foxp3$^+$ Tregs were enumerated by flow cytometry. (F) Tumor-infiltrating CD8$^+$ T lymphocytes were isolated, *ex vivo* stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. IFNγ production was quantified by flow cytometry.

GARP is a novel therapeutic target in cancer

We next determined whether GARP could serve as a novel therapeutic target in cancer, using an antibody-based strategy. The antibody was generated by Dr. Bill X Wu in Dr. Zihai Li's laboratory. In brief, for the generation of anti-GARP monoclonal antibodies (mAbs), mice were immunized with recombinant human GARP, followed by boosting with irradiated whole myeloma SP2/0 cells stably expressing human GARP, with the aim of generating mAbs against GARP that were conformation-specific. More than 20 mAbs were generated that specifically recognize human GARP as determined by flow cytometry. All of these clones were specific for human GARP including clone 4D3 (Figure 4-5A) which also had a low level of cross-reactivity against mouse GARP (data not shown). Importantly, 4D3 but none of the other clones, was able to block the binding of exogenous human LTGF-β1 (huLTGF-β1) to surface GARP (Figure 4-5B). To examine if GARP antibody had any direct anti-tumor activities *in vivo*, BALB/c mice were inoculated orthotopically with 4T1-GARP cells. Mice were then treated with either 4D3 (IgG1) or with isotype control antibody (ISO), either with or without a single dose of CY. This regimen was chosen because it was shown previously that a TGF-β-neutralizing antibody 1D11 was able to potentiate the ability of CY to control 4T1 [116]. We found that 4D3 did not inhibit the primary tumor growth (Figure 4-5C), but it significantly blunted lung metastasis compared with the isotype-treated group (Figure 4-5D). The reduction of lung metastasis was associated with decreased Tregs in the blood. Concomitant treatment with chemotherapy and 4D3 resulted

in significant better control of primary tumors (Figure 4-5F and 4-6G), as well as the lung metastasis (Figure 4-5H).


A
EV
hGARP
GARP
B
No hLTGFβ
hLTGFβ
hLTGFβ+ISO
hLTGFβ+4D3
Isotype
hLTGFb
hLTGFβ
C
hGARP+ 4D3
hGARP+ ISO
EV+ 4D3
EV+ ISO
hGARP+ G14D9
hGARP+ ISO
EV+ G14D9
EV+ISO
G14D9 (5)
G14D9 (1)
No block
ISO
GARP
D
ISO
4D3
MTT (OD595)
Days
E
ISO
4D3
ns
Tumore volume (mm3)
Days
F
Lungs macro mets
ISO
4D3
G
CD25+Foxp3+/CD4+ (%)
ISO
4D3
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CY+ 4D3
Tumore volume (mm3)
Days
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Tumor weight (g)
CY+ISO
CY+4D3
L
Lungs macro mets
CY+ISO
CY+4D3

Figure 4-5. GARP-specific antibody has therapeutic value against preclinical model of breast cancer. (A) Surface staining of pre-B cells stably expressing human GARP (pre-B-hGARP) by 4D3 GARP antibody. Grey histogram represents staining with isotype control antibody**.** (B) pre-B-hGARP cells were incubated without or with human LTGFβ (huLTGF-β), in the presence of GARP antibodies or Isotype control antibody, followed by staining for cell surface hLTGF-β, in order to determine the activity of the antibody. (C) BALB/c mice were injected with $5x10^5$ 4T1-hGARP mammary tumors orthotopically in the 4^{th} mammary fat pad, followed by IP injection of 100 µg/ml of 4D3 GARP antibody every three days. The primary tumor growth kinetics were measured three times a week. Significance is indicated at various time points along the tumor curves. (D) Four weeks after tumor injection, mice were sacrificed. Lungs were isolated and the numbers of visual metastatic nodules were counted. (E) Spleens were harvested and percentage of Treg ($FoxP3^+CD25^+CD4^+$) cells in the spleen of antibody treated mice versus Isotype antibody groups were determined by flow cytometry (F) BALB/c mice were injected with $5x10^5$ 4T1-hGARP mammary tumors orthotopically in the 4^{th} mammary fat pad, followed by one dose of cyclophosphamide and IP injection of 200 µl of 4D3 GARP antibody every three days. The primary tumor growth kinetics was measured three times a week. Significance is indicated at various time points along the tumor curves. (G) Five weeks after tumor injection, mice were sacrificed, tumors were excised and weighted. (H) Lungs were isolated and numbers of visual metastatic nodules were counted. *P<0.05; **P<0.01; ***P<0.001. Significance was determined by two-tailed T-test. Results are representative of two independent experiments.

Broad GARP expression in human cancers

Finally, we reasoned that if GARP expression is an important mechanism for immune tolerance and oncogenesis, aberrant GARP expression shall not be restricted to the breast cancer. The recent cancer genomic revolution, including The Cancer Genome Atlas (TCGA) effort, indeed unveiled that *Lrrc32* is amplified in up to 30% of patients with many human cancer types, including ovarian, lung, breast, and head and neck cancers (data not shown). By IHC, normal colonic epithelial cells showed no significant GARP positivity (Figure 4-6A). However, the primary colon cancers) and lymph node (LN) metastatic lesions stained variably positive for GARP (uniformly negative with isotype control antibody, data not shown) (Figure 4-6A). On a scale of 0 to 4, GARP intensity score ranged between 0 and 3, averaging at 0.78 (p=1.1x10^{-8}) in primary colon cancers and 1.18 (p=0.003) in LN metastasis. More importantly, GARP levels correlated inversely with overall survival in patients with colon cancer, regardless of the pathological grade of tumors or lymph node status of the disease (Figure 4-6B). Similarly, we found significantly increased GARP levels in primary cancers of the lung and lymph node metastasis (Figure 4-6C), and higher GARP expression correlated with worse survival. Overall, we demonstrated for the first time that GARP is widely expressed in human cancers, suggesting that it is a general mechanism in oncogenesis and immune tolerance.

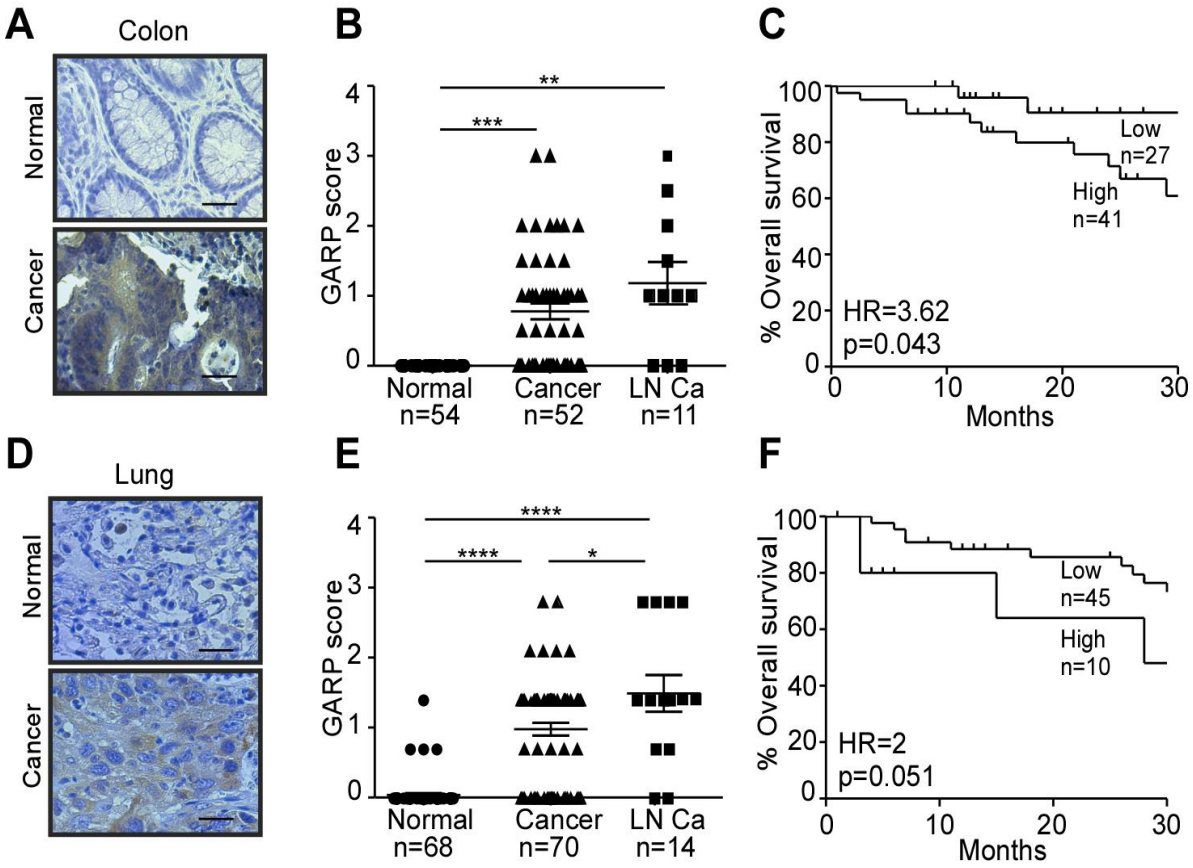


Figure 4-6. GARP upregulation in human colon and lung cancer correlates with poor prognosis (A) Representative images of GARP IHC (brown color) of colon cancers with their respective patient matched normal tissue. Each patient specimen in these TMAs was represented in two cores on the slide and each core measured 1 mm in diameter. Expression intensity of GARP-positive cells in colon cancer is shown. (B) Correlation between GARP expression and overall survival of colon cancer. (C) Representative images of GARP IHC (brown color) of lung cancers with their respective patient matched normal tissue. Each patient specimen in these TMAs was represented in two cores on the slide and each core measured 1 mm in diameter. Expression intensity of GARP-positive cells in lung cancer is shown. (D) Correlation between GARP expression and overall survival of lung cancer. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Statistical significance was analyzed by two-tailed T-test or Kaplan Meier Analysis; the number of samples (n) and are indicated

GARP is over-expressed in prostate adenocarcinoma and its levels positively correlate with Gleason score

