GARP: a key receptor controlling FOXP3 in human regulatory T cells

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Abstract

Recent evidence suggests that regulatory pathways might control sustained high levels of FOXP3 in regulatory CD4+CD25hi T (Treg) cells. Based on transcriptional profiling of ex vivo activated Treg and helper CD4+CD25− T (Th) cells we have identified GARP (glycoprotein-A repetitions predominant), LGALS3 (lectin, galactoside-binding, soluble, 3) and LGMN (legumain) as novel genes implicated in human Treg cell function, which are induced upon T-cell receptor stimulation. Retroviral overexpression of GARP in antigen-specific Th cells leads to an efficient and stable re-programming of an effector T cell towards a regulatory T cell, which involves up-regulation of FOXP3, LGALS3, LGMN and other Treg-associated markers. In contrast, overexpression of LGALS3 and LGMN enhance FOXP3 and GARP expression, but only partially induced a regulatory phenotype. Lentiviral down-regulation of GARP in Treg cells significantly impaired the suppressor function and was associated with down-regulation of FOXP3. Moreover, down-regulation of FOXP3 resulted in similar phenotypic changes and down-regulation of GARP. This provides compelling evidence for a GARP-FOXP3 positive feedback loop and provides a rational molecular basis for the known difference between natural and transforming growth factor-β induced Treg cells as we show here that the latter do not up-regulate GARP. In summary, we have identified GARP as a key receptor controlling FOXP3 in Treg cells following T-cell activation in a positive feedback loop assisted by LGALS3 and LGMN, which represents a promising new system for the therapeutic manipulation of T cells in human disease.

Keywords: positive feedback loop • regulatory circuit • FOXP3

Introduction

Naturally occurring regulatory CD4+CD25hi T (Treg) cells are able to actively suppress immune responses and play an essential role in the homeostasis of immune reactions and tolerance [1]. Treg cell development and function crucially depend on the transcription factor FOXP3. This is compellingly illustrated by the fact that lack of FOXP3 leads to the development of fatal autoimmune
lymphoproliferative disorder in mouse and man [1]. Consequently, FOXP3-deficient recombinant mice or the natural mutant scurfy exhibit an analogous immune pathology due to a lack of regulatory T cells that are able to actively suppress immune responses against self antigens [1–3]. In agreement, FOXP3 expression is found in Treg cells with the onset of their development in thymus.

FOXP3, a member of the winged-helix/forkhead transcription factors, has been well characterized as transcriptional repressor of effector cytokines like interleukin-2 (IL-2) [4]. This suppressor function depends not only on direct promoter occupancy but also on interactions with other proteins, including histone acetyltransferase and class II histone deacetylase [5], runt-related transcription factors, has been well characterized as transcriptional repressor of development in thymus.

For transwell experiments Th cells were stimulated in 96 flat-bottom plates out IL-2 in 96 flat-bottom microtiter plates (Nunc, Wiesbaden, Germany). Human transforming growth factor-β (TGF-β) (R&D Systems, Inc., Minneapolis, MN, USA) was used at 10 ng/ml.

**Antibodies (Abs) and immunization**

For immunostaining PE-, FITC-, APC- and CyChrom-conjugated mAbs against CD4 (RPA-T4), CD8 (53–6.7), CD25 (M-A251), CTLA4 (BNI3), CD83, CD33, lectin, galactoside-binding, soluble, 3 (LGALS3) (B2C10; all from BD Bioscience, San Jose, CA, USA), PE-conjugated and ALEXA-Fluor467-conjugated mAb against FOXP3 (206D) and respective isotype controls (MOPC; BioLegend, Inc., San Diego, CA, USA) were used according to the manufacturer's instructions. Anti-CD3ε (TR66, produced from hybridoma supernatants; and HIT3a, BD) and anti-CD28 (CD28.2, BD) were used for T-cell stimulation. Monoclonal antibodies against human glycoprotein-A repetitions predominant (GARP) were generated according to standard protocols (www.sysy.com/mabservice.html). Briefly, His-tagged human GARP ectodomain (pos. 23 to 612) was bacterially expressed in E. coli, purified by affinity chromatography and used for immunization of three to 8 weeks old Balb/c females. Draining lymph node cells were isolated and fused with the mouse myeloma cell line P3 × 63Ag.653 (ATCC CRL-1580). Clones used in this study, mAb 272G6 and 50G10, were cloned two times by limiting dilution. Similarly, an epitope specific mAb 272G6 was raised by immunization against a synthetic GARP peptide (position 296–308: GWSALPLSAPSGN, kindly provided by Dr. W. Tege, HZI). For cell surface detection of GARP mAb 272G6, a goat antimouse Ig-Biotin, and streptavidin-PE (both Southern Biotechnology Associates, Inc., Birmingham, AL, USA) were used. Loading control was performed with anti-tubulin mAb 3A2 (Savntic Systems, Göettingen, Germany) or Comassie staining of the blot. Western blot detection of legumain (LGMN) was detected using anti-LGMN mAb as described (clone 6E3; provided by C. Watts, Department of Biochemistry, University of Dundee, UK) [17].

**Materials and methods**

**Cell isolation**

Treg cell line derived from sorted CD4⁺CD25hi Treg cells and alloantigen-specific Th cells from peripheral blood of healthy donors used in this study have been characterized and described previously [15]. Thymic tissue was obtained from otherwise healthy children undergoing cardiac surgery (n = 7, age range 4 days to 3 years) and freshly processed. Thymocytes were isolated as described recently [16]. In brief, thymocytes were released by mechanical homogenization and CD4⁺CD8⁻CD25⁺, CD4⁺CD8⁺CD25⁻, CD4⁺CD8⁻CD25⁻, CD4⁺CD8⁻CD25⁻ T-cell subsets were separated by magnetic beads or on a FACSaria (Becton Dickinson, San Jose, CA, USA). The study was approved by the ethical committee of the Helsinki University Hospital. Informed consent was obtained from the parents of the children.

