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REVIEW

Pharmacological modulation of GITRL/GITR system: therapeutic perspectives

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Glucocorticoid-induced TNFR-related (*gitr*) is a gene coding for a member of the TNF receptor superfamily. GITR activation by its ligand (GITRL) influences the activity of effector and regulatory T cells, thus participating in the development of immune response against tumours and infectious agents, as well as in autoimmune and inflammatory diseases. Notably, treating animals with GITR-Fc fusion protein ameliorates autoimmune/inflammatory diseases while GITR triggering, by treatment with anti-GITR mAb, is effective in treating viral, bacterial and parasitic infections, as well in boosting immune response against tumours. GITR modulation has been indicated as one of the top 25 most promising research areas by the American National Cancer Institute, and a clinical trial testing the efficacy of an anti-GITR mAb in melanoma patients has been started. In this review, we summarize results regarding: (i) the mechanisms by which GITRL/GITR system modulates immune response; (ii) the structural and functional studies clearly demonstrating differences between GITRL/GITR systems of mice and humans; (iii) the molecules with pharmacological activities including anti-GITR mAbs, GITR-Fc and GITRL-Fc fusion proteins, GITRL in monomer or multimer conformation; and (iv) the possible risks deriving from GITRL/GITR system pharmacological modulation. In conclusion, GITR triggering and inhibition could be useful in treating tumours, infectious diseases, as well as autoimmune and inflammatory diseases. However, differences between mouse and human GITRL/GITR systems suggest that further preclinical studies are needed to better understand how safe therapeutic results can be obtained and to design appropriate clinical trials.

Abbreviations

DC, dendritic cells; GITR, glucocorticoid-induced TNF receptor-related; GITRL, GITR ligand; GVHD, graft-versus-host disease; hGITR, human GITR; hGITRL, human GITR ligand; mGITR, murine GITR; mGITR ligand, murine GITRL; NK, natural killer; Treg, regulatory T cell

Introduction

Murine GITR (mGITR) is a receptor belonging to TNF receptor superfamily (TNFRSF) that has been identified 14 years ago (Nocentini *et al.*, 1997). Two years later, the human GITR (hGITR) ortholog and its ligand (GITRL) have been identified (Gurney *et al.*, 1999; Kwon *et al.*, 1999). Most of the research work on the GITRL/GITR system has been devoted to its role in development and function of the immune system. GITRL/GITR system contribution to immune system regulation appears to be very complex and important in several immune-related diseases, as demonstrated by experimental models of autoimmunity, inflammation and tumour. After the initial results obtained upon GITR activation in animal tumour models, GITR modulation was listed as one of the top 25 most promising research areas by the NCI (Schaer *et al.*, 2010), and a clinical trial in the United States, testing the efficacy of treatment with anti-GITR mAb in patients suffering from melanoma, gained approval in December 2010 (trial #NCT01239134).

The aim of this review is to discuss the indications from GITRL/GITR mouse models, summarize available

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information about GITRL/GITR system in humans, and discuss how its modulation can be used in therapeutic interventions.

Tissue distribution of mGITR and mGITRL

The mGITR is expressed in a large number of murine cells and tissues including cells of the innate and adaptive immunity (Nocentini and Riccardi, 2009; Azuma, 2010; Placke *et al.*, 2010). In some cell subsets, such as effector T cells, GITR acts as a co-stimulating receptor and is up-regulated upon cell activation (Figure 1) (Ronchetti *et al.*, 2007). Interestingly enough, mGITR is shed after mGITR ligand (mGITRL) binding (van Olffen *et al.*, 2009). Moreover, non-haematological tissues like bone, skin, brain and lung have been found to express mGITR (Gurney *et al.*, 1999; Wang *et al.*, 2005; O'Keeffe *et al.*, 2008; Nocentini and Riccardi, 2009). These findings may be relevant for the pharmacological profile of GITRL/GITR modifiers and appropriate studies are necessary to evaluate the actual possibility for therapeutic treatments in humans.

mGITRL is a type II trans-membrane protein that can be released by shedding (Nocentini and Riccardi, 2009; Azuma, 2010; Placke *et al.*, 2010). In particular, antigen presenting cells (APC) and endothelial cells express high levels of mGITRL. mGITRL is up-regulated upon activation of effector T cells, thus suggesting an autocrine contribution to mGITR triggering and T cell activation (Nocentini and Riccardi, 2009). mGITRL is able to activate signal transduction eliciting

