FOXP3 Expression in GARP-Transduced Helper T Cells Is Not Associated with *FOXP3 TSDR* Demethylation

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Keywords

Regulatory T cells · FOXP3 · GARP · LRRC32 · TSDR

Summary

Aim: Glycoprotein A repetitions predominant (GARP or LRRC32) represents a human regulatory CD4⁺ CD25^{hi} FOXP3⁺ T (T_{reg}) cell-specific receptor that controls FOXP3. Ectopic expression of GARP in helper T (T_h) cells has been shown to be sufficient for the induction of FOXP3 and generation of a stable regulatory phenotype. Since expression of FOXP3 in T_{reg} cells is epigenetically controlled by a conserved motif, the so-called T_{reg}-specific demethylated region (*TSDR*), we asked whether GARP-mediated upregulation of FOXP3 in T_h cells is similarly accompanied by demethylation of the *TSDR*.

Methods: DNA methylation of the *FOXP3 TSDR* was analyzed by direct sequencing of polymerase chain reaction (PCR) products from bisulfite-treated genomic DNA.

Results: Although GARP-transduced T_h cells exhibit constitutive FOXP3 expression and a regulatory phenotype, the *FOXP3 TSDR* is completely methylated as in naive T_h cells. GARP-mediated FOXP3 upregulation in T_h cells is not associated with T_{reg}-specific demethylation of the *FOXP3 TSDR*. **Conclusion:** Although GARP-engineered T_h cells exhibit stable FOXP3 expression and a phenotypic reprogramming towards T_{reg} cells in vitro, these cells do not completely mimic the epigenotype of natural T_{reg} cells. Thus, concepts based on the genetic modification of T_h cells as cellular therapies to treat autoimmune diseases or to control transplantation tolerance should be critically tested before any clinical application.

Schlüsselwörter

Regulatorische T-Zellen · FOXP3 · GARP · LRRC32 · TSDR

Zusammenfassung

Hintergrund: Glycoprotein A repetitions predominant (GARP, LRRC32) ist ein spezifischer Rezeptor von humanen, regulatorischen CD4⁺ CD25^{hi} FOXP3⁺ T (T_{reg})-Zellen, welcher FOXP3 kontrolliert. So konnte gezeigt werden, dass die ektopische Expression von GARP in T-Helfer (T_h)-Zellen ausreichend für die Induktion von FOXP3 und einem stabilen regulatorischen Phänotyp ist. Da die Expression von FOXP3 in T_{reg}-Zellen epigenetisch durch ein konserviertes genomisches Motiv, die «T_{reg}-spezifische demethylated region» (*TSDR*), kontrolliert wird, stellt sich uns die Frage, ob die GARP-induzierte Expression von FOXP3 in T_h-Zellen ebenfalls von einer Demethylierung der *TSDR* begleitet ist. **Methoden:** Die DNA-Methylierung der *TSDR* wurde durch direkte Sequenzierung von PCR(polymerase chain reaction)-Produkten Bisulfit-behandelter genomischer DNA von GARP-, FOXP3-, und Kontroll-transduzierten T_h-Zellen im Vergleich zu natürlichen T_{reg}-Zellen analysiert. **Ergebnisse:** Obwohl GARP-transduzierte T_h-Zellen eine konstitutive FOXP3-Expression und einen regulatorischen Phänotyp aufweisen, ist die *FOXP3 TSDR* wie in normalen T_h-Zellen komplett methyliert. Die GARP-vermittelte FOXP3-Induktion in T_h-Zellen ist nicht mit einer T_{reg}-spezifischen Demethylierung der *FOXP3 TSDR* verbunden. **Schlussfolgerung:** Obwohl durch GARP-veränderte T_h-Zellen in vitro eine stabile FOXP3-Expression mit einer phänotypischen Reprogrammierung zu T_{reg}-Zellen aufweisen, wird in diesen Zellen nicht der komplette Epigenotyp von natürlichen T_{reg}-Zellen erreicht. Aus diesem Grund sollten Therapiekonzepte basierend auf der genetischen Modifikation von T_h-Zellen zur Behandlung von Autoimmunerkrankungen oder der Kontrolle einer Transplantationstoleranz einer kritischen Untersuchung vor der klinischen Anwendung unterliegen.