**Culture medium**

IMDM, supplemented with 10% foetal calf serum, 100 U/ml penicillin/streptomycin, non-essential amino acids and 2 mM glutamine (PAA Laboratories, Linz, Austria) was used for established T-cell cultures. Stimulation of human freshly sorted CD4⁺CD25hi and CD4⁺CD25⁻ T cells for array analysis was done using IMDM, supplemented with 1% heat-inactivated human serum, 50 μg/ml gentamicin and 2 mM glutamine (PAA Laboratories). Recombinant human IL-2 was used at indicated concentrations between 5 and 100 U/ml. Human transforming growth factor-β (TGF-β) (R&D Systems, Inc., Minneapolis, MN, USA) was used at 10 ng/ml.

**T-cell functional assays**

T-cell proliferation and suppressor activity were assessed by stimulating 3 × 10⁵ T cells in triplicates with irradiated L92-EBV B cells with or without IL-2 in 96 flat-bottom microtiter plates (Nunc, Wiesbaden, Germany). For transwell experiments Treg cells were stimulated in 96 flat-bottom plates separated by 0.2-μm-pore transwell inserts (Greiner bio-one, Frickenhausen, Germany) from the T cells above the transwell. Cells were pulsed with 1 μCi/well of [³H]-thymidine after 72 hrs for the final 16 hrs.

**Retroviral transduction of human alloantigen-specific Th cells**

The cDNA encoding human GARP was amplified from plasmid cDNA (kindly provide by Dr. Birnbaum [18]), human LGMN and LGALS3 were
amplified from human T<sub>reg</sub> cell cDNA using high fidelity PFU polymerase (Promega, Madison, WI, USA) and specific primers (see supplemental Experimental Procedures). PCR products were cloned into pCR4.1 TOPO (Invitrogen, Carlsbad, CA, USA), sequenced and inserted into a pMSCV-based retroviral vector encoding an enhanced green fluorescent protein (GFP) under the control of an IRES sequence. The FOXP3 construct as well as production of retroviral supernatants and T-cell transduction have been described recently [15]. Successfully transduced T cells (GFP<sup>+</sup>) were sorted and expanded in culture by antigen-specific re-stimulation using irradiated LG2-EBV B cells and IL-2 for several months as described [15]; in general, after sorting up to four rounds of re-stimulations were needed to expand sufficient numbers of transduced cells until first functional and phenotypic testing were done.

Lentiviral down-regulation of GARP and FOXP3 in human T<sub>reg</sub> cells

GARP and FOXP3 specific targeting sequences (siGARP<sup>5</sup>, 5'-GCC ATA CCC TCT CAG T-3'; siFOXP3<sup>6</sup>, 5'-GCC TGC ATA CCC TCT CAG T-3') were cloned into pSuper as recommended by the manufacturer (OligoEngine, Seattle, WA, USA), Digestion with Sma I and Hinc II released an H1-promoter driven small-interfering RNA (siRNA) cassette, which was cloned into SnB I site of plasmid pHR-SIN-S carrying GFP as selection marker; an irrelevant control siRNA (siGL4) served as control (kindly provided by Dr. M. Scherr, Department Hematology and Oncology, Hannover Medical School, Hannover, Germany) [19]. VSV.G-pseudo-typed lentiviral particles were generated by calcium-phosphate co-transfection of 293T cells. T<sub>reg</sub> cells were infected by spin-infection with lentiviral supernatants and 8 µg/ml polybrene. Successfully transduced T<sub>reg</sub> cells (GFP<sup>+</sup>) were sorted and kept in culture by antigen-specific re-stimulation using irradiated LG2-EBV B cells and IL-2.

GARP structural analysis

Described in detail in supplemental Experimental Procedures.

GeneChip assays and microarray data analysis

Transcriptome analysis of freshly isolated and anti-CD3/IL-2 or anti-CD3/anti-CD28/IL-2-stimulated CD4<sup>+</sup> CD25<sup>hi</sup>-derived T<sub>reg</sub> cells and CD4<sup>+</sup> CD25<sup>-</sup> T cells of individual donors were analysed following RNA amplification as described recently [8] using human genome U133 A and B arrays (Affymetrix, Santa Clara, CA, USA). Selection criteria for differentially expressed genes were: (1) signal fold-change of more than twofold or (2) similar signal change as being either similarly increased (I, including marginal increased, MI) or decreased (D, including marginal decreased, MD) in anti-CD3/IL-2 and anti-CD3/anti-CD28/IL-2 stimulated CD4<sup>+</sup> CD25<sup>hi</sup>- T<sub>reg</sub> cells compared to their respective controls according to MAS 5.0 software algorithms (www.affymetrix.com/support/technical/manuals.affx: Microarray Suite User’s Guide, Version 5.0). Differentially expressed genes of T<sub>reg</sub> cells, and GARP-, FOXP3- or GFP-transduced T<sub>reg</sub> cells were analysed on U133 PLUS 2.0 arrays after stimulation of the cells for 3 days with anti-CD3/IL-2. All data were compared to T<sub>reg</sub>GFP cells. Analysis of microarray data was performed with the Affymetrix GCOS 1.2 software (MAS v5 algorithm, Affymetrix). For normalization all array experiments were scaled to a target intensity of 150, otherwise using the default values of GCOS 1.2. Data selection criteria for cluster analysis were: (1) signal intensities were transformed into log2 values and differences between signal log2 intensities (signal log2 ratio, SLR) for a specific ProbeSet should be equal to or exceed at least once the value 2 and (2) the signal intensity should exceed at least once 100. The next cycle of selection excludes genes were their distance between maximal and minimal SLR is below two. K-means clustering was used to group similar expression changes based on SLR together.

Accession code

Microarray data have been deposited at Geo database (GSE13017 and GSE13234).