Lrrc32 gene amplification has been found in prostate cancers that progressed form hormone-sensitive to hormone-resistance[117]. Interestingly, prostate tumors secrete high amount of TGF-β, raising the possibility that similarly to breast, colon and lung cancer, prostate cancer also might express GARP. In line with this hypothesis plasma TGF-β positively correlates with tumor burden and presence of metastasis in prostate cancer patients[118]. Additionally the inhibition of TGF-β pathway specifically on prostate cancer cells suppresses the tumor progression to higher stage, suggesting that TGF-β exerts a pro-tumorigenic role on the same cells in an autocrine fashion[119]. To test this hypothesis we tested for GARP expression in prostate cancer; 38 malignant and 15 adjacent normal specimens were immunohistochemically stained for GARP. In the adjacent normal group, 6 of 15 samples were negative and the rest only showed faint stain (Intensity=1). In turn, 6 of 38 cancer samples stained negative, and the remaining samples ranged from faint (Intensity=1) to intense (Intensity=4). Therefore, the average intensity was 0.6 in the normal group compared to 1.29 in cancer ($p=0.0016$). As for the average percent of positive cells, it was 26.7% in normal samples and 61.8% in cancer ($p=0.0012$) (Figure 4-7A and 4-7B)

GARP expression also correlated with Gleason score, which is a measure of the grade of de-differentiation of the tumor; a higher Gleason score indicates a higher-grade tumor. Tumors with Gleason scores 6-7 ($n=16$) had an average intensity of 1.125, whereas those with Gleason scores 8-10 ($n=22$) averaged at 1.643

(p=0.036). In terms of tumor stage, we found a trend towards higher expression of GARP in stage III disease (n=30, Intensity=1.4) compared to stage II (n=8, Intensity=0.875), although the results did not reach statistical significance, likely due to the small number in the stage II group (p=0.18) (Figure 4-7C)

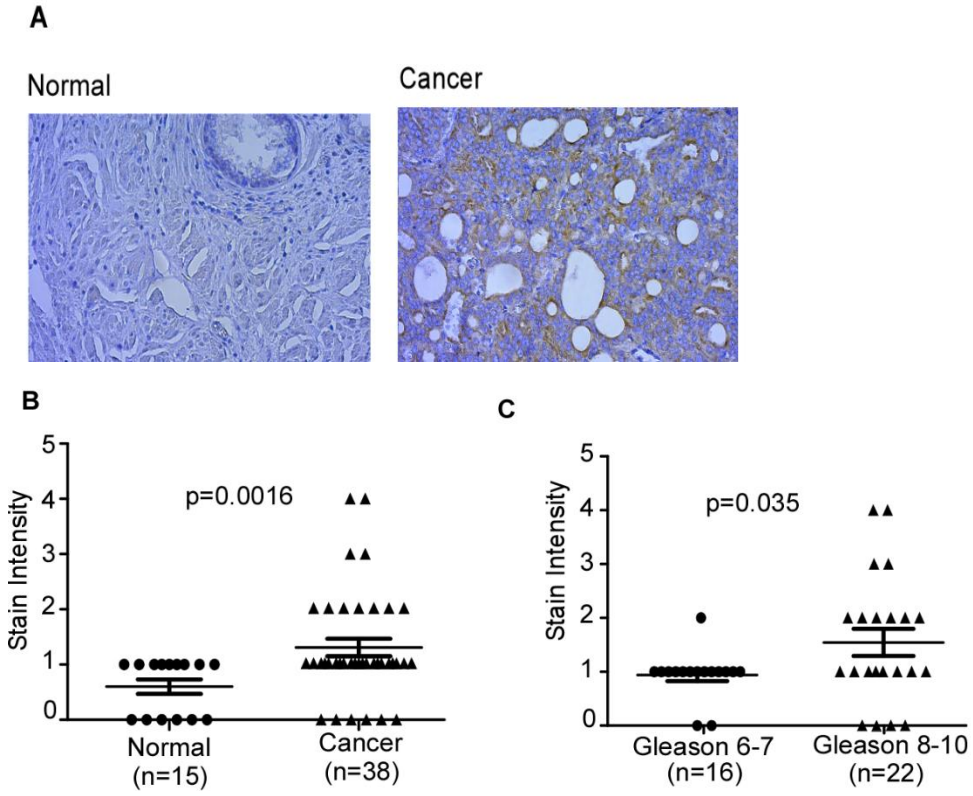


Figure 4-7: GARP is over-expressed in prostate adenocarcinoma and its levels positively correlate with Gleason score Representative images of GARP IHC (brown color) of prostate cancer with their matched normal prostate tissues. Each patient specimen in these TMAs was represented in two cores on the slide and each core measured 1 mm in diameter. Scale bar: 20 μm B. Expression intensity of GARP-positive cells in prostate cancer specimens and patient matched adjacent normal tissue. C**.** Patient's matched correlation between expression intensity of GARP-positive cells and Gleason Score.

Soluble GARP is present in the plasma of prostate cancer patients and correlates with high PSA and presence of metastasis

We also estimated the concentration of the soluble form of GARP protein (sGARP) in plasma of 48 prostate cancer patients and 6 healthy controls and found an increase in cancer patients (325.6 pg/ml ± 37.88) as compared to healthy controls (143.2 pg/ml ± 30.82) (Figure 4-8A). We then analyzed prostate cancer patients with sGARP concentration higher than 150 pg/ml and observed that 78.5% of these patients had PSA higher than 10, and 57% presented secondary tumors (Figure 4-8B).

Finally, we developed a unique sandwich ELISA to evaluate serum concentration of sGARP in complex with TGF-β (sGARP/TGF-β) and found that the concentration of the complex was higher in serum collected from prostate cancer patients compared to healthy controls (p=0.0305) (Figure 3-8C).

Our results on prostate cancer show that histological presence of GARP expression on prostate tissue is a powerful diagnostic tool to use in combination and Gleason Score to ameliorate the current grading system for patients' stratification. Moreover, soluble GARP present in plasma represents a useful and non-invasive diagnostic and prognostic tool.

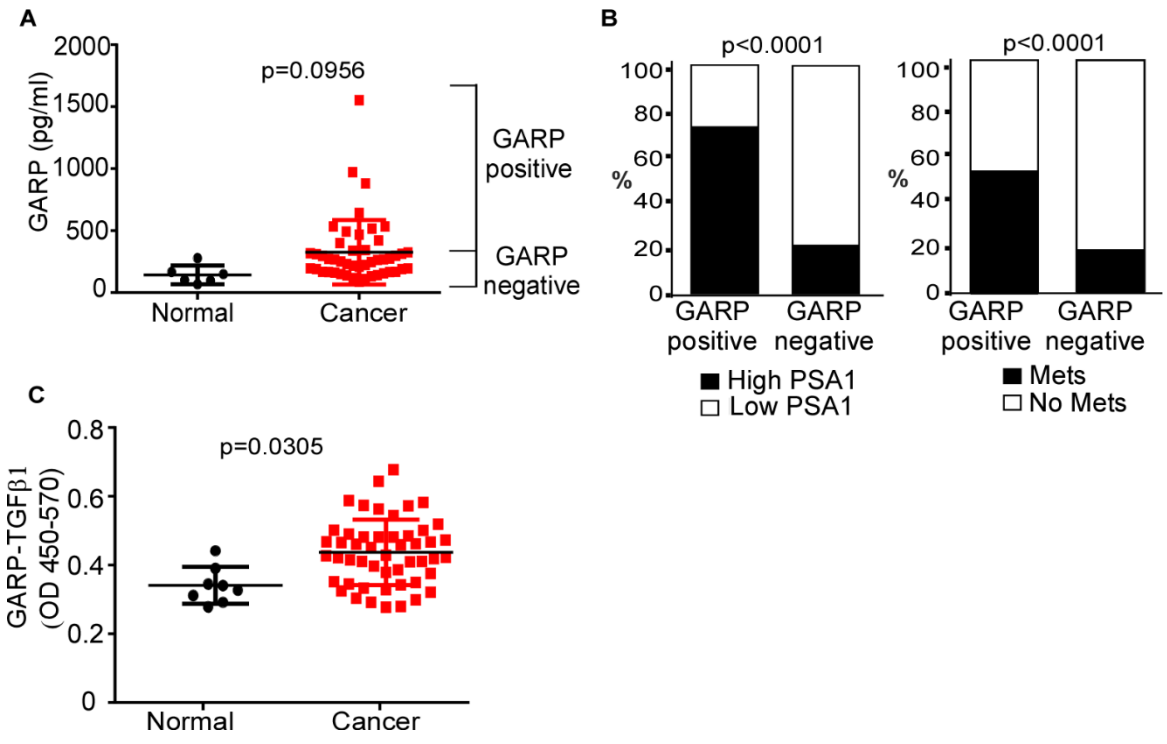


Figure 4-8: Soluble GARP is present in the plasma of prostate cancer patients and correlates with high PSA and presence of metastasis Soluble GARP ELISA performed on plasma samples collected in heparin coated tubes of prostate cancer patients and healthy controls. B. Correlation analysis between GARP positive or negative plasma samples with and high or low PSA1 (left panel), and percentage of metastasis (right panel). C. GARP/TGF-β sandwich ELISA performed on plasma samples collected in heparin coated tubes of prostate cancer patients and healthy controls.

Discussion

Surface expression of TGF-β in cancer has been recognized[120,121], but its biological significance has remained elusive. This is in part due to lack of understanding of the molecular basis for membrane-bound TGF-β. The discovery of GARP to be the sole cell surface docking receptor for latent TGF-β finally created an experimental opportunity to manipulate the level of surface TGF-β through altering GARP. In this study, we demonstrated that the GARP/TGF-β axis plays both cancer-intrinsic and extrinsic roles in promoting oncogenesis.

Using both human and mouse systems with a combination of genetic and immunological tools, we have made several fundamental discoveries in this study: (i) GARP is widely expressed by human cancer cells, but less so by normal epithelial cells; (ii) GARP expression and presence in plasma of cancer patients correlates with advanced stage of cancer and poor prognosis; (iii) GARP itself has a transformation potential, which renders normal mammary gland epithelial cells tumorigenic; (vi) GARP expression in cancer cells leads to increased TGF-β activity, likely due to its ability to concentrate LTGF-β in cis as well as in trans, contributing to cancer aggressiveness and metastasis; (v) GARP expression in the tumor microenvironment promotes the induction of Treg cells and thus blunts the function of effector T cells against cancers; and (vi) neutralizing GARP by blocking its ability to bind to TGF-β reduces tumor metastasis, even in the absence of chemotherapy or shrinkage of primary tumors.

