Molecular BioSystems

REVIEW

View Article Online
View Journal

Cite this: DOI: 10.1039/c7mb00251c

Role of GARP in the activation of latent TGF-β1

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Received 28th April 2017,
Accepted 26th July 2017

DOI: 10.1039/c7mb00251c

rsc.li/molecular-biosystems

TGF-β1, 2 and 3 cytokines are involved in many cellular processes including cell proliferation, differentiation, migration and survival. Whereas TGF-β2 and 3 play important roles in embryonic development, TGF-β1 is mostly implicated in controlling immune responses after birth. The production of TGF-β1 is a tightly regulated process, occurring mostly at a post-translational level. Virtually all cells produce the latent, inactive form of TGF-β1. In latent TGF-β1, the mature TGF-β1 dimer is non-covalently associated to the Latency Associated Peptide, or LAP, which prevents binding to the TGF-β1 receptor. Activation of the cytokine implies release of mature TGF-β1 from LAP. Only a few cell types activate latent TGF-β1, *via* mechanisms that are cell type specific. Proteins such as integrins, proteases and thrombospondin-1 activate TGF-β1 in epithelial cells, fibroblasts and dendritic cells. More recently, the protein GARP was shown to be involved in TGF-β1 activation by regulatory T cells (Treg), a subset of $CD4^+$ T lymphocytes specialized in suppression of immune responses. GARP is a transmembrane protein that binds latent-TGF-β1 and tethers it on the Treg surface. The role of GARP was studied mostly in Tregs, and this was recently reviewed in L. Sun, H. Jin and H. Li, *Oncotarget*, 2016, **7**, 42826–42836. However, GARP is also expressed in non-immune cells. This review focuses on the roles of GARP in latent TGF-β1 activation by immune and non-immune cells.

The TGF-β1 cytokine

Three TGF-β isoforms exist in all mammals and are encoded by 3 different genes (*TGFB1*, *TGFB2*, *TGFB3*). They bind to the same TGF-β receptor and exert pleiotropic effects on cell proliferation, differentiation, migration and survival. The production of TGF-β1, 2 and 3 is tightly regulated, mostly at a post-translational level.[2] We will focus our discussion on TGF-β1, as it is the only isoform that was described to associate with GARP in human or mouse cells expressing these proteins naturally (*i.e.* not as a result of transfection). It can be noted however that recombinant TGF-β2 and transfected TGF-β2 and TGF-β3 can also bind GARP (ref. 3 and our own, unpublished results) but whether this bears any biological relevance is not known.

TGF-β1 is expressed by most human and mouse cells. It is first produced as a precursor called pre-pro-TGF-β1 (Fig. 1).

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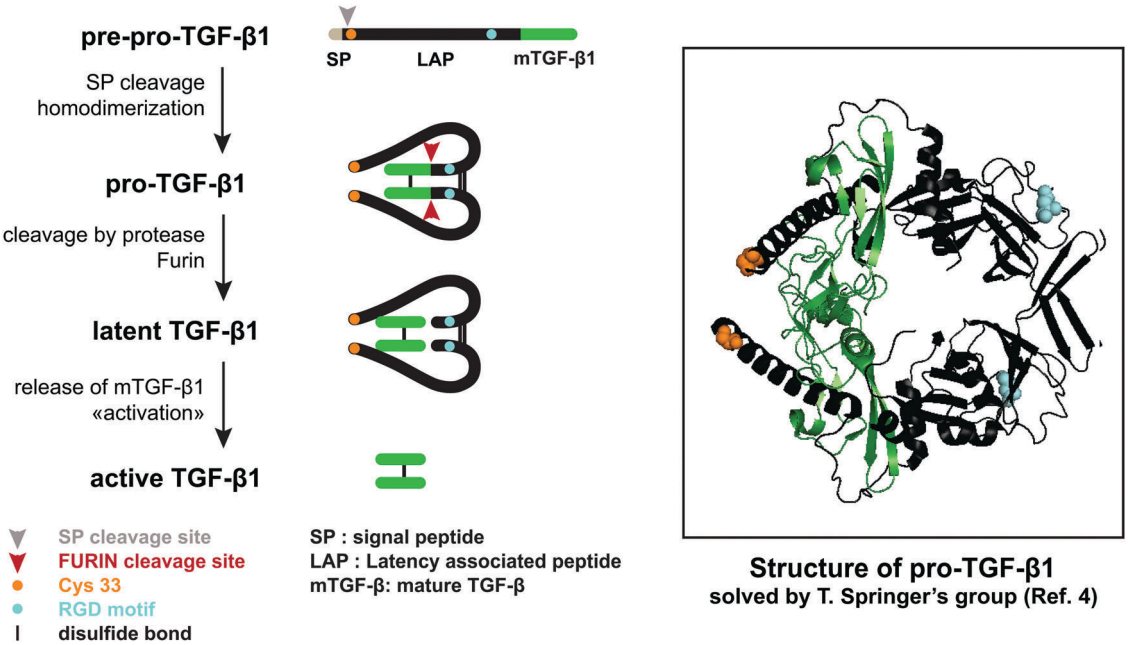

Fig. 1 Schematic representation of TGF-β processing. TGF-β1 is first produced in the form of pre-pro-TGF-β1. After signal peptide removal and homodimerisation *via* disulfide bonds, pro-TGF-β1 is cleaved by the pro-protein convertase Furin. The carboxy-terminal dimer, or mature TGF-β1, remains non-covalently associated to the amino-terminal dimer, the LAP, forming a complex called latent TGF-β1. Latent TGF-β1 is inactive. It can be secreted alone or in association with latent TGF-β1 binding proteins (*via* Cys 33). Mature TGF-β1 needs to be released form the LAP in order to be active. RGD motifs (blue) found in the LAP have been shown to interact with integrins able to activate TGF-β1.