Results

GARP, an early-induced gene of human CD4<sup>+</sup> CD25<sup>hi</sup> T<sub>reg</sub> cells

We isolated CD4<sup>+</sup> CD25<sup>hi</sup> T<sub>reg</sub> cells and CD4<sup>+</sup> CD25<sup>-</sup> Th cells from human peripheral blood of healthy donors by cell sorting. Isolated T cells were either directly analysed or stimulated for 1 day with anti-CD3/IL-2 or anti-CD3/anti-CD28/IL-2 before gene expression profiling. With this approach we identified the orphan receptor GARP (also known as LRRC32) as gene exclusively induced in CD4<sup>+</sup> CD25<sup>hi</sup> T<sub>reg</sub> cells upon stimulation (Fig. 1A, Table S1). To confirm these results CD4<sup>+</sup> CD25<sup>hi</sup> T<sub>reg</sub> cells and CD4<sup>+</sup> CD25<sup>-</sup> Th cells were isolated from independent donors and analysed directly, 12, 48 hrs and more than 1 week after stimulation with anti-CD3/anti-CD28/IL-2 by quantitative real-time RT-PCR. This analysis revealed the exclusive induction of GARP in CD4<sup>+</sup> CD25<sup>hi</sup> T<sub>reg</sub> cells over 12 to 48 hrs following TCR stimulation and sustained mRNA expression thereafter, accompanied by up-regulation of FOXP3 (Fig. 1B). These GARP<sup>+</sup> T<sub>reg</sub> cells were functionally confirmed to be suppressor cells (Fig. 1C).

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reconstruction together with a ribbon representation of the model of human GARP. The asparagine residues of putative glycosylation sites are indicated as space-filling spheres coloured according to atom type (carbon light brown, oxygen red, nitrogen blue). (F) Western blot analysis of alloantigen-specific T<sub>reg</sub> and T<sub>H</sub> cells without or with retroviral overexpression of GARP, FOXP3, LGMN, LGALS3, and GFP under resting and activated conditions using anti-CD3/IL-2 stimulation for 3 days using anti-GARP specific mAb 50G10; anti-Tubulin served as loading control. (G) Detection of cell surface expression of wt GARP and mutant GARP<sup>S/D2</sup> expressed in 293 cells (left panel) using mAb 272G6 compared to WB detection in the same cells (insert) using mAb 50G10. (H) T<sub>reg</sub> and T<sub>H</sub> cells as in (F) were treated for 4 hrs with PMA (40 ng/ml) and ionomycin (0.5 µg/ml) to induce up-regulation of the early-induced gene CD83 (lower panel) and tested for surface expression of GARP (upper panel).
GARP represents a leucine-rich repeat receptor of 662 amino acids. The extracellular portion of GARP is almost entirely composed of leucine-rich repeats [18] with high homology to the ectodomain of Toll-like receptor (TLR)-3 (Fig. S1A). The structure of the ectodomain of TLR3 is a horseshoe-shaped solenoid, largely masked by carbohydrate. One face of TLR3 remains glycosylation free, suggesting a potential role in ligand binding and oligomerization [20]. Interestingly, modelling of GARP using the structural coordinates of TLR3 reveals that three of five potential glycosylation sites of GARP are positioned on the concave face, similar to TLR3. Based on this simulation, a glycosylation-free area and two prominent loops at residues 296–308 and 421–432 are predicted in GARP as potential ligand binding and oligomerization sites (Fig. S1B) [20]. We used electron microscopy and 3D image reconstruction to confirm the horseshoe-shaped appearance of GARP (Fig. 1D and E). Whereas TLRs share a cytoplasmic Toll/IL-1 receptor domain, GARP does not contain such a well-defined signalling domain within its 14 aa short cytoplasmic tail. However, the four C-terminal amino acids Gln-Tyr-Lys-Ala of GARP exhibit homology to the PDZ (post-synaptic density protein-95, post-synaptic disc large and zona occludens-1) class II binding motif, a modular interaction domain [21].

To confirm expression of GARP protein in human activated Treg cells, we immunized mice with bacterially expressed human GARP protein or a synthetic peptide GARP296-308, respectively, to generate anti-GARP specific mAbs. Immunoblot analysis using anti-GARP mAb 50G10 revealed GARP protein expression in activated Treg cells but not in alloantigen-specific Th cells (Fig. 1F). But unlike cell surface expression in epithelial 293 cells transfected with GARP using with the epitope specific anti-GARP mAb 272G6 (Fig. 1G), detection on Jurkat T cell transduced with GARP nor on Treg cells was detected (data not shown). Even stimulation using PMA/ionomycin for 4 hrs, effectively up-regulating the early-induced gene CD83 that is differentially induced in Treg versus Th cells [22], did not let to detectable surface expression of GARP using mAb 272G6 on Treg cells. Because mAb 272G6 also did not detect surface expression on human platelets, which have been reported recently to have GARP surface expression [23], the respective GARP epitope recognized by mAb 272G6 might in some way be mask at least on human T cells and platelets. Moreover, the amount surface expression of GARP in 293 cells depended on the presence of an intact C-terminal PDZ domain, because mutation of the C-terminal Ala towards a Ser impaired protein expression of GARP (Fig. 1G).

Thus, GARP represents an orphan TLR specifically induced in human CD4+CD25hi-derived Treg cells following TCR activation suggesting a potential contribution of GARP to the regulatory phenotype.

**Ectopic expression of GARP in human antigen-specific Th cells confers sustained expression of FOXP3**

To investigate the potential contribution of GARP to the regulatory phenotype, we used a retroviral overexpression system in a human alloantigen-specific effector Th cells with a construct that included enhanced GFP as marker linked by an internal ribosome entry site to the gene of interest as described recently [15]. Successfully transduced Treg cells (ThGARP) were isolated according to their expression of GFP by cell-sorting and expanded in culture by antigen-specific re-stimulation (as described in ‘Materials and methods’). The same alloantigen-specific Th cells retrovirally transduced in parallel with GFP alone (ThGFP) served as negative control. Transduction with FOXP3 (T~h~ FOXP3) and an established and well-characterized Treg cell line [15] were included for comparison. Immunoblot analysis confirmed the ectopic expression of GARP in ThGARP cells (Fig. 1E).

Flow-cytometry of ThGARP cells under resting conditions revealed sustained high levels of FOXP3 protein expression comparable to FOXP3-transduced allo-reactive Th cells and Treg cells (Fig. 2A). This was accompanied by up-regulation of the Treg markers CD25, CTLA4, β-galactoside binding protein LGALS3 [15] and CD27 [24] in ThGARP and ThFOXP3 cells, whereas CD33 (see below), GITR and CD83 [22] were not similarly affected (Fig. 2B).

We further wanted to test whether TCR activation of ThGARP cells would enhance FOXP3 expression. As presented in Fig. 2C, antigen-specific stimulation using allogeneic EBV B cells and IL-2 profoundly increased FOXP3 as well as LGALS3 expression in Th cells that ectopically expressed GARP or FOXP3 but not ThGFP cells. Important to note, that the conversion of an effector towards a regulatory phenotype observed represents a process that needs repeated rounds of TCR stimulations before being established (data not shown).