Mechanistically, we discovered that GARP expression enhances TGF-β activation. We demonstrated this in both NMuMG and 4T1 that enhanced TGF-β activation translated into increased canonical signaling, such as phosphorylation of Smad-2/3. Importantly, the accumulation of active TGF-β within the tumor microenvironment impairs anti-tumor immunity through multiple mechanisms including the induction of Treg cells[122,123]. We investigated the latter utilizing a syngeneic tumor model, namely 4T1 mammary carcinoma (BALB/c). Our data indicate that GARP expression in 4T1 induces Treg cells which blunts the ability of effector T cells to control cancer. Thus, by positively regulating TGF-β in the tumor microenvironment, GARP promotes oncogenesis through cancer-intrinsic as well as cancer-extrinsic mechanism.

The oncogenic roles of TGF-β span from promoting invasion, metastasis and angiogenesis, to maintaining stemness and inducing immune tolerance [48]. Thus, TGF-β remains an attractive target for the treatment of cancer. However, the development of therapeutics that target TGF-β has thus far been hampered by multiple factors, not the least of which is the presence of multiple ligands in multiple forms and the context-dependent function of this pleotropic cytokine [124]. Surface expression of GARP provides cancer cells a means to concentrate TGF-β locally and thus influence TGF-β-dependent growth, transformation and invasion through activation of both integrins and the TGF-β receptors. GARP therefore represents a unique alternative target for blocking the TGF-β pathway in the tumor microenvironment. In this study, we generated a panel of highly specific mAbs

against human GARP, and demonstrated that 1 of these mAbs, 5C5, with or without cyclophosphamide, successfully treated 4T1-hGARP tumors. We believe that GARP-targeted mAbs may exert their therapeutic benefits via three mechanisms: 1) inhibition of LTGF-β binding to GARP and thus blockade of TGF-β biogenesis, 2) tumor-killing through antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cell cytotoxicity (CDCC) and, 3) Ablation of immunosuppressive Treg cells. Interestingly, 4D3 clone is murine IgG1 isotype which has very limited ADCC and CDCC activities. Thus, the therapeutic effect of 4D3 is likely through its ability to block the binding between LTGF-β and GARP, a speculation that is in part supported by reduction of Tregs in the tumor microenvironment. More importantly, unlike the systemic effect of anti-TGF-β blocking antibody, anti-GARP antibody would prevent the activation of TGF-β on the surface of GARP expressing cells, such as Treg and cancer cells.

In conclusion, we have discovered that GARP is a novel oncogene due to its cancer-intrinsic roles in promoting invasion and metastasis, as well as its cancer-extrinsic roles in inducing immune tolerance. It is a novel diagnostic and therapeutic target for cancer.

Chapter 5

GARP/TGF-β axis on activated platelets supports tumor progression

Rationale

Depending on the type and the aggressiveness of the tumor, the incidence of pulmonary embolism and deep venous thrombosis in cancer patients are twice the frequency of the same events in non-cancer patients[125]. This phenomenon was first described in the pioneering work of Armand Trousseau in 1865 when a close association was observed between deep vein thrombosis and advanced gastric malignancy[126,127]. Later studies named this process TCIPA: tumor cells-induced platelets aggregation. Tumor cells indeed produce high level of blood coagulation factors like thromboxane A2, serine proteases, matrix metalloproteinase, prostacyclin, IL-6 and nitric oxide (NO) that stimulate platelets production, aggregation, activation and degranulation[128,129]. Platelets, in turn, confer to cancer cells a selective advantage by forming a “cloak” of fibrin that protects the tumor by the tumoricidal attack of NK cells[130], neutrophils[131], macrophages and CTLs[132].

Critical mediators of platelets-induced tumor growth are the α-granules released by platelets upon activation. By mass spectrometry analyses of Platelets Releasate (PR), we observed that one of the most abundant soluble factors secreted by platelets was TGF-β[2]. This was not surprising knowing that platelets are considered reservoirs of several bioactive molecules, however TGF-β was by far the most abundant among all the molecules present in PR. Interestingly, L-

lactate concentration was significantly high in PR. We observed that PR contained both the latent and active form of TGF-β, and that the latter was the main suppressor of T cell mediated anti-cancer immunity[2]. The mechanism employed by thrombin stimulated platelets to release active TGF-β is not completed elucidated. In the attempt to identify the enzyme involved in TGF-β maturation, Blakytny and colleagues, inhibited all the known latent TGF- β activators as serine proteinases, cysteine proteinases, thrombospondin1 and plasmin, yet none among those enzyme was responsible for mature TGF-β release in PR[81;133]. This might suggest that LAP and TGF-β dissociation is not mediated by proteases.

Other than being the major reservoir of TGF-β, platelets are the only entity that constitutively express surface GARP, suggesting that the pool of platelet TGF-β is made by two fractions: one is stored in the α granules and released upon activation, the other is exposed on platelets surface through GARP. Of notes, it has been already described that two pools of TGF-β are secreted by active platelets, one pool is found in serum as large latent complex (LLC), while the other pool is initially retained in the fibrin clot as bound to LAP, subsequently when the plaque dissolves TGF-β gradually is activated and released[134]. Whether GARP is specifically involved in release and/or activation of TGF-β contained in one of the two pools, is not known.

As an important modulator for the final step of TGF-β maturation[14], we reasoned that GARP might participate in platelet derived latent TGF-β activation. Previous studies showed that $GARP^{+}Foxp3^{+}$ Treg correlates with immunosuppressive and

more aggressive phenotype in advance hepatocellular carcinoma[72] and ovarian cancer[29], yet the physiological function of platelet GARP in anti-cancer host immunity is completely unknown.

In this chapter we employed a novel mouse model where the gene encoding for GARP was specifically knock-out in megakaryocytes and platelets. Additionally, the clinical potential of a targeted pharmacological therapy which reduces surface GARP on platelets was investigated.

Anti-platelets regimens have been largely explored in cancer prevention and treatment. Acetyl salicylic acid (Aspirin), for example showed in several clinical trials to lower the short-term risk of cancer[135,136]. We have recently demonstrated that Aspirin in combination with Clopidogrel potentiate adoptive T cell therapy in B16 melanoma, yet alone these anti-platelets agents do not have significant anti-tumor activity in our model[2].

Aspirin and Clopidogrel inhibit platelets activation by blocking the conversion of arachidonic acid to Thromboxane A2 and by acting as ADP receptor antagonist, respectively[137].

Here, to improve the efficacy of the anti-platelets therapy in cancer treatment we decided to block the most potent platelets activator, thrombin[138], by using the thrombin inhibitor Dabigatran Etexilate, known as Pradaxa. Dabigatran has been FDA approved in October 2010 for the treatment and reduction of risk of deep vein thrombosis and pulmonary embolism. Its mechanism of action relies on the competitive and reversible binding to thrombin active site, thus impeding

coagulation factor-mediated thrombin activation. Interestingly, Dabigatran has anti-tumor properties in preclinical model of breast and pancreatic cancer[139,140], yet the mechanism remains elusive.

When compared to aspirin and Clopidogrel, Dabigatran presents several advantages: first by blocking directly thrombin, Dabigatran blocks the main mediator of the platelets activation[141]; second, Dabigatran directly inhibits thrombin-mediated conversion of fibrinogen to fibrin that leads to clot formation. Most importantly, dabigatran can still inactivate thrombin even when thrombin is fibrin-bound[142,143]. This latter function is critical in cancer because by blocking fibrin-bound thrombin, Dabigatran restrains the accumulation of active TGF-β in the fibrin plaque that surrounds and protects the tumor.

In this chapter platelet GARP/latent TGF-β complex was studied and manipulated through Dabigatran to understand its function in host immunity against cancer *in vivo.*

Results

GARP deletion on platelets does not alter platelets activation and number

For the generation of mice with specific deletion of GARP on platelets the Cre-Lox recombination system was employed. *Pf4*creGARPf/f mice and their littermates were obtained by crossing GARP flox/flox with *Pf4cre* mice. Both mice have been previously described[24,88]. PCR analysis of genomic DNA demonstrates the deletion of Exon1 in both alleles of GARP gene in *Pf4*creGARPf/f mice (Figure 5-1A). Phenotypical characterization of WT and *Pf4*creGARPf/f mice was performed by flow-cytometry analysis of GARP expression on $CD41^+$ activated platelets. In *Pf4*creGARPf/f mice, GARP expression on platelets was totally abrogated (Figure 5-1B). One study performed on *Danio rerio* (zebrafish) demonstrated that GARP is important for thrombus initiation and tissue homeostasis[85]. To assess whether the function of murine platelets was jeopardized by GARP abrogation, we performed a bleeding time test on *Pf4*creGARPf/f and WT littermates. There was no statistically difference between the two groups (Figure 5-1C). This confirmed the finding of another group that in parallel with us showed that absence of GARP from platelets surface did not affect the ability of platelets to control hemostasis[31]. In our system, GARP deletion occurs in the megakaryocytes, platelets precursors[144]. To determine whether this might affect platelets generation, platelets count was performed, yet normal platelet number was observed in *Pf4*creGARPf/f mice (Figure 5-1D). Finally, thrombin-mediated platelets activation was evaluated by p-Selectin expression. Upon thrombin *ex-vivo* stimulation both platelet GARP KO and WT upregulated p-

Selectin as marker of platelets activation (Figure 5-1E). Overall these results indicate that GARP is not essential for normal platelet biogenesis and hemostasis.