After signal peptide removal and homodimerisation *via* formation of interchain disulfide bonds, the resulting pro-TGF-β1 dimer is cleaved by the pro-protein convertase Furin to generate two homodimeric fragments. The carboxy-terminal dimer, or mature TGF-β1, remains non-covalently associated to the amino-terminal dimer, or Latency Associated Peptide (LAP), forming a complex called latent TGF-β1. Latent TGF-β1 is inactive because LAP prevents binding of mature TGF-β1 to its receptor, in a manner nicely illustrated by the crystal structure of latent TGF-β1[4] (Fig. 1). An extended loop of LAP, called the latency lasso, encircles the tip of each mature TGF-β monomer and prevents contact with the type II TGF-β receptor chain, while several other LAP segments also block interaction with the type I receptor chain.

Virtually all cells, including most immune cells, secrete TGF-β1 in their latent form. Some cell types, such as epithelial cells, secrete latent TGF-β1 in association with the Latent TGF-β1 Binding Proteins (LTBPs), to which it is linked *via* disulfide bonds. LTBPs, in turn, associate *via* covalent linkage to proteins in the extracellular matrix, resulting in the deposition of latent TGF-β1 in the matrix. TGF-β1 bioactivity requires release of mature TGF-β1 from LAP, a process referred to as TGF-β activation.[2] Only a few cell types activate TGF-β1, in response to specific stimuli and *via* mechanisms that are cell type specific. To date, mechanisms of TGF-β1 activation shown to be the most relevant *in vivo* involve integrins that bind an Arg-Gly-Asp (RGD) motif found in the LAP (Fig. 1). TGF-β1-activating integrins comprise αVβ1 in fibroblasts,[5] αVβ6 in epithelial cells,[6] and αVβ8 in dendritic cells, glial cells or fibroblasts.[7–11] Binding of integrin αVβ6 to latent TGF-β1 was shown to induce mechanical deformation of LAP, leading to release of mature TGF-β1.[2] Disulfide linkage of the LAP to LTBPs was shown to be crucial for the activation process, and was proposed to oppose the pulling forces exerted by integrin αVβ6 on latent TGF-β. This mechanism of activation based on induction of a conformational change in LAP may also operate when αVβ8 is implicated, although in this case, it was also suggested to require the intervention of a protease to cleave LAP.[12]

Once activated, the mature TGF-β1 dimer can bind with high affinity to the TGF-β receptor. The signal then propagates by

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Sophie Lucas obtained an MD degree in 1994, then a PhD degree in 2001 for her work on the identification of genes encoding tumor specific antigens under the supervision of Prof. Thierry Boon, at the University of Louvain (UCLouvain) in Belgium. After a post-doc dedicated to studying new cytokines and cytokine receptors at Genentech in California, she founded here own research group at the de Duve Institute (UCLouvain) in 2004, studying human Tregs and their immunosuppressive mechanisms. The group currently focuses on a mechanism that implies GARP and the production of active TGF-β1.

phosphorylation of SMAD2 and SMAD3 transcription factors, which interact with SMAD4. Activated SMAD complexes translocate to the nucleus where they will act as transcriptional activators or repressors of target genes.[2] The identity of the TGF-β1 target genes is dependent on the identity of the co-factors that will associate and cooperate with SMAD complexes to regulate transcription. Therefore the effects of TGF-β1 may be different from one cell type to another because various cell types express various SMAD co-factors.

The most striking and non-redundant function of TGF-β1 signaling is immunosuppression. Indeed, mice with a germline deletion of the *Tgfb1* gene die early in life from severe multi-organ inflammation due to the uncontrolled activity of autoreactive T lymphocytes.[13,14] The same early lethal autoimmune phenotype is observed in mice with a T-cell specific deletion of the *Tfgbr1* or *Tgfbr2* genes. It is thus clear that TGF-β1 signaling in T cells is required to maintain immune tolerance and that the phenotype of *Tgfb1* KO mice is predominantly due to the lack of TGF-β1 signals in T cells.[15,16] The cellular sources of the active TGF-β1 required to maintain tolerance are still unclear. Tregs are a subset of $CD4^+$ cells that are also indispensable to maintain tolerance. Their development and function require transcription factor FOXP3 and *Foxp3* KO mice die very early in life from severe autoimmunity, resembling that observed in *Tgfb1* KO mice. Tregs could therefore represent an important source of active TGF-β1, which could play a critical role in mediating their immunosuppressive function.

Whether production of active TGF-β1 is an important mechanism of immunosuppression by Tregs has been a matter of debate. Mice with a conditional deletion of *Tgfb1* in Tregs do not suffer from the severe multi-organ inflammation seen in germline *Tgfb1* KO mice, suggesting that active TGF-β1 production by Tregs is not the main mechanism involved in maintenance of immune tolerance by Tregs.[17] However, it is still possible that Tregs lacking the *Tgfb1* gene activate latent TGF-β1 produced by another cell type to suppress target cells. Several *in vivo* observations do support a role for active TGF-β1 production by Tregs in immunosuppression. It was shown that *Tgfb1*-deficient Tregs are not as suppressive as WT Tregs in a model of colitis induced by transfer of naïve T cells in *Scid* mice. Interestingly, the residual ability of *Tgfb1*-deficient Tregs to suppress colitis was abrogated by anti-TGF-β1 antibodies.[18,19] Other studies showed that T helper (Th) cells unable to respond to TGF-β1, due to deletion of a TGF-βR gene or to expression of a dominant negative TGF-βR, cannot be suppressed by WT Tregs in colitis models induced by naïve T cells.[15,19,20] These data strongly suggest that active TGF-β1 production by Tregs participates in their immunosuppressive function. But how Tregs activate TGF-β1 was not known until recently. Tregs were known for a while to present latent TGF-β1 on their surface.[21] In 2009, this surface presentation of latent TGF-β1 was reported to result from its association with the transmembrane protein GARP,[1,3,22] and it was recently shown that GARP is implied in TGF-β1 activation by human Tregs.