Thus, ectopic expression of GARP in antigen-specific Th cells modulated the TCR-dependent signalling of effector T cells towards the conversion into regulatory T cells including sustained expression of FOXP3 und up-regulation of Treg markers CD25, CTLA4, LGALS3 and CD27. Transduction of FOXP3, in contrast, did not result in a similar Treg-like expression of GARP while CD25, CTLA4, LGALS3 and CD27 were similarly induced.

**GARP induces a Treg-signature of transcriptional control**

ThGARP cells exhibited a phenotype that resembled activated Treg cells. We, therefore, wanted to establish whether such cells would show also impaired IL-2 transcription like Treg cells. Following stimulation of ThGFP cells with anti-CD3/IL-2, transcription of IL-2 was effectively induced (Fig. 3A). In contrast, ThGARP cells revealed severe repression of IL-2 transcription similar to Treg and ThFOXP3 cells (Fig. 3A).

We further tested genes known to be up-regulated in Treg cells and alloantigen-specific Th cells transduced with FOXP3 using semi-quantitative RT-PCR including the endosomal cysteine-protease LGMN, the ubiquitin-like gene diubiquitin (UBD) and IL-1 receptor 2 (IL1R2) [15, 25]. Similar to FOXP3-transduced Th cells, GARP-transduction led to the up-regulation of such genes (Fig. S2A). The induction of LGMN was further confirmed at the protein level by immunoblot analysis (Fig. S2B).
Because ectopic expression of GARP in allantigen-specific Th cells redirected TCR signalling towards transcriptional regulation normally associated with FOXP3 in CD4\(^+\)CD25\(^{hi}\)-derived Treg cells, we extended this analysis by gene expression profiling. Cluster analysis of expression profiles derived from anti-CD3/IL-2-stimulated Treg cells compared to Th cells transduced with GARP, FOXP3 or GFP revealed that 1286 out of 8973 differentially expressed transcripts were similarly up-regulated in ThGARP, ThFOXP3 and Treg cells compared to ThGFP cells (cluster 5 in Fig. 3B, Table S2). These genes represent a Treg-signature, because many of these genes are known to be expressed at high levels in Treg cells or induced by FOXP3 in allantigen-specific Th cells, i.e. FOXP3, GARP, LGALS3 [15, 25], CTLA4, LGAM [26], CD28 [15], CD47 [27], CD62L, CD27 (TNFRSF7) [24], TNFRSF4 (CD134) [28], TNFRSF9 (CD137) [29], IL1R2 [15], LGMN [15] and DICER [30] (Table S2, Fig. 3B).

Besides known genes of Treg-signature induced by GARP, novel genes were revealed. These included the intracellular Ca\(^{2+}\) channel ryanodine receptor-1 (RYR-1), NAD\(^+\)-linked 15-hydroxyprostaglandin dehydrogenase (HPGD), protein tyrosine phosphatase type IVA member 3 (PTP4A3), TOB1 (transducer of ERBB2, 1), ISG20 (interferon stimulated exonuclease gene 20 kD), Kruppel-like factors 2 and 8 (KLF-2, -8), inhibitor of DNA binding 3 (ID3), the early growth response gene-1 and CD33 (Table S2). These genes were all expressed at significant higher levels in Treg, ThFOXP3 and ThGARP cells compared to the ThGFP control. A confirmation of the differential expression of the selected genes CPE (carboxypeptidase E), RYR-1 and HPGD under resting and activated conditions by semi-quantitative RT-PCR is presented in Fig. S3A.

This extended Treg-signature revealed up-regulation of KLF-2, known to be sufficient to program T-cell quiescence [31]. In agreement, the genes PTP4A3, KLF-8 and ISG20, which have been recently described to be induced by KLF-2 [32] were found up-regulated. By quantitative real-time RT-PCR we confirmed that retroviral overexpression of GARP and FOXP3 in allantigen-specific Th cells indeed induced higher levels of KLF-2 expression compared to ThGFP cells (Fig. 3C). Thus, a potential role of KLF-2 in the regulation of Treg cell quiescence is suggested.

The sialic-acid binding myeloid receptor CD33 represents a negative regulator binding SHP-1/SHP-2 protein tyrosine phosphatases [33]. Although CD33 is predominantly expressed in Treg cells (Fig. 2B), a minor portion of antigen-specific Th cells reveal similar high levels. Thus, we separated antigen-specific Th cells according to their different levels of CD33 expressing by cell sorting. Such CD33\(^{hi}\) Th cells express higher levels of FOXP3 compared to their CD33\(^{lo}\) Th cell counterparts following anti-CD3/-CD28/IL-2 (Fig. 3D), although they do not show any difference in their proliferative capacity (data not shown). Similarly, CD33\(^{hi}\) Th cells up-regulate higher levels of CD83 following activation (Fig. 3E) although not reaching Treg levels, suggesting an interrelation between CD33 expression in T cells with FOXP3 and FOXP3-regulating gene products like CD83 [22].

Importantly, some genes in the transductants did not reach the high level typical for Treg cells, e.g. CPE (Fig. S3A) or GITR and CD83 (Fig. 2B). Therefore, differential expression of such genes might explain why the phenotype of GARP- and FOXP3-transduced Th cells is not totally equivalent to Treg cells.
Together these results clearly show that ectopic expression of GARP in alloantigen-specific \( T_h \) cells was sufficient to modulate effector-type TCR signalling towards \( T_{reg} \)-like sustained FOXP3 expression and with that induced most features of the transcriptional signature of \( T_{reg} \) cells.

GARP impairs ionomycin-induced activation of NFAT independent of FOXP3

Because \( T_h \)GARP cells show sustained high levels of FOXP3, potential FOXP3-independent effects of GARP cannot be assessed. Thus,
we transduced A5 hybridoma T cells, which express a GFP-reporter under control of a basal NFAT promoter described recently [34], with GARP, mutant GARPΔPDZ or control vector. After stimulation with ionomycin for 2 and 4 hrs, flow-cytometry revealed a severe impairment of NFAT-dependent GFP induction (Fig. 3F), showing that GARP mainly affects NFAT activation in the absence of FOXP3. Moreover, cross-linking GARP via plate-bound mAb 272G6 did not block nor enhance these results in A5GARP cells (data not shown).