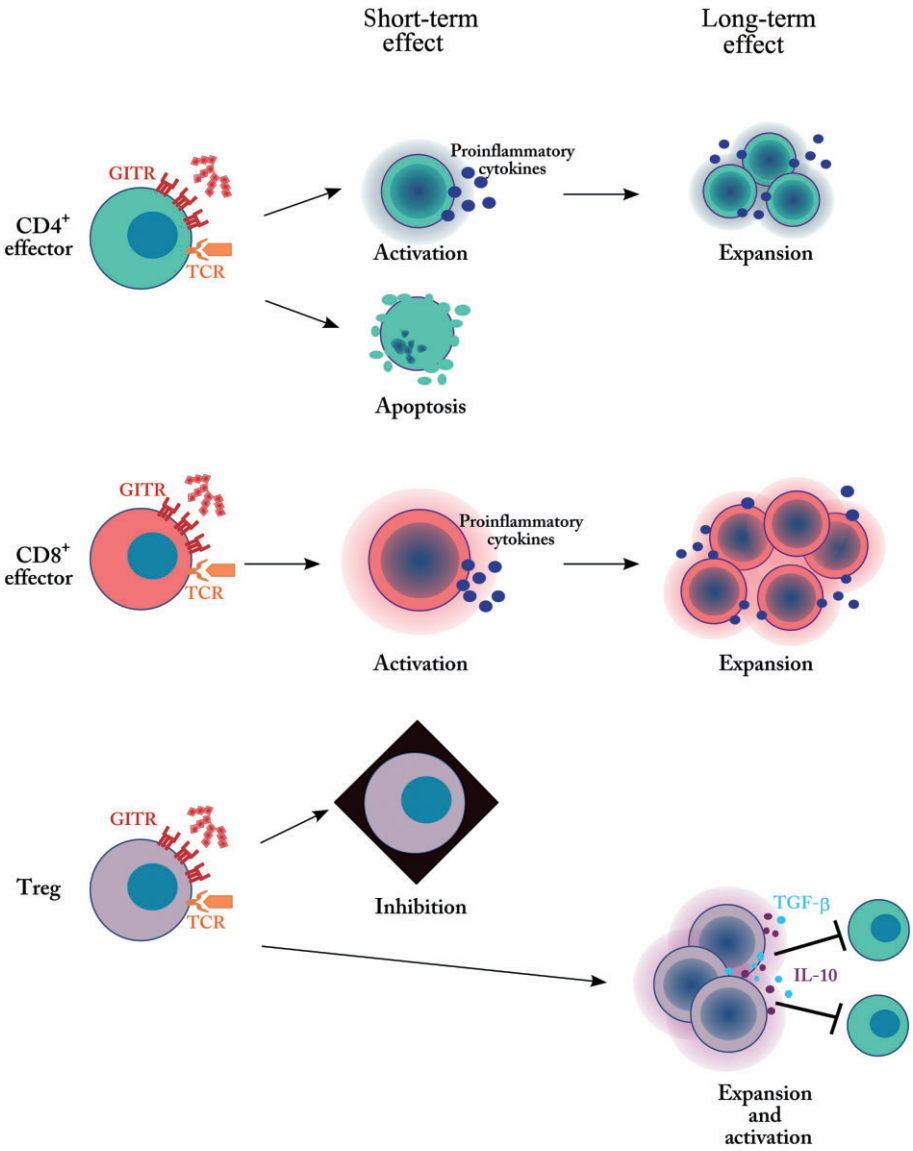

Figure 1
Effect of GITR triggering in effector T cell and Treg regulation and proliferation.