Discovery of the *GARP* gene

The gene encoding protein GARP has two alternative names: *GARP* (Glycoprotein A Repetition Predominant) and *LRRC32* (Leucin Rich Repeats Containin protein 32). In 1992, Ollendorff

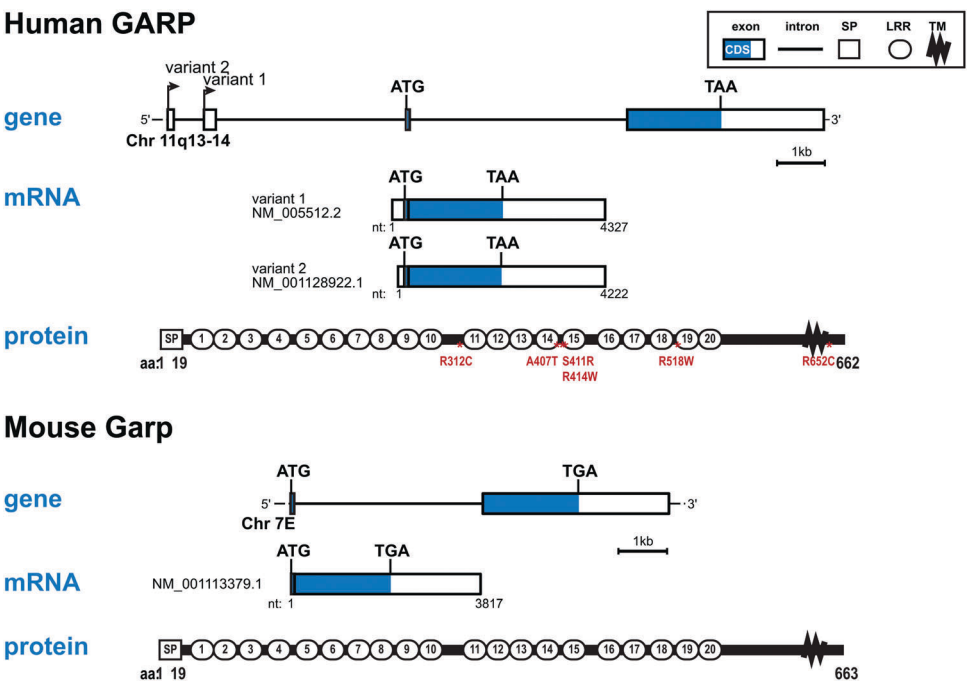

Fig. 2 Schematic representation of *GARP* genes, mRNAs and proteins in humans and mice. Mutants described by Manz *et al.*,[33] which are associated with a higher risk of atopic dermatitis, are represented in red under the human protein. nt: nucleotides; aa: amino acids; SP: signal peptide; LRR: leucine rich-repeat; TM: transmembrane domain; CDS: coding sequence.

et al. reported the sequence of human *GARP*, which they identified in the q13–14 region of Chromosome 11, under scrutiny because it is amplified in several cancers.[23] They thus suggested GARP to be a potential oncogene, but its role in oncogenesis was not formally demonstrated, as other potential oncogenes, including members of the Cyclin family, were also found in this region.[24] Based on sequence homology with human GARP, mouse *Garp* was mapped to the Chromosome 7 E–F region.[23] The encoded human and mouse GARP proteins share 80% identity at the amino acid level. Human and mouse *GARP* genes have a similar structure, with only two coding exons. Human *GARP* gives rise to two reported RefSeq transcripts that differ in their 5′UTR, whereas only one transcript was reported for mouse *Garp* (Fig. 2).

Single nucleotide polymorphisms (SNP) located in non-coding regions of human *GARP* were associated with higher risks of developing several diseases, including immune-related diseases such as asthma, allergic rhinitis, atopic dermatitis or Crohn's disease[25–31] and some types of cancers.[32] Whether and how these non-coding SNPs could influence the pathogenesis of these diseases was not investigated. Rare variants in the *GARP* coding region were also proposed to significantly contribute to the risk of atopic dermatitis.[33] The most frequent of these variants (rs79525962, C/T, T allele frequency in the 1000 genomes project = 0.04) leads to a change of A to T in position 407 (Fig. 2). On transfected cells, the surface levels of GARP A407T were lower than that of wild-type GARP, suggesting that the A407T variation may affect GARP function. Finally, homozygosity for another variant (rs201431152, G/A, A allele frequency = 0.00023), encoding a R414W change, was shown to segregate with Usher syndrome in an isolated inbred population cohort.[34] This syndrome is characterized by profound congenital sensorineural deafness, vestibular dysfunction, and progressive visual loss. Whether GARP is involved in the pathogenesis of this disease is unknown.

Expression pattern of the *GARP* mRNA

Early studies described *GARP* mRNA expression in the placenta, lungs and kidneys, and to a lesser extent in the heart, liver, skeletal muscle and pancreas, but which cell type(s) expressed *GARP* in these tissues was not known.[35] *GARP* mRNA was then studied in different types of primary cell lines. First detected in mouse and human megakaryocytes,[36,37] it was soon found to be expressed in human and mouse regulatory T cells.[38,39] Later, *GARP* expression was also detected in human and mouse mesenchymal stem cells,[40,41] endothelial cells[41,42] and hepatic stellate cells.[43] It was more recently found in human embryonic stem cells and fibroblasts.[44] Interestingly, the expression of the *GARP* mRNA does not always correlate with the expression of the GARP protein. It has been shown that miRNAs such as *miR-142-3p*, *miR-181*, and *miR-185* inhibit expression of the GARP protein in cells such as non-Treg T cells that can express high *GARP* mRNA levels but no GARP protein.[45,46] GARP expression is thus highly regulated, including at post-transcriptional levels.