**GARP confers regulatory function to human antigen-specific Th cells**

The above results indicate that GARP is sufficient to cause Treg-like phenotypic and transcriptional changes in antigen-specific Th cells. Because the hallmark of T_{reg} cells is their anergic proliferative response and suppressor function, we stimulated T_{reg}GARP cells to assess their functional properties. Ectopic expression of GARP in alloantigen-specific Th cells severely reduced their proliferative capacity (Fig. 4A), which could be partially reversed by exogenous IL-2 (data not shown). Such an anergic proliferative response was also observed in ThFOXP3 cells but not in ThGFP control cells (Fig. 4A).

Moreover, T_{reg}GARP cells acquired a strong suppressor activity equivalent to the activity found in T_{reg} cells (Fig. 4B). Suppression was cell-contact dependent, as it was blocked by a transwell membrane (Fig. 4B). Importantly, both, anergy and suppressor function, were more pronounced in T_{reg}GARP cells compared to ThFOXP3 cells.

![Fig. 4 Anergy and suppressor function induced by overexpression of GARP in human alloantigen-specific Th cells. (A) T_{reg} cells and Th cells as in Figs 1–3 were stimulated for proliferation using irradiated allogeneic EBV B cells (stim.); bkg. = background proliferation. Proliferation was assessed at day 3 by measuring incorporation of H^3-thymidin (cpm). (B) T_{reg} and Th cells as in (A) were tested for suppressor function of alloantigen-stimulated ThGFP cells at a ratio of 1 to 1 either separated by a transwell membrane (no contact, upper panel) or without separation (cell contact, middle panel); lower panel represents induced ThGFP cell proliferation without the addition of a potential suppressor or control cell population. Proliferation was assessed at day 3. Similar results were obtained using antigen-specific Th cells as responder cells instead of ThGFP cells (Fig. S6B). (C) Single donor platelets as natural source of GARP^+ cells was tested for suppressor function of alloantigen-stimulated Th cells at indicated ratios as in (B); addition of Th and T_{reg} cells as in (B) at a ratio of 1:1 were included as negative and positive control of suppressor function, respectively.](image-url)
To investigate a potential direct role of GARP in the suppression of T<sub>c</sub> cell proliferation, we used platelets as natural source of GARP<sup>+</sup> cells [23] in a suppressor assay as in Fig. 4. No suppression of T<sub>c</sub> cell proliferation was observed (Fig. 4C). Similar results were obtained with GARP expressing 293 or Jurkat T cells (data not shown). Thus, ectopic expression of GARP in antigen-specific T<sub>c</sub> cells was sufficient to reprogram TCR signalling to induce anergy and suppressor functions similar to T<sub>reg</sub> cells via induction of T<sub>reg</sub>-signature of transcriptional control and induction and maintenance of a regulatory program.

**Positive feedback loop between GARP and FOXP3 in human T<sub>reg</sub> cells**

The above results indicate that overexpression of GARP controls FOXP3 in T<sub>c</sub> cells. Similarly, overexpression of FOXP3 induces GARP. This suggests interdependence via a positive feedback loop. To prove the relevance of this feedback loop we down-regulated GARP and FOXP3 in the established CD4<sup>+</sup>CD25<sup>hi</sup>-derived T<sub>reg</sub> cell line described above [15] with siRNA using a lentiviral vector system. Confirmation of FOXP3 expression, lack of IL-2 induction, anergy, and suppressor function of this T<sub>reg</sub> cell line is presented Fig. S4. T<sub>reg</sub> cells transduced with specific siRNA for GARP, FOXP3 or an irrelevant control were isolated by cell-sorting for GFP included as marker in the lentiviral vector. Quantitative real-time RT-PCR analysis of sorted transductants revealed that irrelevant siRNA did affect neither FOXP3 and GARP expression nor impaired regulatory function of T<sub>reg</sub> cells (data not shown). In contrast, GARP-specific siRNA mediated down-regulation of GARP in T<sub>reg</sub> cells (T<sub>reg</sub>siGARP) as demonstrated in Fig. 5A. This down-regulation was associated with concurrent down-regulation of FOXP3 mRNA (Fig. 5A). This down-regulation of GARP and FOXP3 was associated with some phenotypic changes, including impaired induction of CD83 and CD27, both known to regulate FOXP3 [22, 35], suggesting an interrelated network of FOXP3-regulating systems in T<sub>reg</sub> cells, and down-regulation of CD25 (Fig. 5B). More importantly, T<sub>reg</sub>siGARP and T<sub>reg</sub>siFOXP3 cells revealed comparable impairments of their suppressor activity (Fig. 5C) and improvement of proliferative capacity in the presence of IL-2 (Fig. 5C). Together, these results demonstrate compellingly a positive feedback loop between GARP and FOXP3 in human T<sub>reg</sub> cells, which is an essential component of a higher-order regulation for the maintenance of the regulatory phenotype.

**GARP represents a specific marker of activated CD4<sup>+</sup>CD25<sup>hi</sup>-derived T<sub>reg</sub> cells**

Natural T<sub>reg</sub> cells and TGF-β-induced T<sub>reg</sub> cells differ in some aspects [36] although many phenotypic and functional features are common, including high expression of FOXP3 [37]. We therefore ask whether enhanced FOXP3 expression in TGF-β-induced T<sub>reg</sub> cells would lead to an up-regulation of GARP. Sorted CD4<sup>+</sup>CD25<sup>-</sup> T<sub>c</sub> cells were stimulated with anti-CD3/anti-CD28/IL-2 in the presence of TGF-β1. Quantitative real-time RT-PCR analysis revealed that GARP was not up-regulated in such TGF-β1-induced T<sub>c</sub> cells although FOXP3 was present at high levels (Fig. SSA) and the cells displayed suppressor function (Fig. SS8). Thus, GARP is not required for the function of induced T<sub>reg</sub> cells arguing that natural and induced T<sub>reg</sub> cell might represent alternative differentiation stages of suppressor cells. Moreover, lack of GARP induction might also explain the only transient and partial phenotype of such TGF-β-1-induced regulatory T<sub>c</sub> cells [38].