Introduction

The forkhead box protein P3 (FOXP3) is a transcription factor essential for the development and function of CD4⁺ CD25^{hi} regulatory T (T_{reg}) cells. FOXP3⁺ T_{reg} cells are an essential component for the maintenance of tolerance in transplantation and autoimmunity. Thus, isolation and expansion of natural T_{reg} cells or their design via molecular engineering of antigen-specific T cells for regulatory T cell therapies represent a major focus for adoptive T cell transfer concepts in different disease settings [1, 2]. The molecular mechanism maintaining stable FOXP3 expression in T_{reg} cells has been elucidated recently by the identification of an evolutionarily conserved region with CpG motifs within the non-coding part of the gene. This genomic region is completely unmethylated in T_{reg} cells but methylated in naive and effector T cells and thus has been designated the T_{reg}-specific demethylated region (*TSDR*) [3–6]. The significance of this epigenetic marker is obvious, because the methylation status of the *FOXP3* gene is an even better marker of suppressor function than the differential expression of FOXP3 at the mRNA or protein level [7, 8]. Moreover, 4 CpG motifs within the *TSDR* have been identified as binding sites for essential transcription factors, such as CREB/ATF, NF-κB, and Ets-1. Ets-1 binds only to the unmethylated *TSDR*, a finding suggesting that restricted binding of a transcription factor complex controls stable FOXP3 expression in T_{reg} cells [9]. Therefore, the *TSDR* is an essential control region and marker of natural T_{reg} cells.

Further insights into T_{reg}-specific control mechanisms involved in the maintenance of sustained high levels of FOXP3 have been recently gained with the characterization of glycoprotein A repetitions predominant (GARP or LRRC32) [10–14] (reviewed in [2]). GARP is a T_{reg}-specific protein that is induced after T-cell receptor (TCR) activation and controls FOXP3 via a positive feedback loop [10, 12, 15]. Moreover, GARP functions as a cell surface receptor for latency-associated peptide (LAP)/latent transforming growth factor β (TGF-β) [13, 14] (reviewed in [16]). T_{reg}-specific expression of GARP has been corroborated by the identification of a genomic region in intron 1 of GARP with a T_{reg} cell-specific hypomethylation [17]. This finding suggests lineage-specific transcription of GARP in T_{reg} cells similar to FOXP3. More importantly, retroviral overexpression of GARP in helper T (T_h) cells leads to an efficient and stable reprogramming of effector T cells into T_{reg} cells, and this reprogramming is associated with the induction of the constitutive expression of FOXP3 and a regulatory phenotype [10, 12].

In the murine system, partial demethylation of the *FOXP3* locus has been observed in CD4⁺ CD25[–] FoxP3[–] T cells activated in the presence of TGF-β. This partial demethylation is associated with the upregulation of FoxP3, a finding suggesting that the epigenetic control of murine FoxP3 is mediated by external stimuli that manipulate T cell fate [3].

Recently it has been demonstrated that peroxisome proliferator-activated receptor α (PPARα) and PPARγ agonists, in combination with TGF-β, induce demethylation of the *FOXP3* locus in human peripheral CD4⁺ CD25[–] T_h cells through a downregulation of DNA methyltransferases. This demethylation leads to the stable expression of FOXP3, which results in the generation of functional induced T_{reg} cells [18].

Since GARP represents a potential tool to engineer disease-associated T_h cells to generate FOXP3-expressing regulatory T cells for regulatory T cell therapies [2], the critical risk-benefit assessment for such an approach remains whether stable regulatory functions of GARP-engineered T_h cells observed in vitro would persist after adoptive T cell transfer in vivo. Based on the clear association of *TSDR* demethylation, which permits constitutive expression of FOXP3 and stable regulatory function of natural T_{reg} cells, our hypothesis was that analysis of *TSDR* would enable such a risk-benefit assessment. For this reason we analyzed *TSDR*-methylation of GARP-transduced T_h cells, characterized by T_{reg}-like FOXP3 expression and stable regulatory function and FOXP3-transduced T_h cells with T_{reg}-like FOXP3 expression but only partial regulatory function compared to control-transduced T_h cells and natural T_{reg} cells. In the case of T_{reg}-like *TSDR* epigenotype in GARP-transduced T_h cells, the hypothesis of having guaranteed stable regulatory function of such cells in vivo would have been confirmed.