Structure of the GARP protein

The human *GARP* gene encodes a 662 aa-long, type I transmembrane protein comprising a very short (14 aa) cytoplasmic domain and a large (608 aa) extracellular domain (Fig. 2). No signaling activity was described for the cytoplasmic tail of GARP. It contains a PDZ-like domain, which was suggested to regulate GARP trafficking on the cell surface because mutation of this domain decreases surface GARP levels.[47] The extracellular domain of GARP is composed of two series of 10 so-called Leucine Rich Repeats (LRRs). LRRs are conserved 20–30 aa motifs found in thousands of proteins of prokaryotic and eukaryotic origin.[48] A typical LRR containing protein comprises 2 to 45 LRRs. Each LRR is composed of a highly conserved region with a consensus sequence LxxLxLxxNxL or LxxLxLxxCxxL.[49] LRR proteins present a typical horseshoe-like tridimensional structure. Interestingly, LRR containing domains were described to support protein–protein interactions.[50] Human LRR-containing proteins exert very diverse functions, from the Toll-Like Receptors (TLR), involved in innate immune responses, to proteins involved in nuclear mRNA transport such as NXF1 or present at the neuronal synapse like the LRRTM proteins.[49,51]

GARP is an LRR-containing protein and to date, all its known functions are linked to its ability to interact with latent TGF-β1.

Structure of the GARP/latent TGF-β1 complex

Most probably immediately after protein synthesis in the ER, disulfide bonds are formed that link one molecule of GARP to one pro- or latent TGF-β1 homodimer.[45,52] These disulfide bonds occur between Cys33 of LAP and Cys192 and 331 of GARP (Fig. 3). Both pro- and latent TGF-β1 are found associated

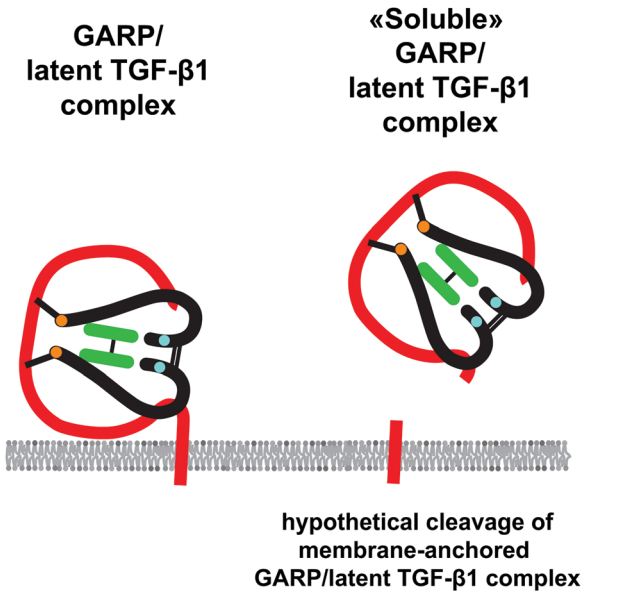

Fig. 3 Schematic representation of membrane-bound and soluble GARP/latent TGF-β1 complexes.

to GARP, suggesting that cleavage of pro-TGF-β1 by Furin may occur on preformed GARP/pro-TGF-β1 complexes.[52] In support of this hypothesis, forced expression of GARP by transfection was shown to enhance the cleavage of pro-TGF-β1 into latent TGF-β1.[45] It must be noted that interaction between recombinant GARP and recombinant latent TGF-β1 can occur in cell-free systems.[3,22] Therefore, stable interaction between GARP and latent TGF-β1 does not require formation of disulfide bonds. However, covalent linkage might be important for activation of TGF-β1 from GARP/latent TGF-β1 complexes, as will be discussed below.[52]

In Tregs, GARP/latent TGF-β1 complexes are retained on the cell surface because GARP has a transmembrane domain. In addition, so-called ''soluble'' GARP/latent TGF-β1 complexes were also described.[45] Soluble GARP/latent TGF-β1 complexes appear to result from proteolytic cleavage of GARP close to its transmembrane domain, but the putative protease implicated in such a shedding has not yet been uncovered (Fig. 3). Soluble GARP/latent TGF-β1 complexes are secreted by some cells types such as human Tregs, or human T cells transduced with GARP. In contrast, HEK 293T cells transfected with GARP do not secrete soluble GARP/latent TGF-β1 complexes, indicating that the putative shedding mechanism does not operate in all types of cells and could be restricted to Tregs.[45,52] This explains the opposite effects of GARP overexpression on the amounts of latent TGF-β1 secreted by different cell types. Indeed, lentiviral-mediated expression of GARP in human T cells results in surface presentation of GARP/latent TGF-β1 complexes and an increase in latent TGF-β1 secretion, probably because GARP expression increases the processing of pro-TGF-β1 into latent TGF-β1, for one part, but also because T cells can shed and secrete GARP/latent TGF-β1 complexes, for another part. In HEK 293T cells, transfection of GARP also results in surface presentation of GARP/TGF-β1 complexes and increases processing of pro-TGF-β1 into latent TGF-β1. However, this results in decreased, rather than increased secretion of TGF-β1, because HEK 293T cells do not shed GARP/latent TGF-β1 complexes and retain them on the cell surface. Whether soluble GARP/latent TGF-β1 complexes secreted by Tregs can serve to produce active TGF-β1 at distant sites is not known. Preparations of soluble recombinant GARP/latent TGF-β1 complexes, in which GARP is truncated from its transmembrane domain, were shown to induce TGF-β1 signals in cell reporter assays.[53] However, these preparations are often shown to be contaminated by active TGF-β1 itself. Finally, soluble GARP/latent TGF-β complexes cannot be activated by integrins.[52] It is thus uncertain whether latent TGF-β1 can be activated from soluble GARP/latent TGF-β1 complexes.