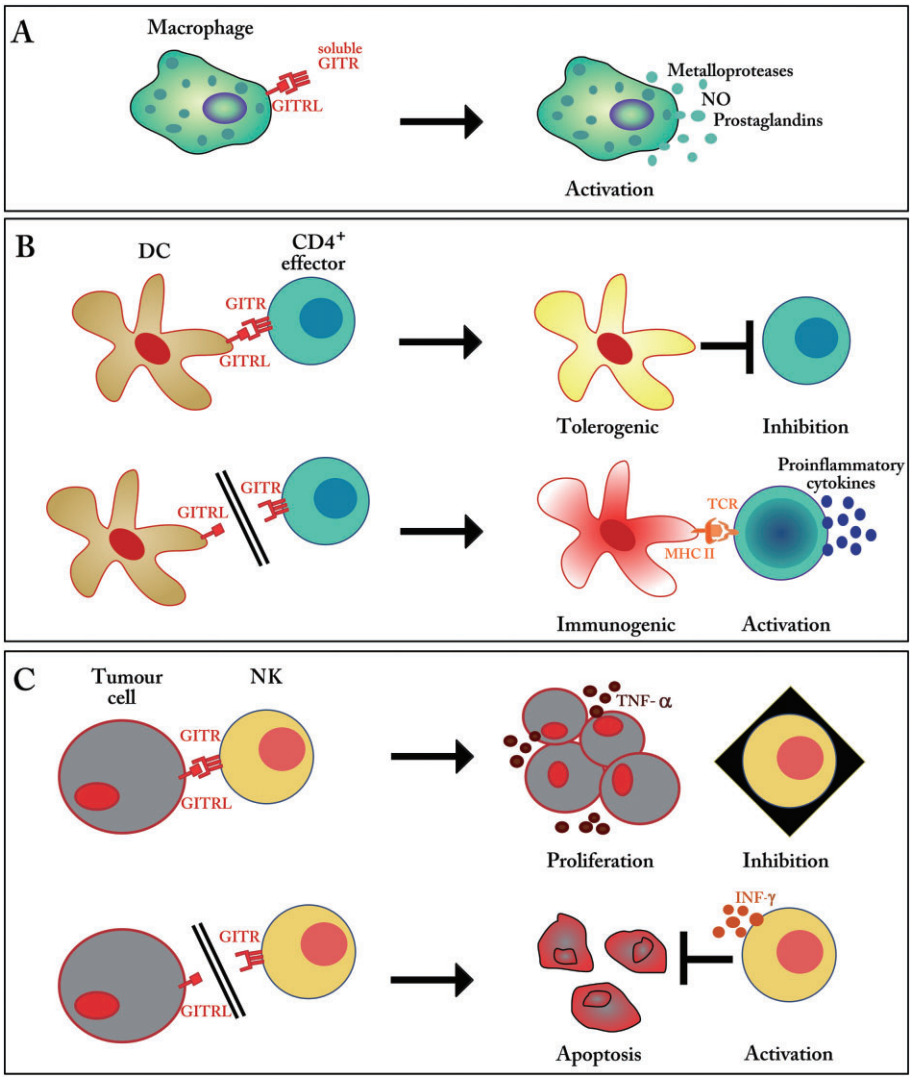

Figure 2

Effect of GITRL triggering or inhibition in macrophages (A), DC (B) and tumour cells (C).

a tolerogenic effect in dendritic cells (DC) and pro-inflammatory stimuli in macrophages (Figure 2) (Kwon *et al.*, 2003; Grohmann *et al.*, 2007; Placke *et al.*, 2010). Given that GITR knock-out ($GITR^{-/-}$) mice are vital while $GITRL^{-/-}$ mice show a strain-dependent embryonic lethal phenotype (Ronchetti *et al.*, 2002; Poulton *et al.*, 2010), it is possible that a GITRL signal, independent of GITR, is essential for embryo development.

Role of GITRL/GITR in murine T cells

GITR triggering exerts co-stimulatory effects in effector T cells that could be due in part to inhibition of TCR/CD3-induced T cell death (Nocentini and Riccardi, 2009). *In vitro* experiments suggest that mGITR-dependent co-stimulation plays a leading role in the activation of $CD8^+$ effector T cells (Ronchetti *et al.*, 2007) while the effects in $CD4^+$ effector T cells depend upon the experimental conditions and can even lead to apoptosis (Spinicelli *et al.*, 2002; Tone *et al.*, 2003). mGITR triggering, by anti-mGITR mAbs, renders effector T cells less susceptible to Treg suppressor activity while transiently inhibits regulatory T cell (Treg) suppressor activity (Schaer *et al.*, 2010). Both mechanisms are operative *in vivo*, as demonstrated in different disease models (Santucci *et al.*, 2007; Cohen *et al.*, 2010). However, mGITR triggering breaks Treg anergy and stimulates their proliferation (Figure 1).

Two genetically modified mice provide information on the role of GITRL/GITR system in the development of T cells in healthy mice. In young (4–8 weeks) $GITR^{-/-}$ mice, the percentage of T cell subsets was similar to wild-type mice, with the exception of $CD4^+CD25^+$ Tregs that were lower in number (Ronchetti *et al.*, 2002; Stephens *et al.*, 2004). In transgenic mice (GITRL TG) over-expressing mGITRL in B cells, T cell subsets were altered (van Olffen *et al.*, 2009). In particular, in the spleen of GITRL TG adult mice (6–24 weeks), Tregs and effector memory $CD4^+$ cells ($CD44^+CD62L^-$) were significantly increased.

Role of GITRL/GITR in modulating T cell response in mouse disease models

Several studies on the role of GITRL/GITR system in infections, autoimmune/inflammatory diseases, transplant rejection and tumours, demonstrate that the immediate effect of mGITR triggering is stimulation of T effector and inhibition of Treg activity, with some interesting exceptions.

In several inflammatory disease models including arthritis, pleurisy, lung fibrosis, inflammatory bowel diseases, splanchnic artery occlusion, spinal cord injury and pancreatitis (Cuzzocrea *et al.*, 2004; 2005; 2007; Nocentini *et al.*, 2008; Nocentini and Riccardi, 2009; Galuppo *et al.*, 2011a,b), mGITR inhibition exerts an anti-inflammatory effect. In accordance, mGITR triggering resulted in an increased inflammatory response (Lee *et al.*, 2006).

mGITR participates also in the development of autoimmune and allergic diseases, as demonstrated in disease models of gastritis, thyroiditis, experimental autoimmune encephalomyelitis and allergic airway inflammation (Shimizu *et al.*, 2002; Kohm *et al.*, 2004; Patel *et al.*, 2005; Morris and Kong, 2006). A role in the autoimmune diabetes has also been demonstrated (Suri *et al.*, 2004; You *et al.*, 2009).

In general, in most of disease models, mGITR activation favours leucocyte extravasation, increases T lymphocyte activation and inhibits Treg activity (Patel *et al.*, 2005; Cuzzocrea *et al.*, 2006; Santucci *et al.*, 2007).

