Perspectives on Regulatory T Cell Therapies

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Key Words

CD4⁺ CD25⁺ regulatory T cells · FOXP3 · GARP · Positive feedback loop

Summary

Adoptive transfer in animal models clearly indicate an essential role of CD4⁺ CD25⁺ FOXP3⁺ regulatory T (T_{reg}) cells in prevention and treatment of autoimmune and graft-versus-host disease. Thus, T_{reg} cell therapies and development of drugs that specifically enhance T_{reg} cell function and development represent promising tools to establish dominant tolerance. So far, lack of specific markers to differentiate human T_{reg} cells from activated CD4⁺ CD25⁺ effector T cells, which also express FOXP3 at different levels, hampered such an approach. Recent identification of the orphan receptor glycoprotein-A repetitions predominant (GARP or LRRC32) as T_{reg} cell-specific key molecule that dominantly controls FOXP3 via a positive feedback loop opens up new perspectives for molecular and cellular therapies. This brief review focuses on the role of GARP as a safeguard of a complex regulatory network of human T_{reg} cells and its implications for regulatory T cell therapies in autoimmunity and graft-versus-host disease.

Schlüsselwörter

CD4⁺ CD25⁺ regulatorischen T-Zellen · FOXP3 · GARP · Positiver «feedback loop»

Zusammenfassung

Tierexperimentelle Arbeiten zur adoptiven T-Zell-Therapie zeigten eindrücklich die bedeutende Funktion von CD4⁺ CD25⁺ FOXP3⁺ regulatorischen T(T_{reg})-Zellen in der Prävention und Behandlung von Autoimmunkrankheiten und der Graft-versus-host-Erkrankung. Hierdurch stellt die Anwendung einer adoptiven T_{reg}-Zelltherapie bzw. die Entwicklung von Medikamenten, die die Funktion von T_{reg}-Zellen spezifisch verstärken, eine neue Perspektive zur Etablierung einer dominanten Toleranz dar. Bisher wurde diese Entwicklung durch den Mangel spezifischer Oberflächenmarker zur sicheren Unterscheidung von T_{reg}-Zellen und aktivierten CD4⁺ CD25⁺ Effektor-T-Zellen, die ebenfalls FOXP3 in unterschiedlichem Maße exprimieren, gehemmt. Die Entdeckung des Rezeptors «glycoprotein-A repetitions predominant» (GARP bzw. LRRC32) als T_{reg}-Zell-spezifisches Schlüsselmolekül, das dominant FOXP3 in Verbindung mit einem positiven «feedback loop» kontrolliert, eröffnet neue Perspektiven für die zelluläre und molekulare T_{reg}-Zell-Therapie. Diese Übersicht befasst sich mit den Perspektiven, die die Entdeckung von GARP als «safeguard» eines komplexen regulatorischen Netzwerks humaner T_{reg}-Zellen mit sich bringen, sowie mit deren Bedeutung für regulatorische T-Zell-Therapien bei Autoimmunkrankheiten und der Graft-versus-host-Erkrankung.

Introduction

Negative selection in thymus does represent an imperfect process and thus self-reacting T cell clones do escape central tolerance. Therefore, to keep these potential dangerous self-

reactive T cells in check, a multitude of peripheral tolerance mechanisms exist. Dominant tolerance exerted by CD4⁺ regulatory T (T_{reg}) cells characterized by the expression of CD25 (IL2 receptor alpha) [1] does represent a major mechanism for immune homeostasis. A major breakthrough in the under-

Fig. 1. CK1 inhibition affects FOXP3 expression in human T_{reg} cells, suggesting the potential use of small molecules to interfere with T_{reg} cell functions. Human alloantigen-specific T_{reg} cells were pretreated over night with lactose that impairs the lectin binding function of LGALS3 [11], CK1 kinase inhibitor (D4476, TOCRIS bioscience, Bristol, UK), and solvent control (DMSO) or irrelevant kinase inhibitors (PD98059 = MAP kinase inhibitor; SB202190 = p38 kinase inhibitor) and analyzed for intracellular FOXP3 as described [13].