Role of membrane-bound GARP in TGF-β1 activation

As stated previously, latent TGF-β1 can be produced by virtually all cell types but only a few are able to transform it into the active cytokine in response to stimulation and *via* mechanisms that are cell-type specific. Tregs activate latent TGF-β1 in response to T cell receptor stimulation (TCR), *via* a mechanism that requires GARP. This is supported or demonstrated by the following observations:

(i) GARP tethers TGF-β1 on the Treg surface, where TGF-β1 activation is thought to occur

We and others showed that TCR-stimulated Tregs produce active TGF-β1, which can exert autocrine activity on the Tregs themselves, as well as paracrine activity on non-Treg T cells (Th cells) in contact with Tregs. Tregs alone can activate latent TGF-β1, as autocrine TGF-β1 activity was observed when Treg clones were stimulated *via* their TCR in the absence of any other cell type.[54] Notably, we found that the active cytokine is not released as a soluble form in Treg supernatants: no phosphorylation of SMAD2 is detected in Th cells incubated in conditioned medium from TCR-stimulated Tregs. The paracrine activity of Treg-derived TGF-β1 in Th cells can only be detected if Treg-to-Th cell contacts are allowed. This was shown by measuring phosphorylation of SMAD2 in Th cells that were isolated by FACS after co-culture with Tregs. We thus concluded that active TGF-β1 is produced close to the surface of TCR-stimulated Tregs.[21,54,55] Interestingly, it is known that suppression of Th cell proliferation by Tregs *in vitro* also requires Treg-to-Th cell contacts: Tregs cannot suppress Th cells if they are separated by a semi-permeable membrane in a transwell assay. This supports again the hypothesis that suppression by Tregs implies active TGF-β1 production. GARP tethers latent TGF-β1 on the Treg surface. GARP/latent TGF-β1 complexes therefore represent a plausible source of "activable" TGF-β1 close to the Treg surface. This indicates that GARP is at the right place at the right time, without however proving that it plays an active role in TGF-β1 activation by Tregs.

(ii) Downregulation of GARP with siRNAs reduces the ability of Tregs to suppress the proliferation of Th cells and to induce FOXP3 expression in Th cells

In co-culture experiments *in vitro*, inhibition of Th cell proliferation by human Tregs was reduced, although not totally abrogated, when Tregs were transfected with a siRNA targeting *GARP*.[3] Induction of FOXP3 expression in naïve human Th cells co-cultured with Tregs was also decreased if Tregs were transfected with a siRNA targeting GARP.[3] These two functions of Tregs (*in vitro* suppression and FOXP3 induction in Th cells) appear to depend, at least partially, on TGF-β1 signals,[56] thus supporting the hypothesis that GARP is important for TGF-β1 production by Tregs. It must be noted however that the contribution of Treg-derived TGF-β1 to the suppressive activity of Tregs is still a matter of debate in the literature. According to the Shevach and Li laboratories, TGF-β1 does not appear to play a role in suppression assays with mouse Tregs *in vitro*,[18,57] whereas human Tregs were shown to be less suppressive after siTGFB1 by the Shevach laboratory.[3] These discrepancies may reflect differences between human and mouse Tregs.

(iii) Anti-GARP monoclonal antibodies block TGF-β1 activation by human Tregs

To formally determine whether GARP/latent TGF-β1 complexes are involved in active TGF-β1 production by Tregs, our lab

derived a large series of anti-GARP monoclonal antibodies (mAbs). Two of these mAbs blocked TGF-β1 activation by human Tregs *in vitro*, as shown by measuring phosphorylation of SMAD2 in TCR-stimulated Tregs, taken as an indicator of autocrine TGF-β1 signals.[58] We identified the epitope bound by the blocking anti-GARP mAbs. We found that it is only present in GARP/latent TGF-β1 complexes, and absent from free GARP (*i.e.* GARP not bound to TGF-β1), and that it is different from those bound by non-blocking antibodies.

Altogether, the findings described above show that GARP is required for TGF-β1 activation by Tregs. However, GARP is not sufficient: transfection of GARP in Th cells or in HEK 293T cells results in presentation of latent TGF-β1 on their surface, but does not lead to active TGF-β1 production.[3,22,52] This indicates that an additional protein, present in Tregs but absent in Th cells and HEK 293T cells, is implicated in TGF-β1 activation from GARP/latent TGF-β1 complexes. Integrins represent attractive candidates, because they are known to activate TGF-β1 in non-T cells.

Role of integrins in GARP-mediated activation of TGF-β1 by Tregs

Integrins belong to a family of heterodimeric proteins composed of one α and one β chain. In humans, 18 α and 8 β subunits were shown to form 24 integrin heterodimers, which function as adhesion molecules or receptors transmitting information from chemical and mechanical extracellular stimuli.[59] Some, but not all integrins bind RGD motifs in their ligand. As mentioned previously, some of these so-called RGD-binding integrins bind the RGD motif present in the LAP moiety of TGF-β1 and are known to activate TGF-β1 in different cell types. Wang *et al.* tested the ability of the RGD-binding integrins αVβ1, αVβ3, αVβ5, αVβ6 and αVβ8 to activate TGF-β1 from GARP/latent TGF-β1 complexes in transfected HEK 293T cells, using a luciferase-based reporter system to detect TGF-β signals. They found that αVβ6 and αVβ8 integrins, but not αVβ1, αVβ3 and αVβ5, were able to induce TGF-β1 activation from GARP/latent TGF-β1 complexes on the HEK 293T cell surface.[52] However, these integrins failed to activate TGF-β1 from "soluble" GARP/latent TGF-β1, *i.e.* in HEK 293T cells expressing GARP/TGF-β1 complexes lacking the transmembrane domain of GARP. This suggests that anchorage of GARP into the membrane is required for integrin-mediated activation of TGF-β1 from GARP/latent TGF-β1 complexes. Expression of integrin αVβ6 is strictly restricted to epithelial cells, in which GARP expression was never detected thus far. Tregs, on the other hand, do not express the gene encoding β6. Thus, integrin αVβ6 most probably plays no role in TGF-β1 activation by Tregs alone, in the absence of any other cell type. It must be noted that TGF-β1 activation from GARP/latent TGF-β1 complexes on Tregs could occur also in *trans*, *i.e.* with GARP/latent TGF-β1 complexes on Tregs being activated by αVβ6 on another cell type. TGF-β1 activation by Tregs alone, in the absence of any other cell type, was demonstrated using clones of human Tregs,[54] and thus implies that a protein different from αVβ6 is implicated in this activation. Integrin αVβ8 is another candidate. Mouse and human Tregs, but not Th cells, express *Itgb8*, the gene encoding β8, and the gene encoding the αV subunit is expressed by all T cells.[60,61] Edwards *et al.* showed that integrin αVβ8 is required for TGF-β1 activation by mouse Tregs.[60] They showed that *Itgb8*-deficient Tregs (*CD4*-Cre x *Itgb8* floxed) co-cultured with naïve T cells were unable to induce their differentiation into IL-17 expressing Th cells (Th17), yet another process known to depend on TGF-β1 signals. More recently, our lab showed that TGF-β1 activation from GARP/latent TGF-β1 complexes on human Tregs requires αVβ8. We showed that in human Tregs, but not in Th cells, GARP/latent TGF-β1 complexes interact with integrin αVβ8 and that an anti-αVβ8 antibody blocked GARP-dependent TGF-β1 activation by Tregs (Stockis *et al.*, submitted). Altogether, it thus seems that integrin αVβ8 contributes to TGF-β1 activation from GARP/latent TGF-β1 complexes by human and mouse Tregs. Very interestingly, we observed that the anti-GARP antibodies that block active TGF-β1 production by human Tregs do not prevent integrin αVβ8 from interacting with latent TGF-β1, indicating that blocking anti-GARP antibodies act *via* another mechanism (Stockis *et al.*, submitted).