The effect of GITR triggering has been tested on a number of viral and parasitic disease models, demonstrating that mGITR activation results in potentiation of immune response against pathogens, as elsewhere summarized (Nocentini and Riccardi, 2009). Recent studies have confirmed this view in visceral leishmaniasis, influenza and vaccination against Friend virus (Dittmer *et al.*, 2008; Haque *et al.*, 2010; Snell *et al.*, 2010).

A role for GITRL/GITR system has been demonstrated also in graft rejection. mGITR triggering resulted in inhibition of Treg suppressor activity and in acute allograft rejection, in recipients previously tolerized against primary transplant. Moreover, mGITR inhibition favoured Treg activity and prolonged skin graft survival (Bushell and Wood, 2007; Kim *et al.*, 2010). Conversely, GITR triggering favoured expansion of Tregs within the cornea, participating in the maintenance of immune privilege in corneal allografts (Hori *et al.*, 2010). These opposite results suggest that mGITR plays opposite roles in different immune contexts. A similar conclusion can be driven from the graft-versus-host disease (GVHD) studies, in which the role of mGITR triggering is not clear-cut, depending on the kind of GVHD and the T cell subset which is mainly involved in the GVHD reaction (Nocentini and Riccardi, 2009). These results are in agreement with different effects of mGITR triggering on Tregs: mGITR triggering causes a transient inhibition of suppressor activity while, at the same time, stimulates Treg proliferation and expansion so that, after time, there is an increased number of Tregs with recovered suppressor activity (Figure 1).

Recent studies clearly indicate that mGITR triggering can induce an immunostimulatory effect in tumour-bearing mice (Placke *et al.*, 2010; Schaer *et al.*, 2010). In particular, it has been shown that GITR triggering, favouring immune response, enhances tumour rejection, that is specific and depends on both effector activation (Ramirez-Montagut *et al.*, 2006; Boczkowski *et al.*, 2009) and inhibition of Treg suppressor activity (Cohen *et al.*, 2010). Moreover, a recent study demonstrated that anti-mGITR Abs deplete Treg compartment (Coe *et al.*, 2010). This effect could be due to the Treg proliferation increase, consequent to GITR stimulation that can induce Treg death.

The study by Ramirez-Montagut *et al.* demonstrated that GITR triggering does not promote survival of mice when natural killer (NK) and natural killer T (NKT) cells are removed, while a recent communication considered NK cells irrelevant in GITR-dependent tumour rejection (Ramirez-Montagut *et al.*, 2006; Ponte *et al.*, 2010a). Moreover, it has been demonstrated that GITR triggering in B cells is crucial in promoting T-cell-mediated anti-tumour immunity (Zhou *et al.*, 2010).

Some experimental results sound as a warning in considering GITR exclusively as a receptor that stimulates the immune response. Despite the original observation on human NK and murine NKT demonstrating that GITR plays a co-stimulatory role in NK cells (Shin *et al.*, 2005; Wang *et al.*, 2005), several studies on murine and human NK/NKT cells demonstrated that GITR triggering can inhibit NK activity (Baltz *et al.*, 2008; Chen *et al.*, 2008; Liu *et al.*, 2008; Placke *et al.*, 2010). Although this effect has to be considered when planning an immunostimulatory treatment, the effect of T cell response potentiation appears to overcome a possible NK inhibition in almost all disease models, with the exception of *Candida albicans* infection, in which GITR$^{-/-}$ mice survived better to *C. albicans* infection as compared with wild-type mice (Agostini *et al.*, 2005). Authors suggest that the survival of GITR$^{-/-}$ mice resulted from lack of mGITRL reverse signalling in DC, causing an increased production of IL-12 and a prevalent Th1 polarization that favours *C. albicans* clearance. An additional explanation is that GITR$^{-/-}$ mice have an increased NK function, relevant in control of *C. albicans* infection (Murciano *et al.*, 2006).

Tools useful to trigger mGITR

In the majority of studies on disease models, the anti-GITR mAb DTA-1 was used (Nocentini and Riccardi, 2009). As summarized previously, the overall effect is to strengthen effector T cell response, allowing tumour rejection and improvement of immune response against pathogens. Similar effects were obtained by another anti-GITR mAb (2F8) (Ponte *et al.*, 2010a,b).

The effect of anti-mGITR mAb G3c represents a remarkable exception (Nishioka *et al.*, 2008). The G3c mAb has a higher co-stimulatory potential than DTA-1 mAb for both CD4$^+$ effector cells and Tregs, the latter cells being the most sensitive to co-stimulation. Although G3c mAb inhibits Treg activity in *in vitro* experiments, G3c mAb treatment of tumour-bearing mice does not have the curative effects observed with DTA-1 Ab. Indeed, Nishioka *et al.* found that G3c mAb *in vivo* treatment exhibits co-stimulatory effects more on Tregs than on non-Treg cells. A lesson from G3c mAb is that different pharmacological tools promoting GITR

stimulation can differentially activate various cell subsets and the balance of effector cell and Treg co-stimulation can result to be crucial in therapeutic outcome.