**GARP and FOXP3 are co-regulated during thymic T<sub>reg</sub> cell development**

The positive feedback loop between GARP and FOXP3 in T<sub>reg</sub> cells implies that both genes should be co-regulated during thymic T<sub>reg</sub> cell development. Therefore, from healthy thymi of seven human donors, we isolated double-positive (CD4<sup>+</sup>CD8<sup>-</sup>) and single-positive (CD4<sup>+</sup>CD8<sup>+</sup>) thymocytes and separated them into CD25<sup>-</sup> and CD25<sup>+</sup> fractions. Quantitative real-time RT-PCR showed that expression levels of both GARP and FOXP3 were found at significantly higher levels in CD25<sup>-</sup> single- and double-positive thymocytes compared to their CD25<sup>+</sup> counterparts (Fig. 5D). Thus, the positive feedback loop between GARP and FOXP3 might already be active in developing thymic T<sub>reg</sub> cells.

Together, these results show that overexpression of GARP reprograms alloantigen-specific T<sub>c</sub> cells towards a regulatory phenotype via sustained expression of GARP and FOXP3. Unlike retroviral overexpression of FOXP3 [15, 39], overexpression of GARP induces a stable regulatory phenotype in antigen-specific T<sub>c</sub> cells that was followed up for more than three months of in vitro antigen-specific stimulation and expansion. The phenotype was not changed by cryopreservation (Fig. S6). Identical results were obtained with five independent transductions of alloantigen-specific T<sub>c</sub> cell lines from three individual donors. Hence, GARP represents a receptor involved higher-order control of sustained expression of FOXP3 with the potential to convert disease-associated antigen-specific effector T towards regulatory T cells.

**LGNN and LGALS3 are constituents of the GARP-FOXP3 feedback loop**

Our previous results showed that LGALS3 and LGMN [15] were up-regulated in alloantigen-specific T<sub>c</sub> cells transduced with FOXP3. Both proteins were also induced by GARP (Fig. 2B, Fig. S2). Because LGMN and LGALS3 are expressed at sustained levels also in activated T<sub>reg</sub> cells similar to GARP and FOXP3, a potential contribution to the GARP-FOXP3 feedback loop could be suggested. Therefore, we retrovirally transduced LGMN (T<sub>c</sub>LGMN) and LGALS3 (T<sub>c</sub>LGALS3) in antigen-specific T<sub>c</sub> cells as described above.

Flow-cytometry and immunoblot analysis of T<sub>c</sub>LGMN cells revealed that LGMN protein expression was comparable between
T<sub>L</sub>GAMN, T<sub>L</sub>GARP and T<sub>L</sub>FOX3 cells but less up-regulated in T<sub>L</sub>LGALS3 cells (Fig. S2B). LGALS3 protein expression was up-regulated in T<sub>L</sub>LGALS3 cells. Interestingly, LGALS3 protein expression was higher in T<sub>L</sub>GARP, T<sub>L</sub>FOX3 and T<sub>reg</sub> cells and even T<sub>L</sub>LGAMN cells compared to T<sub>L</sub>LGALS3 cells (Fig. 2B). This suggests that LGALS3 acts down-stream of GARP, FOX3 and LGAMN. As serine-6 phosphorylation of LGALS3 has been reported to control its function [40], we tested whether deletion of this casseine-kinase I site would affect its FOX3-inducing capacity. Analyses of FOX3 expression in activated T<sub>reg</sub> cells transduced with siRNA constructs specific for FOX3 (T<sub>reg</sub>siFOX3<sup>4</sup>), GARP (T<sub>reg</sub>siGARP<sup>4</sup>) or non-specific control (T<sub>reg</sub>siGL4), Relative mRNA expression of T<sub>reg</sub> cells was arbitrarily set as 1. (B) The same cells as in (A) were analysed for surface expression of CD83, CD27, and CD25 following antigen-specific stimulation with EBV B cells and IL-2. (C) Impairment of suppressor function of T<sub>reg</sub>siFOX3<sup>4</sup> and T<sub>reg</sub>siGARP<sup>1</sup> cells of compared to T<sub>reg</sub>siGL4 cells was assessed in a suppressor assay at a cell ratio of 1:1 as described in Fig. 4. (D) Relative expression of GARP and FOX3 mRNA in the indicated thymic T-cell subsets of normal donors (open symbols), assessed by TaqMan assay, normalized to the expression of β-actin, is represented; black symbols = mean of relative mRNA expression, rel. = relative, * = P < 0.002 by 2-sided Student’s t-test.

**Fig. 5** Positive feedback loop between GARP and FOX3 in human T<sub>reg</sub> cells. (A) Real-time RT-PCR analysis of GARP and FOX3 expression in human T<sub>reg</sub> cells, lentivirally transduced with siRNA constructs specific for FOX3 (T<sub>reg</sub>siFOX3<sup>4</sup>), GARP (T<sub>reg</sub>siGARP<sup>4</sup>) or non-specific control (T<sub>reg</sub>siGL4). Relative mRNA expression of T<sub>reg</sub> cells was arbitrarily set as 1. (B) The same cells as in (A) were analysed for surface expression of CD83, CD27, and CD25 following antigen-specific stimulation with EBV B cells and IL-2. (C) Impairment of suppressor function of T<sub>reg</sub>siFOX3<sup>4</sup> and T<sub>reg</sub>siGARP<sup>1</sup> cells of compared to T<sub>reg</sub>siGL4 cells was assessed in a suppressor assay at a cell ratio of 1:1 as described in Fig. 4. (D) Relative expression of GARP and FOX3 mRNA in the indicated thymic T-cell subsets of normal donors (open symbols), assessed by TaqMan assay, normalized to the expression of β-actin, is represented; black symbols = mean of relative mRNA expression, rel. = relative, * = P < 0.002 by 2-sided Student’s t-test.
and FOXP3. Analysis under resting conditions revealed that GARP protein expression was only minimally affected in ThLGMN and ThLGALS3 cells (Fig. 1D), while FOXP3 protein was obviously induced but not reaching the levels detected in ThGARP or Treg cells (Fig. 2A). Levels of CD25, CTLA4 and CD33 in ThLGMN and ThLGALS3 cells did also not reach that observed in ThGARP and Treg cells (Fig. 2B), whereas CD27 was obviously induced in ThLGMN similar to ThFOXP3 cells (Fig. 2B). Similar to ThGARP and ThFOXP3 cells, LGMN and LGALS3 overexpression did not affect CD83 or GITR expression (Fig. 2B).