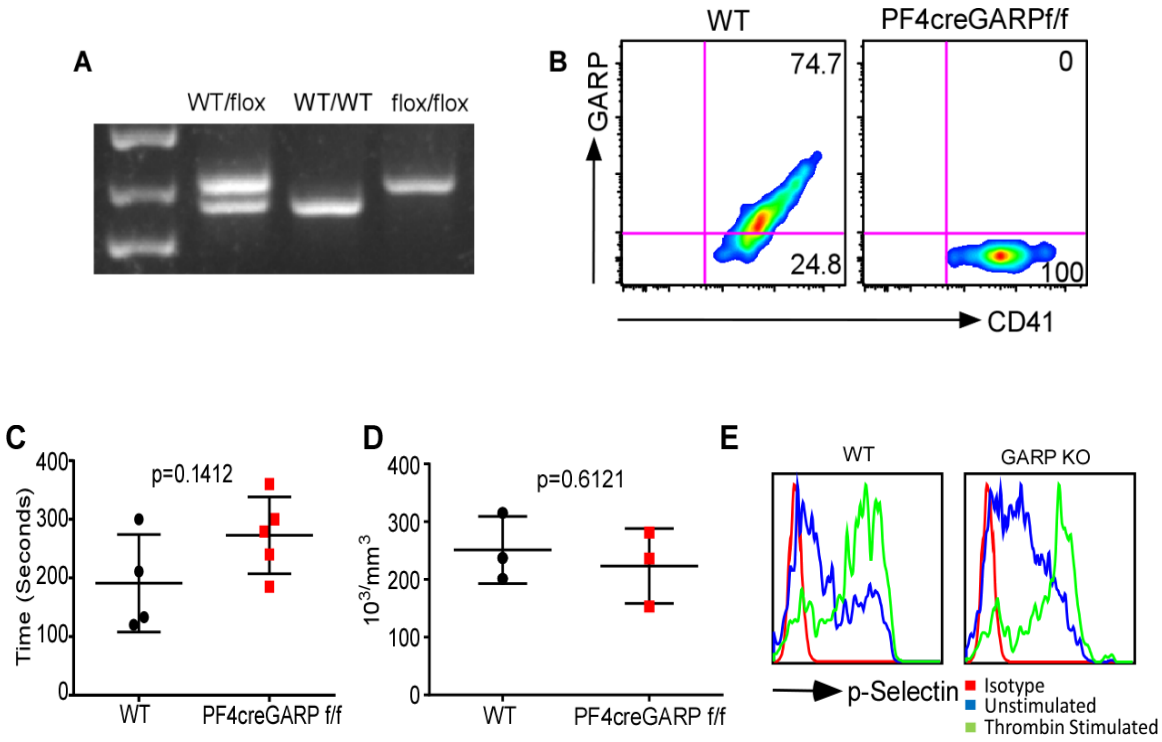


Figure 5-1. GARP deletion on platelets does not alter platelets activation and number. A. Polymerase chain reaction (PCR) showing the excision of exon 1 from GARP gene that results in a smaller DNA fragment. B. Flow cytometry analysis showing the complete lack of expression of GARP in $CD41^{+}$platelets. C. Little incision was performed on mice tail and bleeding time was evaluated. D**.** Periperal blood platelet count performed by scil Vet ABC instrument. E. Flow cytometry analysis to evaluate the p-Selectin expression of WT and GARP KO platelets upon thrombin activation. Two-tailed, independent Student's *t*-test was used in panels C and D.

Platelet-derived GARP/TGF-β complex blunts adoptive T cell therapy of melanoma

So far we have shown that GARP is not critical for normal platelets number and function, however upon thrombin activation GARP is abundantly upregulated on platelets (Figure 5-6A), suggesting that it might play a role in active platelets beside thrombus formation. As explained earlier, platelets derived TGF-β plays a critical role in enhancing tumor progression, for this reason we challenged *Pf4*creGARPf/f and WT littermate mice with B16-F1 melanoma cells to investigate whether GARP, by enhancing TGF-β activation, might promote tumor progression. Surprisingly, tumors in both groups grew at the same rate and with the same aggressiveness (Figure 5-2A). Knowing that B-16-F1 is a poorly immunogenic tumor we asked whether GARP plays critically a roles in the tumor microenvironment by acting on the anti-tumor T cell immunity. This hypothesis was next addressed by comparing the efficacy of adoptive T cell therapy of melanoma in WT and *Pf4*creGARP f/f recipient mice. B16-F1 melanomas were established in either WT or *Pf4*creGARP f/f mice, followed by lymphodepletion with Cy on day 9, and the infusion of *ex vivo* activated Pmel T cells on day 10 (Figure 5-2B). Tumors were controlled much more efficiently in *Pf4*creGARP f/f mice compared with WT mice as represented by the tumor curve growth (Figure 5-2C) and survival (Figure 5-2D). This was associated with longer persistency (Figure 5-2E) and better functionality (Figure 5-2F) of adoptively transferred Pmel1 cells in *Pf4*creGARP f/f mice peripheral blood. These results indicate that GARP on platelets decreases the efficacy of T cell-mediated anti-tumor immunity.

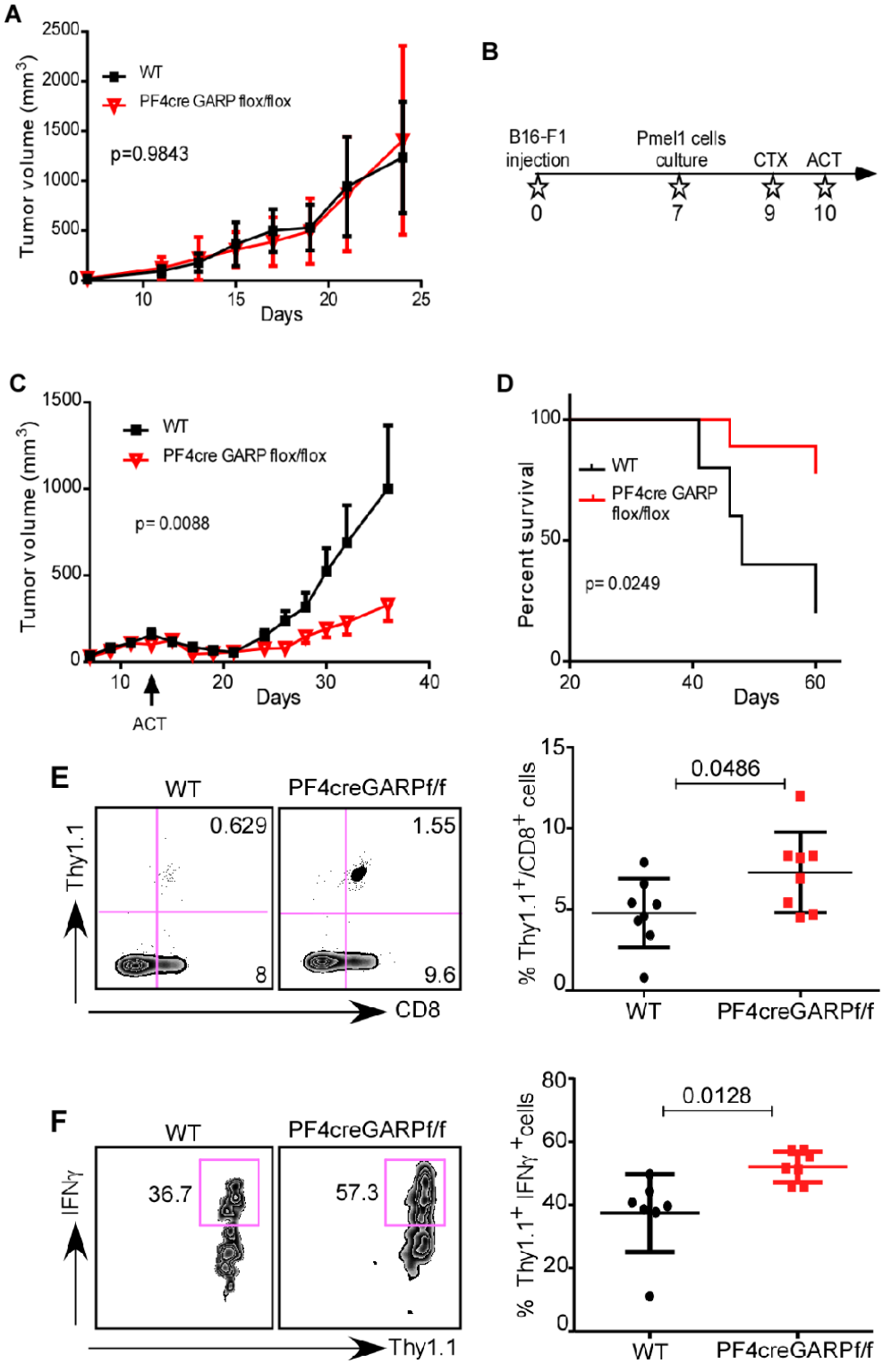

A
Tumor volume (mm3)
WT
PF4cre GARP flox/flox
p=0.9843
Days
B
B16-F1 injection
Pmel1 cells culture
CTX
ACT
0
7
9
10
C
Tumor volume (mm3)
WT
PF4cre GARP flox/flox
p= 0.0088
ACT
Days
D
Percent survival
WT
PF4cre GARP flox/flox
p= 0.0249
Days
E
WT
PF4creGARPf/f
Thy1.1
CD8
0.629
8
1.55
9.6
% Thy1.1+/CD8+ cells
0.0486
WT
PF4creGARPf/f
F
WT
PF4creGARPf/f
IFNγ
Thy1.1
36.7
57.3
% Thy1.1+ IFNγ+cells
0.0128
WT
PF4creGARPf/f

Figure 5-2 Platelet-derived GARP/TGF-β complex blunts adoptive T cell therapy of melanoma. A. Tumor growth of WT and *Pf4*creGARPf/f mice (n=7-8) subcutaneously injected with B16-F1. B. Experiment design: on day 0. Splenocytes from Thy1.1^{+} Pmel transgenic mice were culture for 3 days and injected on day 10 in congenic Thy1.2 mice previously conditioned with Cy on day 9. C**.** Tumor growth curves of mice**.** D. Kaplan-Mayer survival curve in B16-F1 adoptively transferred bearing mice. E**.** The frequency of Pmel cells in mice was enumerated 3 weeks post-adoptive transfer of T cells by flow cytometry in the peripheral blood (CD8+Thy1.1+/total CD8+). F. IFN-γ producing ability of antigen-specific donor T cells (Pmel) from indicated mice 3 weeks after T cell transfer. Repeated measures ANOVA was used in panel A and C. Two-tailed, independent Student's *t*-test was used in panels E and F.

Platelet-intrinsic GARP plays critical roles in generating active TGF-β

Systemic blockade of TGF-β signaling improves the effectiveness of adoptively transferred T cells[145]. Given that GARP enhances TGF-β activation and that platelets are the major reservoir of TGF-β, we decided to investigate TGF-β activation status as potential mechanism to explain the better anti-tumor activity observed in *Pf4*creGARPf/f mice. We observed that KO mice had a severe impairment in the activation of latent-TGF-β since they showed reduced active TGF-β (Figure 5-3A) and increased total TGF-β (Figure 4-3B).