In some experiments, mGITR was stimulated by mGITRL-transfected tumour cells (Calmels *et al.*, 2005; Piao *et al.*, 2009). Interestingly, the expression of mGITRL on tumour cells potentiates $CD8^+$ T cell response only in tumour microenvironment (Cho *et al.*, 2009), decreasing the risk of systemic autoimmune reactions. The transfection/infection approach appears to be the most intriguing even if quite far from the clinics.

Some other studies used soluble proteins containing the extracellular domain of mGITRL to trigger mGITR. A few studies used soluble mGITRL expressed as a monomer, while most studies used mGITRL as a dimer (a fusion protein composed by Fc fragment and mGITRL extracellular domain – mGITRL-Fc), demonstrating that they can stimulate mGITR (Nocentini and Riccardi, 2009). The use of a dimer has a rational basis considering that mGITRL is present in the membrane as a dimer (as discussed next). Another study used a multimer of mGITRL in the hypothesis that this structure would be able to better stimulate mGITR (Stone *et al.*, 2006a). The 4-trimer construct of mGITRL increased both lymphocytes proliferation and antibody responses to Gag protein, but comparison with the effect of mGITRL dimer was not performed. Hu *et al*. demonstrated that a mGITRL-Fc fusion protein in which the extracellular domain of mGITRL was truncated, works better than the full-length mGITRL extracellular domain in the stimulation of $CD8^+$ cells and inhibition of Tregs (Hu *et al.*, 2008). This fusion protein was able to inhibit tumour growth at levels similar to those obtained with DTA-1 antibody. Another study focused on the effect of mGITRL-Fc on Tregs, demonstrating that Tregs expand *in vitro* and *in vivo* following mGITR triggering (Liao *et al.*, 2010). In this case, continuous treatment with mGITRL-Fc favours expansion of Tregs more than effector cells, thus obtaining an effect similar to G3c mAb.

An interesting approach was to prepare a fusion protein binding mGITR and fibroblast activation protein (FAP) (mGITRL/anti-FAP) (Burckhart *et al.*, 2010). When T cells were grown together with FAP-expressing cells, $CD4^+$ and $CD8^+$ T cells were co-stimulated at a concentration of 1 $\mu g\ mL^{-1}$. At the same concentration, Tregs were inhibited. As FAP is expressed on cancer-associated fibroblasts, mGITRL/anti-FAP should concentrate in tumour microenvironment following *in vivo* treatment, minimizing the effects of unwanted mGITR triggering in other tissues.

Localized production of anti-mGITR Ab or mGITRL-Fc fusion protein was also obtained by co-transferring DC with mRNA encoding the heavy and light chains of the anti-mGITR mAb or mGITRL-Fc fusion protein together with tumour antigen-presenting DC (Boczkowski *et al.*, 2009).

Tools useful to inhibit mGITR triggering and/or activate mGITRL

Tools to inhibit mGITR triggering and/or activate mGITRL are: (i) the fusion protein composed of the mGITR extracellular domain and the Fc fragment (mGITR-Fc), allowing dimerization of the mGITR extracellular domain; (ii) the soluble extracellular domain of mGITR as monomer (smGITR); and (iii) the anti-mGITRL Ab.

Several studies on disease models make use of mGITR-Fc and demonstrate mGITR-Fc-dependent anti-inflammatory effects, decreased autoimmune response and prolongation of skin graft survival (Nocentini *et al.*, 2008; Nocentini and Riccardi, 2009; Kim *et al.*, 2010; Galuppo *et al.*, 2011a). As $GITR^{-/-}$ mice show inflammation levels similar to those observed in mGITR-Fc-treated wild-type mice, it can be concluded that, at least in these models, the mechanism of action of mGITR-Fc is inhibition of mGITR triggering. The same effect was also obtained by using anti-mGITRL Ab (Kamimura *et al.*, 2009; Hori *et al.*, 2010).

In *in vitro* experiments and in some *in vivo* models, it has been suggested that the effects of mGITR-Fc or smGITR depend on mGITRL stimulation. In a model focusing on DC, mGITR-Fc promotes anti-inflammatory/tolerogenic effects (Grohmann *et al.*, 2007). By contrast, other studies demonstrate that *in vitro* mGITRL triggering by mGITR-Fc or smGITR activates macrophage, bone-marrow stromal cells or keratinocytes with an increased production of proinflammatory and chemoattractants molecules (Krausz *et al.*, 2007; Bae *et al.*, 2008; Byrne *et al.*, 2009). Enlarged spleen and increased percentage of polymorphonuclears and monocytes in peripheral blood were observed in healthy mouse treated with smGITR (Heikkinen *et al.*, 2004).

Finally, data on the effect of anti-mGITRL Ab are contrasting. In fact, anti-mGITRL Ab has been shown to trigger mGITRL on macrophages, to block mGITRL signalling in keratinocytes and to antagonize mGITR triggering by mGITRL (Bae *et al.*, 2008; Byrne *et al.*, 2009; Kamimura *et al.*, 2009).