Production of active TGF-β1 from GARP/latent TGF-β1 complexes by Tregs *in vivo*

In mice, Tregs differentiate from $CD4^+$ T cell precursors in the thymus or in the periphery, and are called thymus-derived Tregs (tTregs) or peripherally-derived Tregs (pTregs), respectively. Both cell types were shown to express Garp on their surface. Mouse tTregs already bear surface GARP, albeit at low levels, in the thymus and in the periphery without the need for *in vitro* stimulation of their TCR to induce Garp expression.[62] This suggests that resting mouse Tregs express Garp, in contrast with human Tregs, which only express GARP after stimulation of their TCR *in vitro*. Mice deficient for Garp in T cells (*CD4*-Cre x *Garp* floxed mice) were recently described.[62] These mice have normal numbers of tTregs and pTregs and do not show any sign of spontaneous disease. Garp-deficient Tregs are still suppressive *in vitro*. However, a defect in immunosuppression by Garp-deficient Tregs was observed in experiments of oral tolerance. Oral tolerance corresponds to the induction of a state of immune unresponsiveness to orally administrated antigens. Mechanisms of oral tolerance are not completely understood, but have been proposed to depend on the induction of antigen-specific pTregs in the digestive tract. Using mice deficient for Garp in T cells, Edwards *et al.* showed recently that Garp expression on tTregs is required for proper induction of pTregs and efficient induction of oral tolerance against ovalbumine.[63]

Evaluating the role of GARP in immunosuppression by human Tregs *in vivo* is of course challenging. Our group attempted to do this by testing blocking anti-human GARP mAbs in a mouse model of xenogeneic graft-*versus*-host disease (xGVHD) induced by the transfer of human peripheral blood mononuclear cells (PBMC) in profoundly immunocompromised mice.[58] In this model, human T cells engraft readily and colonize the mouse

secondary lymphoid organs. This soon leads to activation of human T cells against mouse tissues, which causes symptoms closely resembling those observed in GVHD after allogeneic hematopoietic stem cell transplantation. Interestingly, this xGVHD is reduced by the co-transfer of autologous Tregs. Reduction of xGVHD by autologous Tregs results from their immunosuppressive activity. In this model, we were able to show that blocking anti-GARP mAbs aggravated xGVHD because they inhibited Treg immunosuppression. Blocking anti-GARP mAbs are currently under pre-clinical development to determine whether they could serve as a new approach for cancer immunotherapy.

GARP/latent TGF-β1 complexes in non-Treg cells

An important aspect of using anti-GARP mAbs to block active TGF-β1 production and immunosuppression by Tregs is that these mAbs are expected to block the activity of Treg-derived TGF-β1, but not that of TGF-β1 derived from other cell types. This is because the mechanisms of TGF-β1 activation are cell-type specific, and Tregs are the only cell type that were formally demonstrated to activate TGF-β1 *via* GARP. This represents a considerable advantage by comparison to antibodies directed against TGF-β1 itself. Indeed, TGF-β1 has dual roles in tumors. On the one hand, it plays tumor suppressive roles by inhibiting the growth of pre-malignant epithelial cells but on the other hand, it can also promote cancer progression by favoring metastasis and immunosuppression.[64] Blocking TGF-β1 signals with anti-TGF-β1 antibodies may thus very well block the tumor suppressive activity of TGF-β1 and favor the proliferation of malignant cells. Specifically blocking active TGF-β1 production by Tregs, but not by other cells, may specifically decrease immunosuppression without interfering with the activity of TGF-β1 produced by other cell types. This may be achievable with anti-GARP antibodies.

The specificity of immunotherapies with blocking anti-GARP mAbs will eventually depend on the pattern of GARP expression among various cell types, and on the ability of non-Treg cells expressing GARP to activate TGF-β1 in a GARP-dependent manner. It is thus important to precisely determine which cell types other than Tregs express GARP/latent TGF-β1 complexes, and which among these activate TGF-β1 in a GARP-dependent manner, as these cells or their function could be targeted by the blocking anti-GARP mAbs that are currently developed towards clinical use.

Table 1 summarizes all cell types known to express GARP in mice and humans. Data summarized in this table are described below.