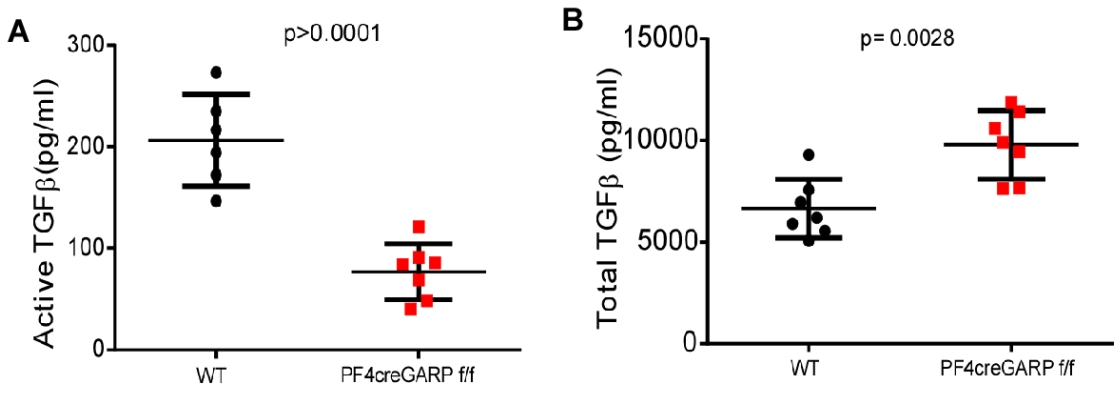


Figure 5-3 Platelet-intrinsic GARP plays critical roles in generating active TGF-β. Serum levels of active A. and total B. TGF-β were measured by ELISA in B16-F1 bearing mice (n=6-8 per group). Comparison was performed using two-tailed, independent Student's *t*-test.

Targeting platelet-derived GARP/TGF-β complex improves MC38 tumor control

To further investigate the TGF-β driven phenotype that we observed using B16-F1 tumor model, we decided to employed MC38 colon carcinoma. In this model, indeed, tumor infiltrated $CD8^+$ T cells functionality is severely impaired by TGF-β signaling[146]. Interestingly the rate of tumor growth was the same in both groups in the first two weeks after tumor injection, only around day 15 the two curves started to separate showing a better tumor control by *Pf4*creGARP f/f mice (Figure 5-4A). This might indicate that platelets derived TGF-β has a potent immunosuppressive effect on the adaptive arm of the anti-tumor immunity. This was reflected also by the longer overall survival experienced by *Pf4*creGARP f/f (Figure 5-4B), along with primary tumor weight (Figure 5-4C and Figure 5-4D). We next measured by ELISA the concentration of serum active and total TGF-β in tumor bearing mice and we observed a drastic reduction of the mature form of TGF-β (Figure 5-4E) in parallel with an increase of the total form of the same cytokine (Figure 5-4F).

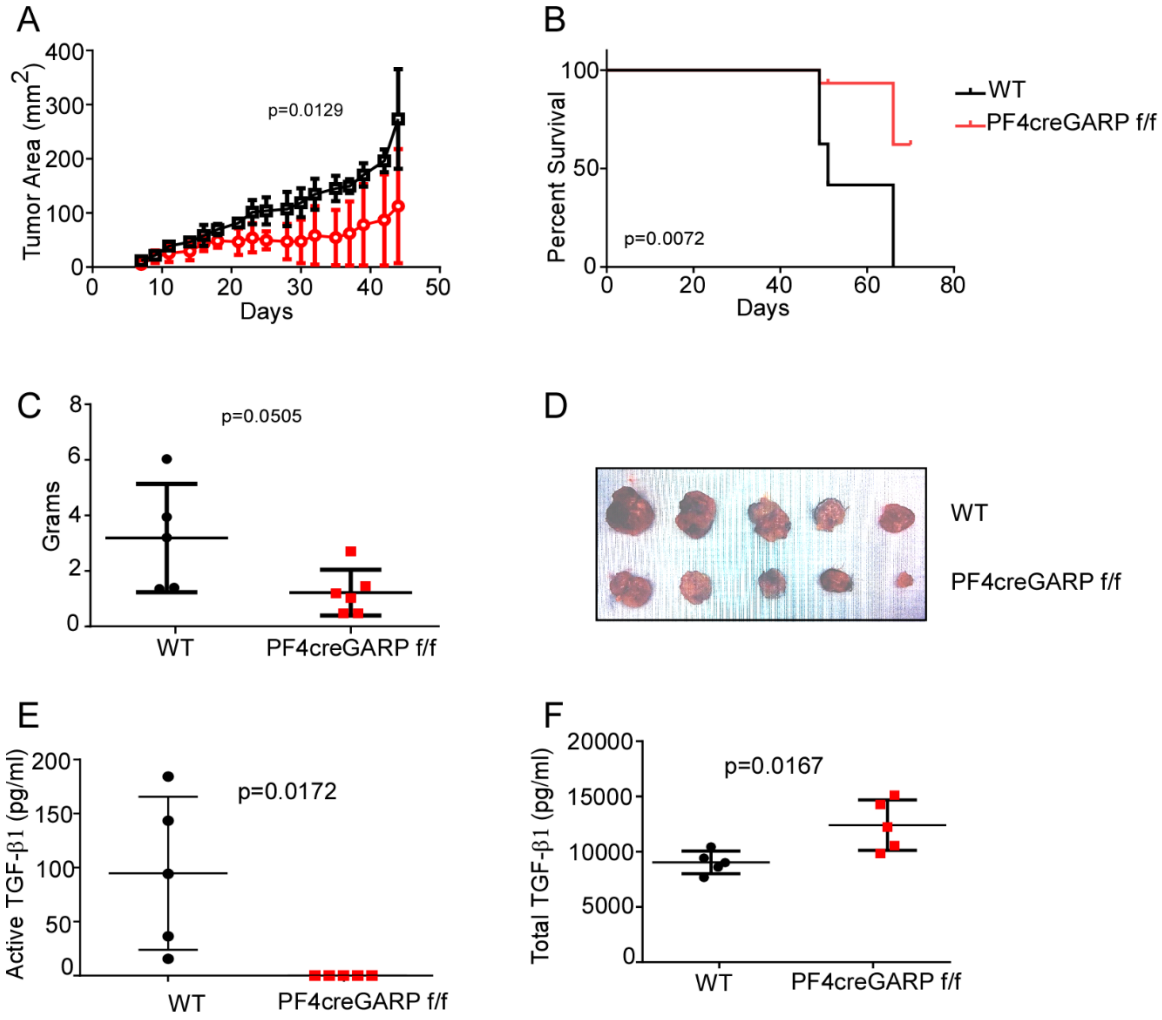


Figure 5-4 Targeting platelet-derived GARP/TGF-β complex improves MC38 tumor control. A. WT or Platelet GARP KO mice (n=5 each group) were injected in the right flank with $1x10^6$ MC38 colon cancer cells. Tumor size was measured every 3 days with digital vernier caliper. B. Kaplan-Mayer survival curve in MC38-bearing mice. C. In a separate experiment, 6 weeks after MC38 injection, mice were sacrificed and the primary tumors were resected and weighed. D. The inset shows the photographs of primary tumors resected from mice 6 weeks after injection. Serum was obtained from mice 6 weeks after MC38 injection and E. active and F. total TGF-β1 was measured by ELISA. Repeated measures two-way ANOVA was used in panel A; Kaplan-Meier curves and log rank tests were used in panel B. Two-tailed, independent Student's *t*-test was used in panels C, E, and F.

Targeting platelet-derived GARP/TGF-β complex results in reduction of TGF-β activity in the tumor microenvironment

Physiologically, cancer represents non-healing wound where the coagulation cascade forms a fibrin cloak where platelets are constantly activated[147]. We therefore reasoned that GARP effect on TGF-β derived platelets would have been obvious within the tumor. For this reason, tumors from WT and *Pf4*creGARPf/f mice were analyzed for active TGF-β signaling pathway. As expected, Smad3 phosphorylation was significantly increased in WT tumor when compared to tumors from *Pf4*creGARPf/f mice as shown by IHC pictures (5-5A) and score (5-5B). Accordingly, GARP abrogation in platelets impaired regulatory T cells expansion (5-5C).

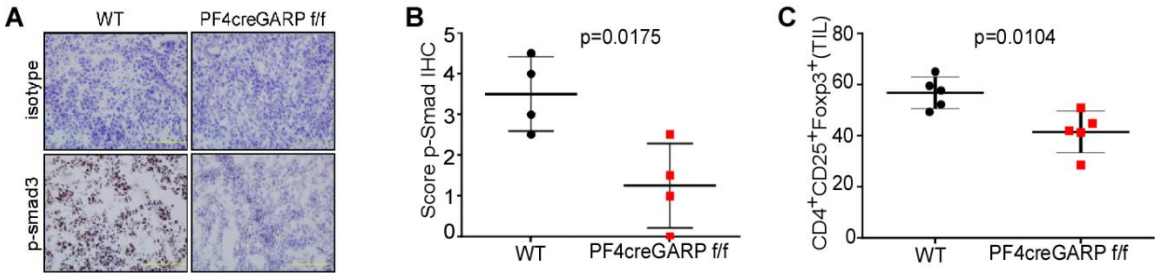


Figure 5-5 Targeting platelet-derived GARP/TGF-β complex results in reduction of TGF-β activity in the tumor microenvironment. A. IHC for p-SMAD-2/3 in MC38 tumors from indicated mice; representative images are shown. Scale bar: 12.5 μm. B. Independent histopathological quantification of p-SMAD-2/3 staining intensity from panel A (n=4 per group). C. Percentage of regulatory T cells $CD25^+$ $FOXP3^+$ in the $CD4^+$ tumor-infiltrating lymphocytes (TIL) from the indicated mice. Two-tailed, independent Student's *t*-test was used in panels B and C.

Platelet GARP is increased upon thrombin stimulation and enhances active TGF-β release in Platelets Releasate

In the PR derived from human platelets stimulated with thrombin, TGF-β is one of the most abundant cytokines[2]. To study how platelet GARP modulates TGF-β release and activation in PR, we stimulated WT and GARP KO platelets with thrombin and analyzed GARP expression and TGF-β activation status. Active platelets increased surface GARP/LAP expression of about 45%. Notably, LAP expression was also increased in activated GARP KO platelets, suggesting the upregulation of other latent TGF-β binding receptors, however the amount of latent TGF-β on GARP KO platelets was still less compared to activate WT platelets (5-6A). Next WT and GARP KO platelets were isolated from peripheral blood and activated in presence or absence of thrombin (Figure 5-6B). Western blot analysis of the resulting PR revealed that upon thrombin stimulation GARP on platelets enhances the release of total as well as active TGF-β. (Figure 4-6C). Furthermore, TGF-β ELISA performed on the PR confirmed that GARP is critical for activation of the TGF-β released in PR (Figure 5-6D left panel), while the amount of total TGF-β was not affected by the lack of GARP (Figure 5-6D right panel). We next hypothesized that just like in Treg, GARP could be also be released from activated platelets as soluble protein in the PR. For this reason, we stimulated murine WT platelets with increasing units of mouse thrombin, collected the PR and analyzed GARP by Western Blot. Strikingly, we observed soluble GARP in PR in response to thrombin in the dose-dependent fashion (Figure 5-6E). Accordingly, further

ELISA data showed that GARP was released in PR only upon thrombin mediated platelet activation (Figur5-6F).