Because activation of Treg cells up-regulates expression of FOXP3, we tested ThLGMN and ThLGALS3 cells for expression of FOXP3, LGALS3 and GARP after antigen-specific stimulation. Activation of ThLGMN and ThLGALS3 cells clearly enhanced FOXP3, LGALS3, but not GARP protein expression (Figs 1E and 2C). Nevertheless, levels of FOXP3 and LGALS3 never reached levels observed in ThGARP and Treg cells. This suggests an involvement of LGMN and LGALS3 in the GARP-FOX3 feedback loop mainly following T-cell activation.

**LGMN and LGALS3 induce a partial Treg-signature**

We extended our analysis to the transcriptional control of genes of the Treg-signature. Quantitative RT-PCR revealed repression of IL-2 transcription in ThLGMN and ThLGALS3 cells (Fig. 3A). Similarly, only minimal induction of IL1R2 and UBD mRNA (Fig. S2A) was observed, indicating partial FOXP3-dependent transcriptional control. Interestingly, although FOXP3 and GARP mRNA was highly elevated in ThLGMN and ThLGALS3 cells (Fig. S3B), the presence of such levels of specific mRNA was not sufficient to induce Treg-like protein expression (Figs 1D and 2B), suggesting post-transcriptional control of GARP and FOXP3 expression.

In line with the lower levels of FOXP3 and GARP expression, genes of the extended Treg-signature were not up-regulated in ThLGMN and ThLGALS3 cells. These included RYR-1, HPGD and CPE (Fig. S3A). A further difference represented the up-regulation of IL17R observed only in ThLGMN cells (Fig. S3A). In contrast, KLF-2 induction was observed only in ThLGALS3 but not ThLGMN cells (Fig. 3C). Because serine-6 phosphorylation of LGALS3 was an essential prerequisite to induce FOXP3, we tested the effects of mutant LGALS3 on the expression of GARP and LGMN. Under resting and activated conditions, the mutant LGALS3 was unable to induce GARP and LGMN, corroborating the proposed interrelataion of LGALS3 with these genes via FOXP3 (Fig. S7B). Interestingly, KLF-2 was not affected by the serine-6 mutation of LGALS3, suggesting that LGALS3 also regulates some gene expression down-stream of FOXP3 independent of its phosphorylation (Fig. S7B).

As expected, the functional changes observed in ThLGMN and ThLGALS3 cells were only minor (Fig. 4A) and no significant suppressive activity was observed (Fig. 4B). Thus, LGMN and LGALS3 appear to play a minor and redundant role in the GARP-FOX3 feedback loop. In line with that is the fact that LGMN and LGALS3 knock-out mice do not show obvious Treg cell deficiencies [41, 42].

**Discussion**

In this study, we demonstrate that GARP represents a key receptor that is specifically induced in human CD4+ CD25hi regulatory T cells upon TCR stimulation. Unlike other Treg markers like FOXP3, CD25 and CTLA4 that are shared between Treg and activated CD4+ CD25- Th cells, GARP is not induced in human naive CD4+ CD25- Th and antigen-specific Treg cells following TCR activation, nor in TGF-β1-induced Treg cells. Thus, GARP represents a specific marker suitable to differentiate natural derived CD4+ CD25hi Treg cells from activated effector Treg and Treg cells. We further demonstrate that GARP dominantly controls FOXP3 via a positive feedback loop able to stably reprogram antigen-specific Treg cells towards Treg cells. Thus, GARP plays a key role in peripheral and most likely thymic Treg cells, and might serve as an intrinsic control mechanism that ensures the proper induction and stabilization of sustained high levels of FOXP3 safeguarding the regulatory program.

GARP encodes a receptor of yet unknown specificity. Thus far, detailed analysis of the spatio-temporal expression in mice suggested a potential role of GARP in organogenesis at the neuromuscular and dermal-epidermal junctions [43]. In lymphoid organs of mice, the expression of GARP was restricted to endothelial and megakaryocytic cells [23, 43], suggesting a role in coagulation that has recently been confirmed in a coagulation model in zebrafish [44]. Because Treg cells represent only a minor population of human CD4+ T cells and GARP protein expression is induced in Treg cells only following TCR stimulation, detection of GARP expression in Treg cells might have been missed thus far [23].

GARP-associated signalling pathway and its potential ligand can only be speculated upon. The C-terminal PDZ motif of GARP might be involved as a protein–protein interaction module besides being involved in regulation of GARP protein level and cell surface expression. Although the short cytoplasmic tail of GARP suggests the existence of a signalling co-receptor as suggested recently [23], mutation of the PDZ motif alone is sufficient to impair FOXP3-independent inhibition of ionomycine-induced NFAT activation assessed in A5 cells. Inhibition of NFAT by GARP might be the reason for the previously reported impairment of IL-2 in human GARP-transduced T cells with concurrent down-regulation of FOXP3 [45]. These authors showed that transduction of GARP or GARP without the cytoplasmic tail were equally efficient in partially inducing FOXP3 in human T cells [45]. In contrast, our results show that GARP itself does not simply represent a suppressor molecule, because platelets that naturally express GARP do not exert suppressor activity.

Whatever the mechanisms, with the functional characterization of LGMN and LGALS3, we could reveal some insights into the complex regulatory network controlled by the GARP-FOXP3 feedback loop. Unlike GARP, overexpression of LGMN and LGALS3 in Th cells did not induce sustained high levels of FOXP3 and GARP but enhanced FOXP3 and GARP expression mainly following TCR stimulation. Thus, LGMN and LGALS3 were not able to induce a stable regulatory phenotype and
LGMN is made as an inactive pro-enzyme that requires autocatalytic sequential cleavage to gain activity, localized in receptor-recycling compartments of endosomes/lysosomes [46, 47]. Although the mechanism of LGMN-mediated FOXP3 regulation following TCR stimulation remains speculative, it should differ from GRAIL, an E3-ubiquitin ligase with a similar localization in receptor-recycling compartments. GRAIL has been reported to be sufficient to convert murine T cells to a regulatory phenotype via induction of TGF-β in the absence of FOXP3 [48]. Because antigen-specific stimulation of our differentiated effector T cells in the presence of TGF-β did not induce up-regulation of FOXP3 (data not shown), a significant impact of TGF-β signalling to the effects of LGMN can be excluded. Nevertheless, LGMN might control some other important cell-surface associated signalling components or their recycling, enhancing the Th cell inherent capacity to induce FOXP3.