GARP in megakaryocytes and platelets

Garp mRNA was first detected at high levels in megakaryocyte from the mouse fetal liver.[37] Later, in an effort to identify new

Table 1 Summary of the cell types in which GARP expression and function have been studied

Cell type	Species	Detection	Function of GARP	Model	Ref.	Comment
Tregs	H	qPCR, FC, WB, IF	– TGF-β1 activation – Suppression of T cell responses	*In vitro*/*in vivo* (xenogeneic)	3, 22 and 58	Upon TCR stimulation
	M	qPCR, FC	– TGF-β1 activation – Th17 differentiation – Induction of oral tolerance	*In vitro*/*in vivo*	60, 62 and 63	Upon TCR stimulation
Hepatic stellate cells	H and M	PCR, FC, WB	– TGF-β1 activation – Suppression of T cell responses	*In vitro*	43	Indirect evidence
Tumor cells (lung colon, breast)	H	IHC	Unknown		71	Associated with worse prognosis
	M	—	– Favors tumor growth – Correlates with TGF-β1 activity	*In vitro*/*in vivo*	71	Overexpression of human GARP in mouse cells
Megakaryocytes and platelets	H and M	ISH, qPCR, FC	– Indirect evidence of TGF-β1 activation – Delayed tumor growth and suppression of anti-tumor T cell responses in platelet-specific Garp knock-out	*In vivo*	36, 37, 66 and 67	
Mesenchymal stromal cells	H and M	qPCR, FC, WB	– Inhibition of TGF-β1 activation	*In vitro*	41	Upon culture
Endothelial cells	H	NB, qPCR	– Not investigated	*In vitro*	37 and 41	
	M	ISH, qPCR, FC	– Indirect evidence of TGF-β1 activation – No phenotype of the conditional deletion of Garp	*In vitro*/*in vivo*	37, 42 and 66	
Fibroblasts	H	WB	– We found no role in TGF-β1 activation	*In vitro*	58	

Abbreviations: H: human; M: mouse; qPCR: quantitative PCR; FC: flow cytometry; WB: western blot; IF: immunofluorescence; IHC: immunohistochemistry; ISH: *in situ* hybridization; NB: northern blot.

activating and inhibitory receptors of human platelets, Macaulay *et al.* identified *GARP* as an mRNA upregulated in human megakaryocytes by comparison to erythroblasts and reported the presence of GARP protein on the surface of human megakaryocyte and platelets.[36] This may suggest a role for GARP in coagulation, or in platelet–endothelium interactions. The potential functions of GARP in coagulation were studied in zebrafish.[65] Human GARP and its zebrafish ortholog share 38% amino acid identity. Using a model of laser-induced arterial injury and inhibition of GARP with antisense morpholino nucleotides, O'Connor *et al.* elegantly evidenced a defect in thrombus growth in zebrafish larvae in which *GARP* had been knocked down. These data were very recently challenged by Vermeersch *et al.* who studied thrombosis and hemostasis in mice bearing a conditional deletion of *Garp* in platelets (*Pf4-Cre x Garp* floxed).[66] Using *ex vivo* analysis to study platelet function, the authors showed that Garp-deficient platelets were as efficient as WT platelets in terms of aggregation, adhesion on collagen and clot retraction. They performed *in vivo* experiments and confirmed that mice deficient for Garp in platelets displayed times for coagulation and thrombus formation that were similar to WT mice. The authors concluded that Garp expression in platelets was not required for thrombosis, hemostasis or thrombo-inflammation. The discrepancy between studies in mice and zebrafish may not only stem from differences in the coagulation processes between the two species but also from the fact that morpholinos in the zebrafish model do not only knockdown *GARP* in platelets. The cell type expressing GARP involved in this process might be platelets, or on any other cell type present in the vessels.

Very recently, Rachidi and colleagues analyzed *Pf4-Cre x Garp* floxed mice in more detail.[67] The authors observed that serum from *Pf4-Cre x Garp* floxed mice contained considerably less active TGF-β1 than control mice, suggesting that platelets are the major source of systemic active TGF-β1, and that its production requires Garp. Interestingly, they also reported delayed tumor growth in *Pf4-Cre x Garp* floxed mice, correlating with increased immune responses against the tumor. Altogether, these data suggest that production of active TGF-β from GARP/Latent TGF-β complexes by platelets may represent an important immunosuppressive mechanism at the tumor site.

GARP in endothelial cells

GARP expression is also detected in another cell type present in vessels, namely in endothelial cells. *Garp* mRNA was first detected in vessels from the mouse placenta and decidua, but not in vessels from other organs.[37] In the aforementioned study in platelets, Vermeersch *et al.* also tested the involvement of Garp expressed on endothelial cells in hemostasis and thrombus formation.[66] They first confirmed Garp expression on endothelial cells from the liver using flow cytometry. They then applied the *in vivo* hemostasis and thrombus formation models in mice deficient for Garp in endothelial cells (*Tie2-Cre x Garp* floxed). Hemostasis and thrombus formation were not different from those observed in WT mice. Altogether these data suggest that Garp expression on platelets or on endothelial cells is not involved in hemostasis in mice. It would however be interesting to see if this also holds true in double KO mice deficient for Garp both in platelets and in endothelial cells.