Material and Methods

T Cells

The T cells analyzed have recently been extensively described and characterized [12, 15, 19, 20]. In brief, CD4⁺ CD25[–] T_h cells and CD4⁺ CD25^{hi} T_{reg} cells were sorted from a healthy donor and stimulated weekly with the allogeneic Epstein-Barr virus (EBV) B-cell line LG2 EBV to generate alloreactive T_h cells (CD4–39) and T_{reg} cells (T_{reg}HG and T_{reg}THU) [19]. T_h cells were transduced with a retrovirus expressing either GARP or FOXP3 coupled with an IRES-driven green fluorescent protein (GFP) (T_hGARP and T_hFOXP3, respectively) and the empty IRES-GFP vector (T_hGFP). Transduced cells were sorted for GFP expression, cultured further in the same manner, and extensively characterized by functional, transcriptional, and protein expression analyses, as previously described [12, 15].

Immunostaining

For cell surface immunostaining, PE- and APC-conjugated monoclonal antibodies (mAbs) against CD25, CD27, CD83, and CD33 with respective isotype-control Abs were used (all from BD Bioscience, San Jose, CA, USA). For intracellular immunostaining, Alexa Fluor 467-conjugated mAb against FOXP3 (206D) and respective isotype control (MOPC; BioLegend Inc., San Diego, CA, USA) were used according to the manufacturer's instructions.

DNA Isolation and Methylation Analysis

DNA isolation was performed with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Bisulfite modification of DNA was performed according to an established protocol with minor modifications [21]. Genomic DNA (1–2 μg in 50 μl) was denatured for 15 min at 37 °C by adding 5.5 μl of 3 mol/l sodium hydroxide

A

(NaOH). For complete denaturation, samples were incubated at 95 °C for 2 min and immediately cooled on ice. The bisulfite solution was freshly prepared by dissolving 4.25 g of sodium bisulfite (Sigma-Aldrich, St. Louis, MO, USA) in 7.5 ml H₂O. Next, 450 µl of 50 mmol/l hydroquinone solution was added, and the pH was adjusted to 5.15 by the addition of 500 µl of 10 mol/l NaOH. The denatured DNA solution was mixed with 1 ml of the bisulfite solution and incubated in the dark at 50 °C for 16–20 h. The DNA was recovered by using the Wizard DNA Clean-Up System (Promega Corp., Madison, WI, USA) followed by elution in 50 µl H₂O (65 °C). Subsequently, 5.5 µl of 3 mol/l NaOH was added, and the samples were incubated for 15 min at 37 °C. The solution was then neutralized by adding 55 µl of 6 mol/l ammonium acetate (NH₄OAc), pH 7.0. The DNA was precipitated with ethanol, washed in 70% ethanol, dried, and resuspended in 15 to 30 µl of water, depending on the initial DNA input. The regions of interest were analyzed by polymerase chain reaction (PCR) using primers that specifically amplify bisulfite-treated DNA of the *TSDR* of *FOXP3* (Foxp3Amp5fw: CTT GCT TCC TGG CAC GAG TGT TTG GGG GTA GAG GAT TT and Foxp3Amp5rev: TGT AAA ACG ACG GCC AGT TAT CAC CCC ACC TAA ACC AA, modified from Baron et al. [22]). The later direct sequence analysis of PCR products was facilitated by the use of PCR tag primers containing additional nucleotides at the 5' end that do not bind to the template and thus compensate for the missing base in PCR products from unmethylated samples during sequence analysis. PCR was performed with a GeneAmp 9700 system (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µl containing 3 µl of

B

Fig. 1. A Fluorescence-activated cell sorting (FACS) analysis of alloantigen-specific T_h cells ectopically expressing GARP, FOXP3, or control GFP alone after retroviral transduction, as recently described [12]. Graphs are representative of intracellular expression of FOXP3 and cell surface expression of CD25, CD83, CD33, and CD27 after stimulation with mAbs against CD3/CD28 and interleukin 2 (IL-2) for 3 days. **B** Methylation status of the same cells as in A of the complete *TSDR* of *FOXP3* was analyzed. Shown is a representative part of the sequence analysis of bisulfite-treated DNA of the *TSDR*. CpG positions are highlighted with grey boxes. The G peak represents the methylated C, and the A peak represents the unmethylated C within the CpG. All CpGs are completely methylated in FOXP3- and GARP-transduced T_h cells but are largely unmethylated in the male T_{reg} (T_{reg}HG) cells analyzed.