Distribution and role of hGITR and hGITRL

Human GITR (CD357) is less characterized than mGITR. At present, data suggest that human expression pattern and function are somehow different from mGITR. Contrary to mGITR, hGITR is not expressed in peripheral $CD4^+$ and $CD8^+$ effector cells while similarly to mGITR, it is expressed at high levels following activation (Kober *et al.*, 2008; Bianchini *et al.*, 2011). Unlike mGITR and supposedly because of hGITR absence in $CD4^+$ and $CD8^+$ un-stimulated effector cells, in some experimental settings hGITR triggering exerts a weak co-stimulatory activity, weaker than that exerted by other TNFR superfamily (TNFRSF) members such as 4-1BB or CD70 (Kober *et al.*, 2008). However, in a model of immune privilege, in which retinal pigmental epithelial (RPE) cells inhibit T cell proliferation, hGITR triggering rescued T cell proliferation while CD28 triggering did not, suggesting that hGITR signals through a pathway distinct from CD28 and that specifically antagonizes RPE-mediated immunosuppression (Mahesh *et al.*, 2006). Notably, this last finding is compatible with the mouse system where hGITR triggering was able to co-stimulate at similar levels wild-type and $CD28^{-/-}$ T cells (Ronchetti *et al.*, 2007).

In humans, a small $CD4^+$ cell population with Treg activity has been recently discovered and found to express hGITR,

low levels of CD25 and FoxP3 (Bianchini *et al.*, 2011). hGITR is expressed also in some, but not all, $CD25^{high}$ Tregs (Condomines *et al.*, 2006; Lau *et al.*, 2007; Satoguina *et al.*, 2008; Gerli *et al.*, 2009; Bianchini *et al.*, 2011), and some studies suggest that $CD4^+CD25^+GITR^+$ can represent the Treg subset with higher suppressor activity. As an example, in myasthenia gravis the percentage of $CD4^+CD25^+GITR^+$ is lower than in healthy donors (Masuda *et al.*, 2010) and the increased suppressive activity of Tregs in patients infected with *Plasmodium vivax* correlates with an increased number of $CD4^+CD25^+GITR^+$ cells (Bueno *et al.*, 2010). hGITR is expressed also on antigen-specific $CD8^+CD25^+$ and thymus-derived $CD8^+CD25^+$ Tregs (Cosmi *et al.*, 2003; Scotto *et al.*, 2004; Mahic *et al.*, 2008). While there is a general agreement that one of the effects of GITR triggering is inhibition of suppressor activity of murine Tregs, the effect of hGITR triggering on human Tregs is a matter of debate (Levings *et al.*, 2002; Cardona *et al.*, 2006; Tuyaerts *et al.*, 2007; Satoguina *et al.*, 2008; Bianchini *et al.*, 2011).

Like in mice, hGITR is expressed on DC, including plasmacitoid DC, monocytes, NK cells and virtually all B-cell subsets, including plasma cells (Kim *et al.*, 2007; Cabezon *et al.*, 2011; Matesanz-Isabel *et al.*, 2011; Ronchetti *et al.*, 2011).

Few studies deal with hGITR expression in non-haematological tissues. hGITR mRNA is expressed in lung and, at lower level, in brain, kidney, liver and a colorectal adenocarcinoma cell line (Gurney *et al.*, 1999; Kwon *et al.*, 1999).

The expression pattern of hGITRL is similar to that of mGITRL. In particular, APC and endothelial cells express high levels of GITRL, particularly when activated (Byrne *et al.*, 2009; Perez Novo *et al.*, 2010; Grosser *et al.*, 2011). Moreover, hGITRL is expressed in tumours, as demonstrated in primary cultures from gastrointestinal and haematological tumours (Baltz *et al.*, 2007; Baessler *et al.*, 2009). Interestingly, hGITRL reverse signalling can play a role in protecting tumour cells from immune response as its triggering down-regulates the expression of the immunostimulatory molecules and induces production of immunosuppressive cytokines (Figure 2) (Baltz *et al.*, 2007; Baessler *et al.*, 2009).

hGITR and GITRL are structurally different from their respective murine orthologs

In a comprehensive study on the interaction between TNFSF members and TNFRSF members, Bossen *et al.* demonstrated that mGITRL does not interact with hGITR receptor and that hGITRL does not interact with the mouse receptor (Bossen *et al.*, 2006). Even if contrasting results have been recently reported (Sonawane *et al.*, 2009), it seems that the GITRL/GITR binding is species specific as also suggested by the lack of similarity between mouse and human GITR in the first cystein pseudorepeat of the extracellular domain (Krausz *et al.*, 2007). Indeed, important differences between the tridimensional structures of hGITRL and mGITR have been demonstrated by two independent groups performing crystallographic studies on both murine and human GITRL (Chattopadhyay *et al.*, 2007; 2008; Zhou *et al.*, 2008a,b). In contrast to all previously characterized homotrimeric TNF family members, mGITRL crystal structure revealed a previously unrecognized dimeric assembly through a unique C terminus arm (Chattopadhyay *et al.*, 2008; Zhou *et al.*, 2008b). Consistent with its crystal structure, mGITRL exists as a stable dimer in solution. Engagement of the mouse receptor and ligand could probably result in an assembly with a 2:2 receptor : ligand stoichiometry (Chattopadhyay *et al.*, 2009), thus having an impact in the activation of the transduction pathways. For example, the dimer could preclude a typical interaction with trimeric TRAFs (Chattopadhyay *et al.*, 2009).