Another group studied Garp in endothelial cells *in vitro*, focusing their work on their potential immunomodulatory properties. They found Garp expression in Mouse Liver Sinusoid Endothelial Cells or MLSEC.[42] Active TGF-β1 is known to participate in the generation of pTregs promoting immunological tolerance in this organ, but the cellular source of active TGF-β1 implicated in pTreg differentiation in the liver has not been identified. Carambia *et al.* showed that in co-cultures of MLSEC with naïve T cells *in vitro*, in the absence of added exogenous TGF-β1, a small percentage of naïve T cells converted into so-called *in vitro* induced Tregs (iTreg) and started to express Foxp3. Induction of iTregs was not seen in co-cultures with the other types of liver cells tested (Kupffer cells and liver dendritic cells). These experiments suggest that mouse MLSECs may produce active TGF-β1, as Foxp3 induction in naïve T cells and differentiation into iTregs *in vitro* is known to depend on TGF-β1 signals. Since these cells were also shown to express Garp and LAP on their surface, it could be speculated that they produce active TGF-β1 from GARP/latent TGF-β1 complexes. But this has not been formally proven. Finally, GARP protein or mRNA expression was also reported by several groups in Human Umbilical Vein Endothelial cells, but its function has not been further studied in this cell type.[37,41,65]

GARP in mesenchymal stromal cells

Mesenchymal stromal cells (MSC) are multipotent, self-renewable cells found in adult tissues. They appear to play important roles in tissue repair, and were shown to differentiate *in vitro* into chondrocytes, osteoblasts and adipocytes.[68] Because of this, they are described as a promising tool for regenerative medicine.[69] Carillo-Galvez *et al.* described GARP and LAP expression in mouse and human MSCs after short *in vitro* culture.[41] Surprisingly in these cells, *GARP* knockdown leads to increased latent TGF-β1 secretion, suggesting that, like in HEK 293T cells, overexpression of GARP retains latent TGF-β1 on the cell surface and reduces its secretion. Reduced levels of active TGF-β1 were also measured in the supernatants of mouse MSCs in which *Garp* was knocked down with shRNAs. In mouse and human MSCs, GARP could thus sequester latent TGF-β1, preventing its activation by a GARP-independent mechanism.

GARP in hepatic stellate cells

Hepatic stellate cells (HSCs) are liver cells of mesenchymal origin that were first known for their ability to store vitamin A, and which were also described to display immunomodulatory activities.[70] They are known to secrete high amounts of latent TGF-β1, but whether they activate TGF-β1 and whether it contributes to their functions was unclear. Li *et al.* studied the ability of murine HSCs to inhibit murine T cell growth in co-cultures *in vitro*.[43] HSCs inhibited T cell proliferation, and this inhibition was reduced by an inhibitor of the TGFβRI kinase. In addition, HSCs were less able to inhibit the growth of Smad3-deficient T cells. These data suggest that active TGF-β1 is produced in these co-cultures, and that it participated

to the inhibition of T cell growth. The authors also observed that both human and mouse HSCs express GARP on their surface, and showed that *Garp* knockdown with shRNA in mouse HSCs decreased their ability to inhibit T cell growth *in vitro*. GARP might thus function in HSCs as it does in Tregs, and participate in TGF-β1 activation and the immunosuppressive activity of HSCs.

GARP in tumor cells

Metelli *et al.* recently studied GARP expression in human tumors by immunohistochemistry (IHC), using the Plato-1 mAb.[71] They detected GARP expression in breast, colon and lung cancer samples. However, because GARP staining is uniformly distributed and found on all cell types (tumor and stromal cells), it is difficult to define precisely which cell types are positive in these samples. In lung cancer patients, the intensity of GARP expression measured by IHC in tumor samples correlated with a significantly worse prognosis. The authors also transfected murine tumor cell lines with GARP and observed that GARP overexpression increased the growth of the tumors that formed upon transplantation of the transfected cells in mice. Since an increased phosphorylation of Smad2/3 was detected in the tumors, the effect of GARP on tumor growth was suggested to result from production of active TGF-β1 by the transfected cells, even if this was not formally proven. Metelli *et al.* also suggested that, in addition, GARP expression by tumor cells may favor tumor growth by inducing Treg differentiation. Indeed, the cultures of naïve T cells *in vitro* with supernatant from GARP transfected tumor cells increased the percentage of Foxp3 expressing cells by a factor of 2. Depletion of Tregs with an anti-CD25 depleting antibody in tumor bearing mice decreased the aggressiveness of GARP-expressing tumors. Finally, the authors developed anti-human GARP mAbs and tested one in mice transplanted with a murine tumor expressing human GARP. The treatment of tumor-bearing mice with this mAb decreased the proportions of Tregs in the spleen, the number of lung metastases and the growth of the primary tumor. This latter result was only obtained when treatment with the antibody was combined with chemotherapy. Altogether, their data suggest that forced Garp expression in tumor cells induces active TGF-β1 production. However, whether and which types of human tumors produce active TGF-β1 in a GARP-dependent manner has not been demonstrated.

Conclusion

Altogether, the work reviewed above shows that GARP expression is not restricted to Tregs and is found in other hematopoietic cells (megakaryocytes and platelets), in cells of mesenchymal origin (fibroblasts, hepatic stellate cells,...), endothelial cells and possibly in some types of human tumors. Only Tregs, and as suggested recently perhaps also platelets, were shown to produce active TGF-β1 from GARP/latent TGF-β1 complexes. Production by other GARP-expressing cells was suggested in other cell types, namely hepatic stellate cells and endothelial cells, although not formally demonstrated.

Future work should address whether all cell types expressing GARP/latent TGF-β1 complexes are able to produce active TGF-β1, at least in some circumstances. We observed that fibroblasts and endothelial cell lines that express GARP/latent TGF-β1 complexes do not activate TGF-β1 (ref. 58 and unpublished observations). However, it cannot be excluded that specific stimuli are required to trigger TGF-β1 activation from complexes on the surface of these cell types. Identifying which stimuli trigger TGF-β1 activation, and which other molecules, *e.g.* which integrins, are implied in the process would be very interesting. In Tregs, TCR stimulation is required to induce GARP expression, presentation of GARP/latent TGF-β1 on the cell surface, integrin β8 expression, and finally, TGF-β1 activation. Although all the actors of this final process are expressed on Tregs, including on Treg clones, it is still not clear whether integrin αVβ8 activates TGF-β1 from GARP/latent TGF-β1 complexes in *cis* (*i.e.* on complexes present on the same Treg cell), or in *trans* (*i.e.* on complexes present on the surface of another Treg cell). This question deserves careful examination, but will be very difficult to address.

Conflict of interest

S. L. is the co-owner of a patent for the use of anti-GARP antibodies.

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