bisulfite-treated DNA, 0.2 mmol/l of each dNTP, 0.8 µmol/l of each primer, 2.5 µl of 10 × PCR buffer, 2.5 mmol/l magnesium chloride (MgCl₂), and 0.5 U Taq Polymerase (AmpliTaQ Gold, Applied Biosystems). A touchdown protocol was adopted as follows [23]: after denaturation at 95 °C for 5 min, the annealing temperature was decreased by 0.5 °C every cycle from 63 °C to 56 °C, at which temperature 35 cycles were carried out. For all cycles, annealing was performed for 1 min, denaturation at 95 °C for 20 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. After agarose gel electrophoresis and purification of the PCR products with the MinElute™ Gel Extraction Kit (Qiagen), sequence analysis was performed with an ABI PRISM 3100 automated capillary genetic analyzer (Applied Biosystems, Carlsbad, CA, USA) using Big Dye 1.1 (ABI, Applied Biosystems) and a primer binding to the tag sequence. The sequence files were further analyzed with Geneious Pro 5.3.4. A cytosine was classified as fully methylated if the A signal was absent or very low at scattered positions, as unmethylated in the absence of a G signal, and as partially methylated if both signals were present.

Results and Discussion

We analyzed the methylation status of the *FOXP3 TSDR* in alloreactive T_h cells that had been transduced with GARP (T_hGARP) or FOXP3 (T_hFOXP3) coupled to an IRES-driven GFP or GFP alone (T_hGFP) [12]. These cells differ in many aspects, as described recently [12]. In brief, T_hGARP cells show T_{reg}-like sustained FOXP3 expression (fig. 1A), an extended T_{reg}-mRNA signature, and T_{reg}-specific function (reviewed in [15]). In contrast, T_h cells transduced with GFP alone exhibited an effector T cell phenotype and limited FOXP3 expression. T_hFOXP3 cells lie somewhere between these 2 extreme phenotypes, because they exhibit high levels of FOXP3 expression and an extended T_{reg}-mRNA signature but also unstable or minor regulatory function.

The methylation status of all CpGs of *FOXP3 TSDR* was analyzed in T_hGARP, T_hFOXP3, and T_hGFP cells and revealed a fully methylated *TSDR* in all cases (fig. 1 B). In contrast, T_{reg} cells showed a typical largely unmethylated *TSDR* (fig. 1 B). Thus, neither the stable expression of FOXP3 nor the regulatory function of GARP-transduced T_h cells in vitro depends on an unmethylated T_{reg}-like *FOXP3 TSDR* despite a phenotypic transdifferentiation towards T_{reg} cells. Because GARP transduction does not influence the epigenotype at the *FOXP3 TSDR*, which is regarded as a specific and reliable marker of natural T_{reg} cells and their suppressor function [8], GARP-transduced T_h cells do not perfectly mimic T_{reg} cells, and thus our hypothesis that a *TSDR* epigenotype of GARP-transduced T_h cells might guarantee their stable regulatory function in vivo has to be rejected.

Interestingly, in transdifferentiation studies of pluripotency, incomplete demethylation of genes has been shown to be characteristic of incompletely reprogrammed cells [24]. Therefore, *FOXP3 TSDR* methylation may be regarded as a marker of incomplete transdifferentiation of T_hGARP cells. Consequently, concepts of GARP [10, 12] or FOXP3 transduction [25] of T_h cells for cellular therapies to treat autoimmune diseases or control transplantation tolerance [2] should be critically tested before any clinical application to ensure stable regulatory function before adoptive transfer of such engineered T cells and to exclude potential hazardous reversion of a potentially disease-controlling regulatory to-

wards a diseases-aggravating effector phenotype. Despite the encouraging preclinical results in many mouse models using either FOXP3 itself [26, 27] or FOXP3-regulating genes like GPR83 [28, 29] and CD83 [30] to generate regulatory T cells for adoptive transfer, based on the known differences between FOXP3 expression of T_{reg} cells in mice and man [31], critical risk-benefit assessment of such cellular therapies to ensure safe clinical application is of utmost importance.

In summary, ectopic expression of GARP in T_h cells can induce sustained FOXP3 expression and a stable regulatory phenotype in vitro similar to that of T_{reg} cells. However, this phenotypic transformation is not reflected by a T_{reg}-specific demethylation of the *TSDR* raising serious questions about the stability of such engineered T cells for cellular therapies to treat autoimmune diseases or control transplantation tolerance in vivo.

Acknowledgments

This work was supported in part by grants from the Mercator-Stiftung.

Disclosure Statement

The authors declared no conflict of interest.

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