In contrast with mGITRL, hGITRL self-assembles into a homotrimer, which is atypical because it possesses a relatively weak tendency to trimerize in solution and displays a monomer–trimer equilibrium never described for other TNFSF members (Chattopadhyay *et al.*, 2007). Zhou *et al.* confirmed that hGITRL forms a loosely associated open trimer and also described the possibility that hGITRL can form a dimer and a tetramer of trimers (i.e. supercluster) (Zhou *et al.*, 2008b).

In summary, structural and functional studies suggest that murine and human GITRL/GITR pairs have a different structure so that may activate different transduction pathways in mouse and humans, further suggesting that the effects of hGITR triggering may be different from that of mGITR. Indeed, TRAF2-dependent NF-κB modulation following GITR triggering is opposite in mice and humans (Kwon *et al.*, 1999; Esparza and Arch, 2005; Krausz *et al.*, 2007).

Tools useful to trigger hGITR

The physiological soluble hGITRL trimer has a dissociation constant (Kd) about 100-fold higher than that of the other family members (Chattopadhyay *et al.*, 2007), suggesting that soluble hGITRL is a weak agonist. Notably, it has been shown that the lower the number of hGITRL molecules in the complex that interact with hGITR is, the lower the activation level of hGITR (i.e. monomer and dimer stimulate hGITR weakly, the trimer stimulates hGITR at an intermediate level, and the supercluster or a stabilized trimer stimulate hGITR at high levels) (Chattopadhyay *et al.*, 2007; Zhou *et al.*, 2008b) will be. Accordingly, Mahesh *et al.* were unable to trigger hGITR by soluble hGITRL (Mahesh *et al.*, 2006). An interesting study by Wyzgol *et al.* demonstrated that hGITR is not triggered by hGITRL monomer and dimer and that the level of stimulation increases with oligomerization. In particular, powerful activation of hGITR is obtained by two stabilized trimers linked by an anti-Flag Ab (Wyzgol *et al.*, 2009). Cui *et al.* added an isoleucine-zipper motif to the N-terminus of the soluble hGITRL and expressed it in *Escherichia coli* (Cui *et al.*, 2010). This fusion protein exhibited a predominant trimer organization and showed significantly higher biological activity compared with soluble hGITRL. Stone *et al.* used a pmacSP-D-GITRL (four trimers of GITRL) construct expressed in 293HEK cells (Stone *et al.*, 2006b). GITRL sequence was that of macaque that codes an extracellular domain identical to that of hGITRL, with the only exception of two amino acids. PmacSP-D-GITRL was able to co-stimulate human $CD4^+$ cells and to inhibit Treg activity.

In this context, the studies from Baltz *et al*. are surprising. In one study, hGITR was triggered by a plastic cross-linked fusion protein formed by the extracellular domain of hGITRL and the Fc fragment (Baltz *et al*., 2007). In another study, shGITRL-containing serum of tumour-affected patients in co-cultures with tumour cells triggered hGITR and significantly reduced NK cell cytotoxicity and IFN-gamma production (Baltz *et al*., 2008). A possible explanation is that serum favours hGITRL multimerization or that hGITR has different assembly and/or transduction pathways when expressed in NK cells.

It may be thought that the straightforward way to stimulate hGITR is to use anti-hGITR Abs, as in the mouse models. However, a few pieces of evidence suggest that anti-hGITR mAbs are unable to stimulate hGITR. Satoguina *et al*. showed that an anti-hGITR mAb (R&D Systems, pers. communication) is unable to trigger hGITR while it inhibits its physiological activation (Satoguina *et al*., 2008). Baltz *et al*. demonstrate that the same anti-hGITR Ab (R&D Systems) does not trigger hGITR expressed in NK cells (Baltz *et al*., 2007). We also used anti-hGITR mAbs in the attempt to co-stimulate purified human $CD4^+$ cells following anti-CD3 Abs and anti-hGITR Abs co-treatment. Monoclonal Abs were used either in solution or cross-linked to the plastic or beads, but we did not observe any co-stimulation (manuscript in preparation).

In other hands and/or using other mAbs, hGITR triggering was observed. Liu *et al*. cross-linked the same Ab used by Baltz *et al*. and considered it as an agonist (Liu *et al*., 2008) and Bae *et al*. used another anti-hGITR mAb (Immunomics) to stimulate human macrophages (Bae *et al*., 2007). Moreover, Rosenzweig *et al*. have recently prepared TRX518, an aglycosyl fully humanized anti-hGITR mAb (Rosenzweig *et al*., 2010). TRX518 blocks the interaction of hGITR with its ligand but also co-stimulates T lymphocytes and enhances the cytotoxicity of NK cells.