standing of this peculiar subpopulation of CD4⁺ T cells was the identification of the forkhead transcription factor FOXP3 that is essential for their development and function [2–4]. The roles of FOXP3 and T_{reg} cells are compellingly demonstrated by the natural loss-of-function, leading to the development of a fatal autoimmune lymphoproliferative disorder in man (IPEX, immunodysregulation, polyendocrinopathy, and enteropathy, X-linked) and mice (scurfy) [5]. Despite advances since the identification of FOXP3 for understanding T_{reg} cell lineage, commitment and function differences between mice and man have become apparent [6]. Contrary to murine T_{reg} cells, which are expressing FOXP3 as lineage-specific marker, consensus has now been reached that T cell receptor (TCR) stimulation of human nonregulatory CD4⁺ CD25[−] T_h cells does induce FOXP3 expression without interfering with the expression of effector cytokines like IL2 and IFN γ or the acquisition of suppressor function [7–10]. Moreover, even antigen-specific T_h lines and clones can express FOXP3 to different extents [11–15]. With that, FOXP3 does not represent a bona fide marker of human T_{reg} cells, suggesting the existence of a T_{reg} cell-specific marker and/or higher-order regulatory networks to explain the qualitative and quantitative differences of FOXP3 expression of human T_{reg} compared to conventional T_h cells [16, 17].

GARP Controls FOXP3 via a Positive Feedback Loop

The question of T_{reg} cell-specific control mechanisms has been answered only recently with the identification of the orphan receptor glycoprotein-A repetitions predominant (GARP or LRRC32). GARP does represent a T_{reg} cell-specific molecule that dominantly controls FOXP3 via a positive feedback loop (see below) [13, 18, 19]. T_{reg} cell-specific expression of GARP has been further confirmed at the DNA level with the identification of a hypomethylated region in intron 1 together with two differentially methylated regions with enhancer function in human T_{reg} cells [20]. Thus, lineage-directing transcription

of GARP in T_{reg} cells is based most likely on differential accessibility of the locus similar to what has been described for FOXP3 [15, 21].

The identification of GARP was enabled by differential gene expression analysis of TCR stimulated CD4⁺ CD25⁺ T_{reg} cells compared to their CD4⁺ CD25[−] T_h cell counterparts (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13017) as TCR stimulation represents the prerequisite to activate their mutually exclusive functions. The functional contribution of GARP to the genetic program and phenotype of T_{reg} cells was elucidated by ectopic overexpression in human alloantigen-specific T_h cells and downregulation of GARP in human alloantigen-specific T_{reg} cells. Retroviral overexpression of GARP in T_h cells led to an efficient and stable re-programming of effector T towards T_{reg} cells. This was associated with induction of constitutive expression of FOXP3, the β -galactoside binding protein LGALS3, the cysteine-endoprotease LGMN, and an extended T_{reg} cell signature similar to T_{reg} cells [13]. Interestingly, LGALS3, recently identified FOXP3-dependent gene [11], and LGMN do represent minor constituents of this feedback loop as they enhance GARP and FOXP3 expression following TCR activation. LGALS represents an interesting regulator of anergy as it has been shown to directly interfere with TCR proximal signaling in human T cells [22]. Furthermore, FOXP3 induction via ectopic expression of LGALS3 depends on an intact serine-6 CK1 phosphorylation site [13], whereas kinase inhibition of CK1 in T_{reg} cells downregulates FOXP3 expression (fig. 1). The result of the GARP-dependent re-programming process is established only after some rounds of antigen-specific TCR-(re-)stimulation. Surprisingly, GARP was more efficient, and the regulatory phenotype induced was stable compared to the overexpression of FOXP3. The final outcome of this complex transcriptional re-programming/transdifferentiation of effector T_h cells towards T_{reg} cells was obvious as most of the T_{reg} cell signature genes showed a sustained high level of expression and induction of effector cytokines was lost [13, 18].

Fig. 2. GARP as safeguard of the regulatory phenotype. Qualitative and quantitative differences of FOXP3 expression between T_{reg} (upper panel) and T_h cells (lower panel) with their mutually exclusive effector and regulatory functions can be explained in part by the existence of T_{reg} -specific control mechanisms. First of all, FOXP3 is expressed ab initio in T_{reg} cells, because of difference in the accessibility of the FOXP3 locus [15, 21], whereas it is induced with delay in T_h cells following TCR stimulation. Lineage-restricted induction of GARP due also to selective hypomethylation of the locus in T_{reg} cells [20] (indicated by lack of black dots) initiates a positive feedback loop that enhances and maintains high levels of FOXP3. This regulatory network is associated with redundant GARP/FOXP3-enhancing molecules, e.g. the β -galactoside-binding protein LGALS3, which also reveals lineage-specific differences of DNA methylation [20], the endoprotease LGMN, receptor CD27 and CD83 suggesting a higher-order regulatory network [13].