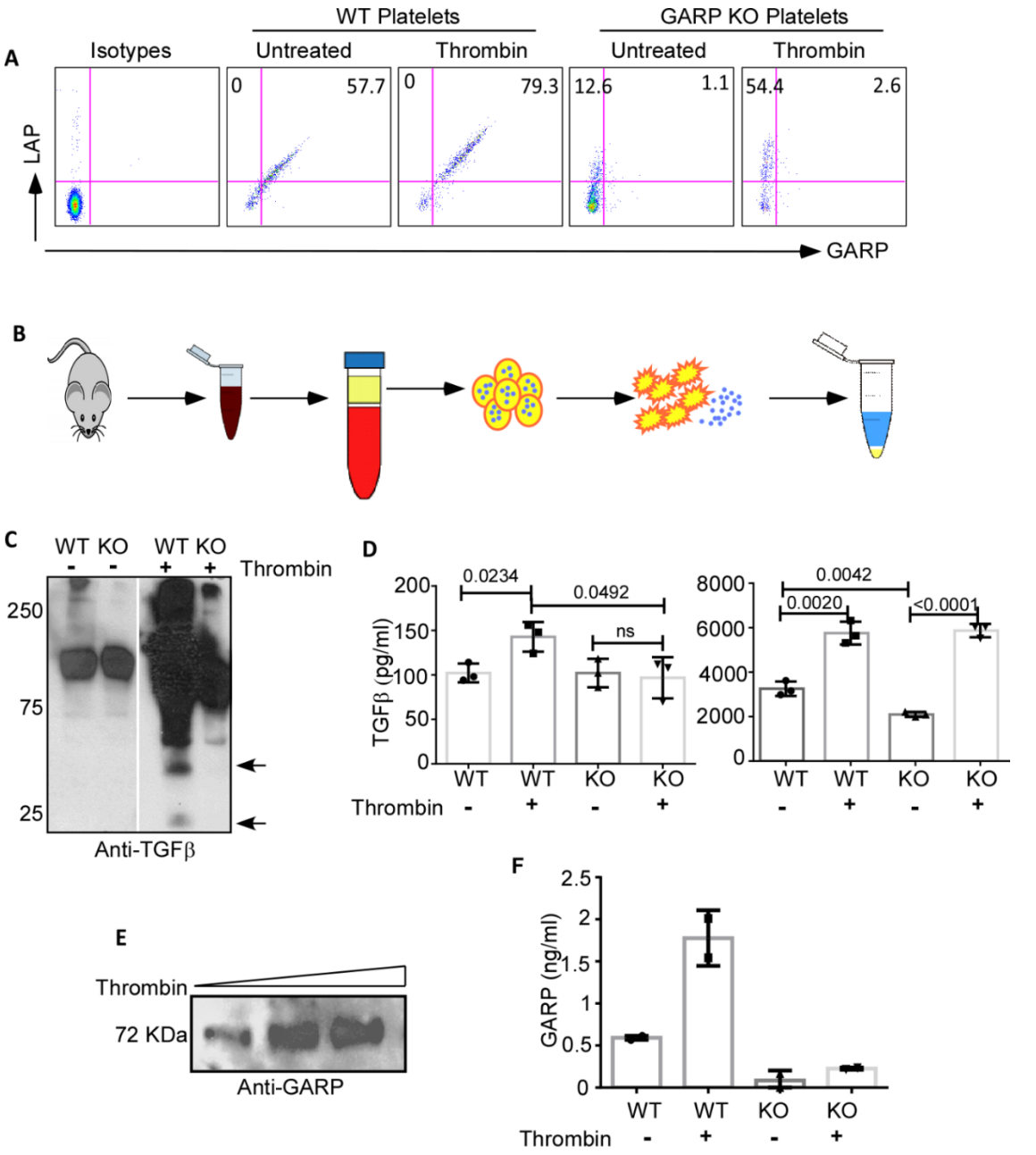

A
WT Platelets
GARP KO Platelets
Isotypes
Untreated
Thrombin
Untreated
Thrombin
0
57.7
0
79.3
12.6
1.1
54.4
2.6
LAP
GARP
B
C
WT KO
WT KO
- - + +
Thrombin
250
75
25
Anti-TGFβ
D
TGFβ (pg/ml)
0.0234
0.0492
ns
0.0042
0.0020
<0.0001
WT WT KO KO
Thrombin - + - +
E
Thrombin
72 KDa
Anti-GARP
F
GARP (ng/ml)
WT WT KO KO
Thrombin - + - +

Figure 5-6 Platelet GARP is increased upon thrombin stimulation and enhances active TGF-β release in Platelets Releasate. A. Flow cytometry analysis of surface GARP/LAP complex on WT and GARP KO platelets with and without thrombin stimulation. B. Schematic representation of the passages required for PR isolation. C. PR from WT and GARP KO platelets was obtained with and without thrombin stimulation. PR was analyzed by western Blot in non-reducing and non-denaturing conditions. D. PR from WT and GARP KO platelets was obtained with and without thrombin stimulation. TGF-β was quantified by ELISA. E. PR from WT animals was stimulated with increasing concentration of Thrombin and analyzed by WB in reducing and denaturing conditions. F. PR from WT and GARP KO platelets was obtained with and without thrombin stimulation. Soluble GARP was quantified by ELISA. Two-tailed, independent Student's *t*-test was used in panels D and F.

Direct thrombin inhibitor Dabigatran Etexilate reduces platelet GARP expression and protects against melanoma and colon cancer

So far we have demonstrated that thrombin-stimulated platelets release active TGF-β through the mediation of GARP. Now, to establish the clinical relevance of the Thrombin-GARP/TGF-β axis we employed the direct thrombin inhibitor, Dabigatran Etexilate. Flow cytometry analysis of thrombin activated platelets shows that the increase of GARP expression on platelets can be neutralized by the inhibitory activity of Dabigatran (Figure 5-7A). We than tested the anti-tumor efficacy of Dabigatran Etexilate in 2 different tumor models. Strikingly we observed that Dabigatran reduced B16-F1 tumor aggressiveness even in absence of adoptive T cells transfer (Figure 5-7B). The efficacy of Dabigatran was then tested with MC38 colon carcinoma where, as well as B16-F1, the sole direct inhibition of thrombin was sufficient to decrease the rate of tumor growth (Figure 5-7C). As a further proof of the mechanism employed by dabigatran to exert its antitumor activity, serum active and total TGF-β, and serum GARP concentrations were assessed by ELISA. Even though not statistically significant the pool of active TGF-β was drastically reduced: the mean observed in untreated mice was 101.3 ± 26.59, versus 42.47 ± 23.73 in Dabigatran treated mice. Similarly, blocking thrombin mediated platelets activation reduced serum total TGF-β (Figure 5-7E). Notably, Dabigatran impairs the released of serum soluble GARP from platelets (Figure 5-7F) and reduces the TGF-β mediated induction of Treg in the tumor microenvironment (Figure 5-7G).

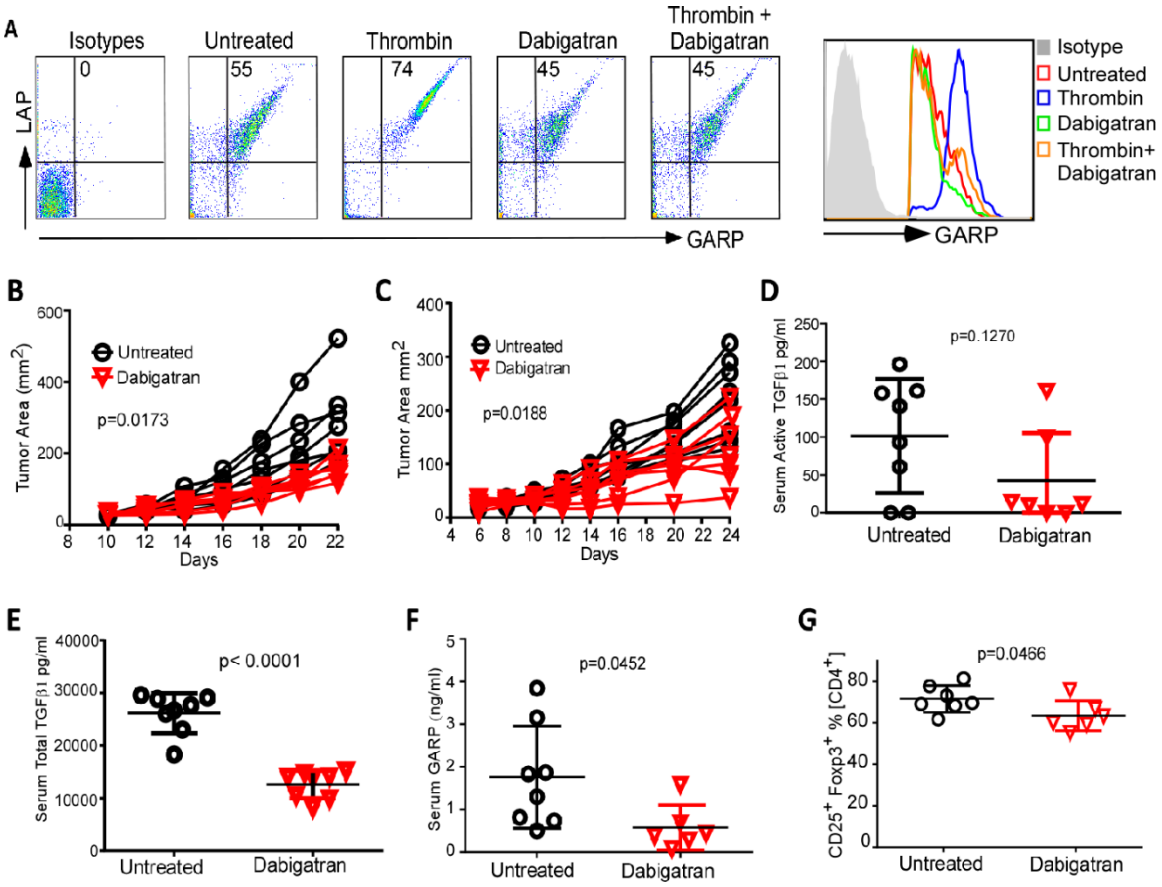


Figure 5-7. Direct thrombin inhibitor Dabigatran Etexilate reduces platelet GARP expression and protects against melanoma and colon cancer.