The different results obtained with anti-hGITR mAb may be due to the kind of the mAb, the experimental conditions and the cells expressing hGITR. However, the possibility that anti-hGITR mAbs are antagonists or weak agonists, weaker than physiological hGITRL, has to be taken into account. The lack of hGITR triggering by anti-hGITR mAbs may be a characteristic of hGITR that is appropriately stimulated only by stabilized trimers or GITRL superclusters.

Tools useful to inhibit hGITR

As discussed previously, anti-hGITR Ab can have antagonistic properties, at least in some experimental conditions. A few studies have tested other reagents that inhibit hGITR activation. Baltz *et al*. reported that anti-hGITRL mAbs (R&D Systems) do not block the interaction of GITR-Fc fusion protein with hGITRL, concluding that anti-hGITRL Abs are not blocking (i.e. do not inhibit either hGITR or hGITRL triggering) (Baltz *et al*., 2007). Conversely, Satoguina *et al*. found that anti-hGITRL mAb (R&D Systems, pers. comm.) has the same effect of antagonistic anti-hGITR mAb in their experimental setting (Satoguina *et al*., 2008) and Mahesh *et al*. (Mahesh *et al*., 2006) found that anti-hGITRL mAb (R&D) has partial blocking properties.

In Baltz *et al*.'s study, hGITR-Fc fusion protein is able to stimulate hGITRL reverse signalling and very likely inhibits hGITR triggering by the ligand (Baltz *et al*., 2007).

Concluding remarks

A number of observations indicate that GITRL/GITR modulation can be useful in the treatment of cancer, infections and autoimmune/inflammatory diseases (Nocentini and Riccardi, 2009; Placke *et al*., 2010; Schaer *et al*., 2010). In particular, the possible usefulness of GITR triggering in cancer treatment is suggested by:

- The striking results observed in mouse models (Ramirez-Montagut *et al*., 2006; Boczkowski *et al*., 2009; Cohen *et al*., 2010; Placke *et al*., 2010; Schaer *et al*., 2010; Zhou *et al*., 2010)
- The possibility to trigger GITR in various cell subsets that can synergize in obtaining tumour clearance. In particular: (i) a powerful co-stimulation of $CD8^+$ and $CD4^+$ effector cells; (ii) an inhibition of Treg suppressor activity; and (iii) an activation of B cells
- The possibility that molecules used to stimulate mGITR (anti-mGITR Abs and/or soluble mGITRL) can inhibit mGITRL reverse signalling that plays a role in the maintenance of a tolerogenic microenvironment inside tumours (Baltz *et al*., 2007; Grohmann *et al*., 2007).

For the same reasons, GITR triggering tools promise to be powerful adjuvants and may result to be crucial in the treatment of chronic infection.

It is widely accepted that mouse disease models do not reproduce human diseases so that preclinical results may not give appropriate indications for clinical trials design. In the case of GITRL/GITR system, differences between mice and humans are evident. In summary:

- There are important structural differences between mouse and human GITRL/GITR systems.
- Tissue distribution appears to be different.
- Signalling of hGITR may be different from that of mGITR and the co-stimulatory effects are weaker.
- Anti-GITR mAbs have much weaker triggering potential in humans than in mice.

Other problems may derive from the possibility that NK cell activity is inhibited following GITR triggering and that, at least in some models, GITR activation favours autoimmune diseases development (Shimizu *et al*., 2002; Morris and Kong, 2006; Baltz *et al*., 2008).

The possible usefulness of GITR inhibition in the treatment of autoimmune/inflammatory diseases, prevention of transplant rejection and GVHD is suggested by:

- the striking results observed in the mouse models (Cuzzocrea *et al*., 2004; 2005; 2007; Bushell and Wood, 2007; Nocentini *et al*., 2008; Nocentini and Riccardi, 2009; Kim *et al*., 2010; Galuppo *et al*., 2011a,b);
- the inhibitory effects on several cells of the immune system, including effector T cells, B cells and macrophages (Krausz *et al*., 2007);

- the inhibition of leucocyte extravasation (Cuzzocrea *et al.*, 2004; 2006); and
- the possible activation of GITRL, favouring a tolerogenic differentiation of DC (Grohmann *et al.*, 2007).

Some data suggest that GITR inhibition can be obtained in humans easily, using tools similar to those effective in mouse models, but more studies are needed. The main worry in the use of GITR-inhibiting drugs is the theoretical possibility that GITR inhibition favours immunosuppression and development of infectious diseases.

In conclusion, modulation of GITRL/GITR interaction has an interesting potential for anti-tumour therapy, autoimmune/inflammatory diseases treatment, prevention of transplant rejection and GVHD. Future studies could clarify which drug can better stimulate or antagonize hGITRL/hGITR.

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Conflict of interest

The authors do not have conflicts of interest.

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