Contrary, downregulation of GARP in human T_{reg} cells, achieved by lentiviral transduction of specific small interfering RNA (siRNA), significantly impaired suppressor function and FOXP3 expression. This was associated with impaired induction of CD83 and CD27, both representing known FOXP3-regulating receptors [13, 23, 24]. Strikingly, lentiviral downregulation of FOXP3 with specific siRNA in T_{reg} cells resulted in similar phenotypic changes and affected induction of GARP, CD83, and CD27. Therefore, a GARP-FOXP3-positive feedback loop in human T_{reg} cells and a close interrelation with other FOXP3-regulating cell surface receptor systems was compellingly established. With that, speculations on a higher-order regulatory network and the necessity on a new molecular definition of human T_{reg} cells [16, 17] reached a robust conceptual framework (fig. 2). Moreover, as TGF β -induced T_{reg} cells do not upregulate GARP, lack of GARP might explain the only transient and partial phenotype of TGF β 1-induced regulatory T_h cells [10], corroborating the need for a safeguard of FOXP3 expression and maintenance

of the regulatory program. In sum, GARP is a key receptor controlling FOXP3 in T_{reg} cells following TCR activation in a positive feedback loop, representing a promising new system for the therapeutic manipulation of T cells in human immune diseases (fig. 3).

Future Directions of T_{reg} Cell Therapies in View of GARP

Because of the encouraging results of adoptive transfer showing an essential role of CD4 $^+$ CD25 $^+$ FOXP3 $^+$ T_{reg} cells in prevention and treatment of autoimmune [25–27] and graft-versus-host disease (GvHD) [28–30] in animal models, first clinical trials have been started with CD4 $^+$ CD25 $^+$ T cells in humans [31, 32]. These trials should also provide insights into the potential effects of T_{reg} cell therapies on the graft-versus-leukemia effect, which is linked in part to GvHD [33]. But keeping in mind the difficulties to achieve pure T_{reg} cell

Fig. 3. Adoptive T_{reg} cell therapy – perspectives. Based on the isolation of either CD4⁺ CD25^{int}/+ T_h cells or CD4⁺ CD25^{hi} T_{reg} cells, two different strategies exist for adoptive regulatory T cell applications. i) Isolation of antigen-specific CD4⁺ T_h cells from different disease settings (e.g. GvHD or autoimmunity) with the use of ‘classical’ antigen-specific stimulation/cloning procedures or by tetramer/multimer separation [15], and re-programming/transdifferentiation of these disease-associated effector towards T_{reg} cells via genetic engineering with GARP as described for alloantigen-specific T_h cells [13]. ii) Isolation of T_{reg} cells and their preferable antigen-specific expansions with the need for re-isolation to exclude potential harmful antigen-specific T_h cells included in the preparation by means of T_{reg} cell-specific marker(s), including e.g. GARP or the recently described strategy of using CD121 (IL1R), recently identified as FOXP3-dependent molecule [11], and LAP as T_{reg} markers [19, 38, 48] (table 1).

preparations with using CD4⁺ CD25⁺ as surrogate marker of human T_{reg} cells [34], more reliable surface markers to exclude potential hazardous T_h cell contaminations from T_{reg} cell preparations have to be used. Such markers should be lineage-specific and indicators of proper regulatory function. GARP fulfills these demands as lineage-specific T_{reg} marker that is responsible for maintenance of the regulatory program (fig. 3). Concerning the limitations of T_{reg} cell availability and the dominant role of GARP leading to stable FOXP3 expression and regulatory function in terminal differentiated alloantigen-specific T_h cells [13], genetically engineering disease-associated T_h cells would represent an attractive alternative (fig. 3).

Clinical Application of T_{reg} Cells

In order to facilitate the transformation of research-based cell cultures and processes into clinical good manufacturing practice (GMP) procedures which are required for clinical application, a number of hurdles have to be taken. A strict standardization of the production is one of the most important parameters. Minimizing risks of contamination, especially microbial contamination during the cultivation process, is another important prerequisite for clinical application. Therefore, the clinical acceptance will be greatly improved with cell-cultivating systems which are modular closed systems and easy to use.

As we described in the first part of this review, T_{reg} cells represent a critical component of the immune regulatory system. Through availability of specific antibodies and improved understanding of functional and genetic differentiation of T_{reg} cells, we are now able to better define these T cells and standardize their isolation and quality control (fig. 3). An important point still represents the limited quantity of T_{reg} cells, representing a rare subset of CD4⁺ T cells estimated to be 2–4% or even lower, concerning proper gating to sort pure T_{reg} cells [35–37]. Thus, ex vivo expansion is necessary but hindered by the fact that T_{reg} cells replicate slowly in comparison to conventional T_h cells [38]. In other words, if a batch of T_{reg} cells is contaminated with other T_h cells, these will in the end overgrow the T_{reg} cell population, necessitating a re-purification step (fig. 3). This problem has been noticed with most surface markers of human T_{reg} cells (table 1). Therefore, combinations of separate markers for primary enrichment of T_{reg} cells ex vivo (e.g. CD4⁺ CD25⁺ CD127^{lo} [39]) and later re-purification using other markers like GARP or other molecules differentially expressed on T_{reg} cells (table 1) seems to be the future concept to get more pure T_{reg} preparations [38].