A. Flow cytometry analysis of surface GARP/LAP complex on murine WT platelets stimulated with and without thrombin and dabigatran etexilate. B. Tumor growth curves of mice subcutaneously injected with B16-F1 melanoma cells and daily treated with 3 mg/mouse of dabigatran etexilate. C. Tumor growth curves of mice subcutaneously injected with MC38 colon cancer cells and daily treated with 3 mg/mouse of dabigatran etexilate. D. Serum was obtained from tumor bearing MC38 colon cancer and active and total TGF-β1 (E) was measured by ELISA. F. Serum was obtained from tumor bearing MC38 colon cancer and soluble GARP was measured by ELISA. G. Percentage of regulatory T cells $CD25^+$ $FOXP3^+$ in the $CD4^+$ tumor-infiltrating CD4+ lymphocytes (TIL) from the indicated mice. Two-tailed, independent Student's *t*-test was used in panel D, E, F, and G. Repeated measures two-way ANOVA was used in panel A

Direct thrombin inhibitor Dabigatran Etexilate in combination with anti-PD1 blockade antibody reduces tumor burden in MC38 tumor model

Checkpoint blockade, alone or in combination with chemotherapy, antibody therapy, radiotherapy or cancer vaccine are demonstrating excellent results in multiple tumor models[148,149]. Interestingly, gene expression analysis of tumors that do not respond to checkpoint therapy show an increase in TGF-β signaling transduction pathways[150]. Additionally, mice harboring 4T1 breast cancer and treated with triple combination of PD1 blockade antibody with TGF-β blockade and radiotherapy achieved complete regression, indicating a synergistic effect of anti-PD1 and anti-TGF-β blockade therapy[151]. These evidences raise the hypothesis that PD1 blockade antibody together with anti TGF-β might be a new and effective combinatorial approach against cancer. We decided to use Dabigatran, and not TGF-β blocking antibody, in combination with PD1 blockade because by blocking thrombin and consequentially platelet activation, we would achieve both a drastic reduction of systemic TGF-β, and the abrogation of platelet-mediated tumor support. Platelets, indeed, secrete many other factors beyond TGF-β that facilitate tumor immune evasion. Animals harboring palpable tumors were daily treated with 3 mg/mouse dabigatran by oral gavage. Additionally, 200 µg of anti-PD1 blockade antibody was administered every 3 days starting on day 8 (Figure 5-8A). Single therapies alone were equally effective in reducing tumor growth, however mice treated with combination of anti-PD1 and Dabigatran achieved total regression (Figure 5-8B) as confirmed by prolonged survival (Figure 5-8C).

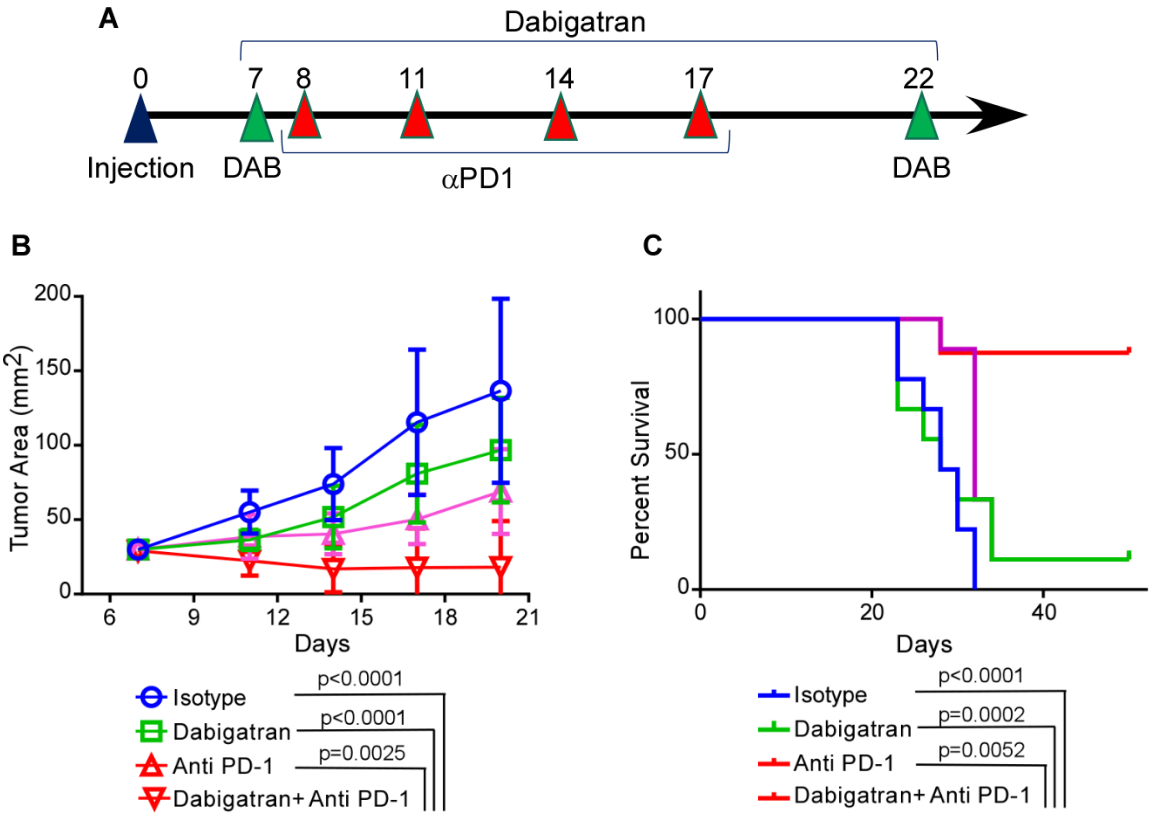


Figure 5-8. Direct thrombin inhibitor Dabigatran Etexilate in combination with anti-PD1 blockade antibody reduces tumor burden in MC38 tumor model

A. Experimental design for Dabigatran and PD1 blocking antibody combination therapy. B. Tumor growth curves of mice subcutaneously injected with MC38 colon cancer cells and treated with Dabigatran alone, with PD1 blocking antibody, with the combination of Dabigatran and PD1 blocking antibody or left untreated. C. Survival curve in MC38-bearing mice treated with Dabigatran alone, with PD1 blocking antibody, with the combination of Dabigatran and PD1 blocking antibody or left untreated. Repeated measurement 2 way ANOVA was performed in B. Log-rank (Mantel-Cox) was performed in C.

Discussion

The role of platelets in promoting cancer invasion has been previously observed[152]. As a chronic unhealed wound, cancer constantly activates the coagulation pathway that culminates with the generation of high concentration of thrombin leading to massive platelets activation[153]. The fibrin clot surrounds and confers a selective advantage to the growing tumor protecting it by the tumoricidal attack of NK[130] neutrophils[131], macrophages and CTLs[132]. One of the critical mediator of the tumorigenic nature of platelets is TGF-β that, with its immunosuppressive properties, dampens both adaptive and innate anti-tumor immunity. Several efforts have been made to describe the release and function of platelet derive TGF-β[81], yet the mechanism behind its maturation is still elusive. In this study we showed that GARP enhances the activation of latent TGF-β released by platelets and in doing so potentiates platelet tumorigenic activity. We also showed for the first time that GARP is released in the PR of thrombin activated platelets. Furthermore, we demonstrated that the release of mature TGF-β is the last step of a new thrombin-GARP/TGF-β axis that can be pharmacologically blocked by direct thrombin inhibitors. Studies with genetic ablation of GARP from platelets selectively have led to a clear conclusion that serum active TGF-β depends on the platelet surface GARP/TGF-β complex. The increased in serum total TGF-β observed in platelet GARP KO, mice might be explained as a compensatory mechanism operated by platelets failing to generate a mature form of the cytokine. This hypothesis is supported by the elevated level of L-Lactate observed in platelets *Pf4*creGARPf/f mice (Supplemental Figure 1). The decreased TGF-β signaling in TME highlighted

by the ablation of Smad3 phosphorylation is a strong evidence of the loss of functional TGF-β in mice with specific deletion of GARP on platelets.

Mechanistically we were able to demonstrate the presence of a pathway where thrombin, enhances GARP/latent TGF-β expression on platelets that in turn results in more release of active TGF-β and GARP in the PR. Not only TGF-β, but soluble GARP also have immunomodulatory function, thus reinforcing the pro-tumorigenic PR activity[70]. Based on these findings, high concentration of circulating soluble GARP and TGF-β1 could be regarded as valuable biomarkers for sustained platelet activation. Accordingly, high level of serum TGF-β is a poor prognostic factor in several malignancies[154,155] and plasma soluble GARP was increased in metastatic prostate cancer patients (See results section 4, Figure 4-8), reinforcing the concept of platelets and cancer bi-directional activation. Based on our results it is reasonable to hypothesize that platelets PR is one of the major contributors to systemic circulating TGF-β and GARP pool in cancer patients.

Consistent with the genetic studies, the pharmacological inhibition of thrombin was sufficient to recapitulate some of the effects seen in the genetic studies. We are aware that Dabigatran may not be able to phenocopy platelet-specific GARP deletion for two reasons: first, by blocking thrombin, Dabigatran reduces the increase, but not GARP baseline expression; second Dabigatran, by blocking thrombin active site, has a direct effect on the ability of the platelets to aggregate and to form the fibrin clot. This difference is evident in the reduction of both active and total serum TGF-β: mice treated with Dabigatran show a drop in both active

and total TGF-β, unlike *Pf4*creGARPf/f mice that experienced a drop in active, yet an increase in serum total TGF-β. Intriguingly, this last difference may shed light on the regulation of the two pools of TGF-β that are released by active platelets. As described earlier, Grainger and colleagues noticed that one pool of TGF-β is released in the serum as part of the LLC complex, the other pool instead is retained in the clot and is gradually released as active form. Since GARP KO platelets have normal thrombotic activity and the only phenotypic manifestation found in *Pf4*creGARPf/f mice is the ratio between total and active TGF-β, it is reasonable to conclude that platelet GARP plays its role in the release of active TGF-β from the fibrin clot. Dabigatran, on the other hand, by blocking both platelets aggregation and clot formation, acts earlier in the coagulation cascade, inhibiting the secretion of both pools of platelet-derived TGF-β. Additionally, it is noteworthy to mention the pro-tumorigenic effect exerted by other platelet soluble and membrane bound components. Dabigatran, indeed, inhibits the upregulation and expression and secretion of multiple oncogenic factors that would occur otherwise are upon platelets activation.

The results from the combination therapy of Dabigatran Etexilate and PD1 blockade strongly supports the notion that the efficacy of re-activated tumor-specific T cells can be further reinforced by decreasing the amount of tumor TGF-β.

Chapter 6

Summary, Conclusions and Future Prospective

Summary and Conclusions

The importance and pleiotropic activity of TGF-β justifies the complexity and the multiplicity of mechanisms involved in its regulation. Multiple diseases, indeed, are a direct consequence or are exacerbated by defects in TGF-β regulation. Because of its tolerogenic properties, TGF-β is involved in infections clearance, cancer initiation, metastasis dissemination, and loss of tolerance to self-antigen in autoimmunity[179].

GARP protein has been previously described as a latent TGF-β receptor expressed on Tregs that enhances TGF-β activation and, as a consequence, impacts Tregs induction and function. This thesis has uncovered novel insights regarding GARP/TGF-β axis which might be potentially exploited in clinic.

Initially results showed in chapter 3 offer two mechanisms to explain soluble GARP formation and signal transduction. In the first mechanism, unbiased informatics analysis revealed the presence of two different thrombin binding sites in GARP protein sequence. Our genetic studies then validated that thrombin is the enzyme that cleaves GARP and enables the formation of two soluble fragments. Mechanistically, we demonstrated that GARP cleavage mediates the release of latent TGF-β. We also observed that thrombin cleaves GARP on isolated platelets, suggesting the *in vivo* physiological relevance of this phenomenon. This novel

finding add insights into the role of thrombin in platelets stimulation: as a protein abundantly expressed on platelets, GARP cleavage might facilitate latent TGF-β release and activation in platelets releasate.

Our functional studies with GFP p-SMAD3 reporter cell line, show that soluble GARP relies on αV integrins to elicit TGF-β signal transduction and, surprisingly, to be internalized. This unexpected phenotype might suggest that soluble GARP signal transduction is mediated by endocytosis with the subsequent release of mature TGF-β in the acidic vesicles.

Finally, we clearly showed that GARP in complex with TGF-β is abundantly secreted in the exosome cargo, unveiling another novel aspect of GARP biology in the process of mediating intercellular communication and tolerance.

In chapter 4, we observed GARP expression in several aggressive human cancers. Mechanistically, we showed that i) GARP/TGF-β axis makes cancer cells acquire oncogenic properties and that ii) enforced GARP expression leads to the amplification of active TGF-β signaling in TME. The accumulation of active TGF-β in TME was evidenced by Smad3 phosphorylation and expansion of tolerogenic Treg cells. Additionally, our data showed that soluble GARP in plasma of cancer patients is elevated and its level directly correlates with cancer stage.

After analyzing function of GARP/TGF-β expression on cancer cells, in chapter 5 we decided to investigate the role played by GARP on platelets. We reasoned that platelets are a perfect model for our research since they abundantly express surface GARP/latent TGF-β1 and because platelets are the biggest TGF-β

reservoir in the body. Strikingly, GARP on platelets regulates the balance between serum active and latent TGF-β: genetic deletion of GARP from platelets ablates active TGF-β while increasing its latent form. This might suggest a constant platelets activation status (as indicated by higher serum lactic acid) to compensate for lacking of mature TGF-β. Using two different cancer models we discovered that GARP exacerbates platelets-induced tumor growth because of enhanced TGF-β activation in platelets releasate. Additionally, for the first time we identified that, upon platelets activation, soluble GARP is released by platelets. This finding might explain why cancer patients, who experience sustained platelets activation, have high concentration of GARP in their plasma. It is also possible that this platelet-derived soluble GARP adds up to the soluble GARP released by cancer cells.

In a clinically relevant system, we then decided to block platelet GARP/TGF-β axis using Dabigatran, a direct thrombin inhibitor approved by the FDA for stroke prevention. Our results show that blocking thrombin was therapeutic beneficial against two cancer models, especially in combination with checkpoint blockade treatment. This last observation is important especially because intratumoral TGF-β has been showen to be one of the reasons responsible for checkpoint blockade therapy failure.

Clinical Translation

The results reported in this thesis offer a wide range of translational options. First, GARP as tissue tumor biomarker is a valuable prospective especially for those tumors, like prostate carcinoma, that do not have consistent tissue biomarkers[180]. Second, we also observed a good correlation between plasma soluble GARP and tumor aggressiveness in prostate cancer patients indicating that the amount of soluble GARP is a good indicator of tumor status and anti-cancer treatment efficacy. Again, this is especially true in prostate cancer where the only existing liquid biomarker PSA1 (Prostate Specific Antigen 1) is known to associate with problems in specificity and sensitivity [181].

Moreover, we envision GARP as an attractive target for tumor antibody based therapy. Anti-GARP antibody, alone and in combination with cyclophosphamide, indeed showed anti-metastatic potentials in murine mammary tumors[1]. Importantly, targeting GARP on cancer cells has the favorable side effect to reduce the immunosuppressive ability of Treg. This effect has been already demonstrated by another investigator showing that monoclonal GARP antibody restrains Treg immunosuppressive activity in GVHD[69]. Additionally, we do not underestimate the importance of the blocking activity that GARP antibody might exerts on soluble and exosomes bound GARP/TGF-β complex.

Another exciting translational potential is represented by the combination of anti-platelets agents and anti-tumor therapy. We have proposed Dabigatran Etexilate as inhibitor of thrombin-mediated platelets activation, in combination with PD1

blockade antibody. Our results demonstrate that Dabigatran Etexilate reduces the accumulation of intratumoral TGF-β which potentiates checkpoint blockade therapy.

Future Directions

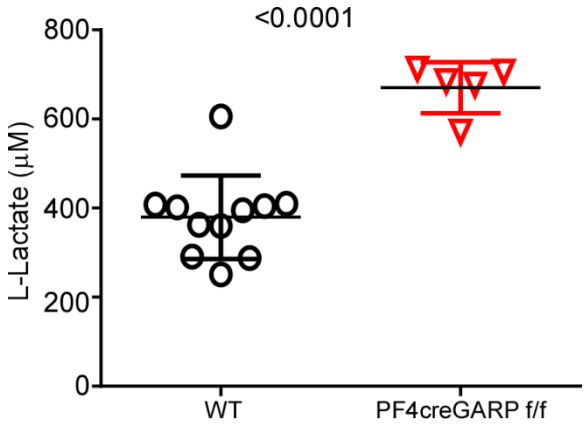
This work, while answering several aspects of GARP biology, raises many unanswered and provoking questions. Several investigators using numerous models largely demonstrated that GARP enhanced TGF-β activation, yet the intimate mechanism for mature TGF-β release is still elusive. The discovery of thrombin mediated GARP cleavage unveils a novel protease-dependent mechanism of TGF-β maturation that requires more investigation to be fully understood. Our results show that the complete activation of TGF-β seen in platelets releasate, is not achievable by the sole GARP/latent TGF-β complex digestion with thrombin. This indicates that another components or other pathways present in platelets are critical for the final step of TGF-β activation. In this regards we plan to explore i) the signal transduction initiated by thrombin receptor engagement, ii) the role of integrins expressed on platelets, iii) the effect of the acid environment in which latent TGF-β is released, and iv) the possibility of a role played by GARP, that with the intracellular domain, might engage an unexplored signaling pathway. This latter possibility has never been evaluated, since the shortness and the lack of obvious binding motifs in the GARP cytoplasmic tail. However, as mentioned earlier, human and mouse GARP share a conserved tyrosine residue that is also present in TLRs where it acts as a part of a signal transduction.

Additionally, we are the first to observe integrin-mediated soluble GARP internalization and signal transduction. This observation indicates the possibility that GARP, other than acting as a surface receptor, plays an intracellular function.

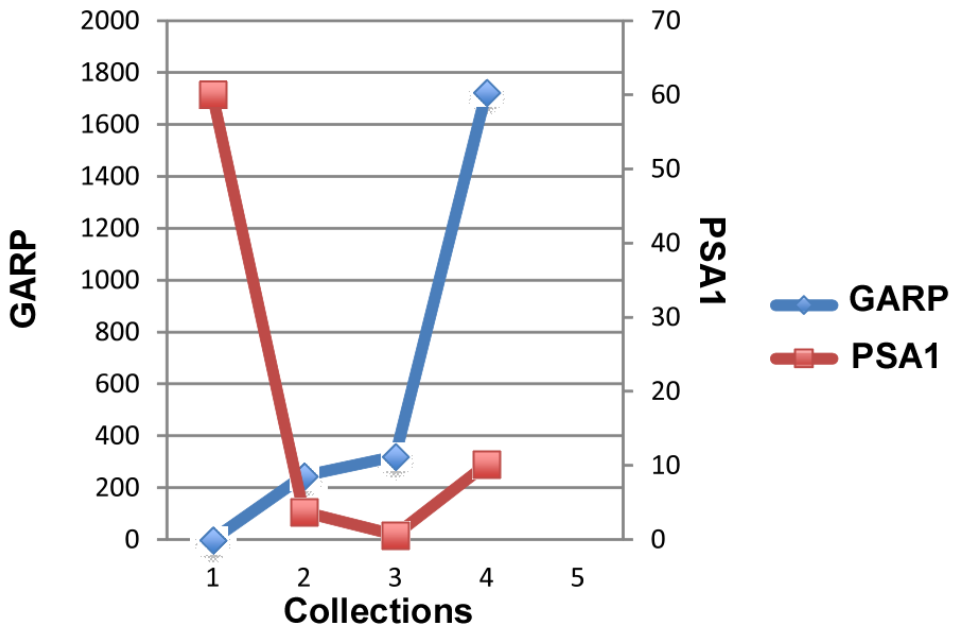
For this reason, we intend to investigate the role of the acidic endosomal compartment in the degradation of GARP/TGF-β complex and in the release of mature TGF-β.

Finally, the presence of GARP/TGF-β complex in exosomes suggests that GARP mediates the intercellular diffusion of TGF-β. The implications of this novel discovery in cancer and immune tolerance will be addressed in the immediate future.

Supplemental Materials



Supplemental Figure 1. Serum L-lactate increases in *Pf4*creGARPf/f mice
L-lactate assay results on serum of WT and *Pf4*creGARP f/f mice.



Supplemental Figure 2. GARP is released in the plasma when the anti-tumor therapy is effective. Comparison of Plasma GARP and PSA1 level during 5 cycles of Androgen deprivation therapy. Similar trend was observed in 16 over 36 patients (44.4 %).

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