In the research situation most of the isolation procedures were performed using FACS sorting, a cell sorting methodology based upon physical properties of the cellular elements and use of fluorescently labelled antibodies against specific cellular membrane receptors [37], which is time consuming. Thus, Riley et al. [31] stated the cumbersome FACS isolation

Table 1. Potential markers of human T_{reg} cells besides GARP

Marker	Comment	Reference
CD25+	not specific; no long-term stability of isolated cells* (if not sorted for CD25 ^{hi} [11]; 60–80% FOXP3+ after MACS isolation [36])	[34]
CD152	ionomycin-stimulation needed, not specific; long-term stability of isolated cells not determined	[49]
MHC-class II	not specific; no long-term stability of isolated cells tested	[50]
CD127 ^{lo}	not specific; no long-term stability of isolated cells* [38]	[51]
CD27+	not specific; long-term stability of isolated cells not determined	[48]
CD39	not specific; long-term stability of isolated cells not determined	[52]
CD121a+ CD121b+	not specific, long-term stability of isolated cells not determined; improved purity of CD25+ CD127 ^{lo} reported (>90%)	[38]
LAP+	not specific, long-term stability of isolated cells not determined; improved purity of CD25+ CD127 ^{lo} reported (>90%); GARP represents receptor of latent TGFβ/LAP [53]	[38]
CD83+	not specific, stimulation needed; long-term stability of isolated cells not determined	[13, 23]
CD33 ^{hi}	not specific, stimulation needed; long-term stability of isolated cells not determined	[13]

MACS = Magnetic-activated cell sorting.
*Outgrowth of nonregulatory T cells.

of this infrequent cell type by the words ‘take a billion or so and call me in the morning’. Yet there is another important point with respect to contamination. FACS sorting is a relative open system with numerous porte d’entrées for microorganisms. This problem gets even greater concerning the need for several separation steps for enrichment and re-purification of antigen-specific T_{reg} cells for cellular therapies, discussed above. In conclusion it will be difficult to obtain sufficient T_{reg} cells under GMP conditions using this approach.

Modern cytopheresis procedures are the basis for harvesting sufficient numbers of mononuclear cells (10^{10} – 10^{11}) within an acceptable amount of time. This is not only possible for healthy donors but can be achieved with patients as well [40–42]. The collected mononuclear cells are harvested under the same conditions as classical blood products such as thrombocytopheresis products and are suitable for further processing in GMP facilities.

Novel immunomagnetic techniques in combination with specific antibodies enable the rapid isolation of cells in cell culture bags. This approach enables isolation and cultivation of a large amount of T_{reg} cells in a GMP setting and therefore facilitates the clinical applicability of these cells [39, 43]. This approach also includes the possibility of ex vivo generation of human alloantigen-specific T_{reg} cells at the step of in vitro expansion by means of antigen-specific stimulation with the use of a primary enrichment and a second re-purification step including GARP and other potential markers of T_{reg} cells (table 1) [39]. Further protocols enable the ex vivo expansion of antigen-specific T_h cells and genetic modifications of these cells [13, 44]. Although attractive, these approaches however are still rarely found in clinical settings.

Other approaches result from the better understanding of the T_{reg} cells and try to modify T_{reg} cells responses pharmacologically, for example by using antibodies, cytokines, or small molecules [45] (fig. 1).

Combining the knowledge we have on T_{reg} cells with efficient medical devices for harvesting (apheresis) and isolation (e.g. clinimacs) we are gradually coming in a position in which we will see clinical applications starting.

Concluding Remarks

The identification of GARP as lineage-restricted key receptor of human T_{reg} cells and the characterization of a GARP-FOXP3 positive feedback loop as essential component for the maintenance of the regulatory phenotype does provide a conceptual framework for a new molecular definition of the regulatory program. Open questions remaining include the signal transduction pathway of GARP and the potential ligand. Development of potential GARP-ligand mimetics to enhance regulatory functions in vivo should take into account expression and function of GARP in platelets [46, 47]. In-depth elucidation of the GARP-FOXP3 regulatory system does represent a major challenge for the future and opens up the possibility for generation of antigen-specific T_{reg} cells for adoptive cellular therapies (fig. 3) and identification of new molecular targets to develop new strategies to enhance T_{reg} cells in autoimmune diseases and transplantation.

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Disclosure

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