Similarly, the mechanism of LGALS3-mediated GARP and FOXP3 induction remains to be elucidated. Importantly, mutagenesis of LGALS3 at position 6 impaired its FOXP3-inducing function, establishing the importance of this potential CK1-phosphorylation site. The molecular mechanisms of LGALS3 action might include the modulation of the transcription factors NFAT and AP-1, reported to be activated by LGALS3 in Jurkat T cells [49], because both factors are involved in the control of FOXP3 transcription in human T cells [50]. As expected, the impaired induction of FOXP3 by the mutant LGALS3 was accompanied by the inability to induce GARP and LGMN. Although serine-6 phosphorylation of LGALS3 has been described as important checkpoint in the control of LGALS3, some phosphorylation independent unique functions have also been reported [40]. In line with that, the serine-6 mutant LGALS3 had nearly the same effect on the expression of KLF-2, suggesting that KLF-2 might be regulated by FOXP3 via LGALS3 independent on its serine-6 phosphorylation.

In conclusion, we discovered GARP as a key receptor controlling FOXP3 in human CD4⁺ CD25⁺ Treg cells following TCR activation. GARP is sufficient to reprogram human alloantigen-specific Th cells towards regulatory T cells via induction of sustained high levels FOXP3, presented as simplified model in Fig. 6. Furthermore, we established a positive feedback loop between GARP and FOXP3, reminiscent of positive auto-regulation ensuring formation and maintenance of high concentrations of important ‘master regulators’ of cell differentiation [51, 52]. With that GARP represents an epigenetic stabilizing system that allows the development of a permanent suppressor cell lineage, as suggested recently [53], and acts as a Treg cell-intrinsic tolerance mechanism as Treg cells are potentially auto-reactive [54]. Together, our findings provide compelling evidence of a GARP-FOXP3 positive feedback loop that is interrelated with a regulatory network including LGALS3, LGMN and other FOXP3-regulating genes like CD33, CD27 and CD83, providing a conceptual framework for the molecular definition of the regulatory program [14]. This opens up the possibility for generation of antigen-specific regulatory T cells for clinical applications. It further will provide the basis to develop new strategies and tools to induce or inhibit Treg cells in chronic infection, tumour immunotherapy, autoimmune diseases and transplantation.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article.

**Fig. S1** Alignment of human GARP with the structure of the ectodomain of human TLR3. (A) Comparison of the ectodomain of TLR3 (pdb-id: 2A02) and GARP, conserved sequences are indicated by blue boxes, strictly conserved residues are highlighted.
white-on-red, and similar residues indicated by red characters. Secondary structure elements of the TLR3 structure are indicated by arrows (β strand), coils (helices) and TT (turns). (B) Ribbon diagram of a hypothetical GARP dimmer model, based on the dimerization proposed for TLR3. The prominent loops at 296–308 and 421–432 of GARP (yellow) could have similar functions for dimerization as proposed for TLR3. Putative glycosylation sites are indicated with space-filling representations.

Fig. S2 GARP induced transcriptional control. (A) Analysis of LGMN, UBD, IL1R2, and GARP mRNA by semi-quantitative RT-PCR in T\(_h\) cells transduced with GARP, FOXP3, LGMN, LGALS3, and GFP as described in Figs 1–4. cDNA was tested in threefold dilutions starting with 1:3 using RPS9 as housekeeping control. (B) Western blot analysis of LGMN protein expression in the same cells as in (A).

Fig. S3 GARP induces genes of the extended T\(_{reg}\) signature. (A) Semi-quantitative RT-PCR analysis of IL7R, CPE, RYR-1 and HPGD in Th cells as in (Figs 1–4). (B) Quantitative real-time RT-PCR analysis of GARP and FOXP3 in T\(_{reg}\) cells as in (A) under resting conditions and 3 days after stimulation as in (A). Relative mRNA expression of T\(_{reg}\) GFP cells was arbitrarily set as 1.

Fig. S4 Characterization of the T\(_{reg}\) cell line used in this study and for siRNA experiments. (A) This alloanigen-reactive T\(_{reg}\) cells line (T\(_{reg}\)THU) used, has been established and characterized in detail recently [15]. These cells constitutively express known T\(_{reg}\)-markers, FOXP3, LGALS3, and CD25 are shown as selected examples. For comparison T cells derived from sorted CD4\(^{+}\)CD25\(^{-}\) T\(_{h}\) cells were stimulated with the same allogeneic EBV B cells and IL-2 in the presence of vehicle or 10 ng/ml TGF-β1. Analysis was done at day 7 after stimulation. (B) The same cells as in (A) were stimulated with anti-CD3/CD28 DynalBeads (Invitrogen) and IL-2 for 6 hrs in the presence of 10 μg/ml brefeldin and tested for intracellular IL-2 and FOXP3 expression. Allo-antigen specific T\(_{h}\) cells served as control. (C) Lentiviral transduction efficacy of the T\(_{reg}\) cells as in (A) is similar as compared to T\(_{h}\) cells. (D) The hallmark feature of T\(_{reg}\) cells, anergy and suppressor function, are preserved over a period of six months of in vitro expansion of the same T\(_{reg}\) cells as in (A) and Figs 1–5.

Fig. S5 GARP is not up-regulated in TGF-β1-induced T\(_{reg}\) cells. (A) Sorted CD4\(^{+}\)CD25\(^{-}\) T\(_{h}\) cells were stimulated with anti-CD3/-CD28/IL-2 without (Th), in the presence of solvent (Th vehicle), and 10 ng/ml human TGF-β1 (Th\(_{TGF-β1}\)) as in Fig. S4. Expression of GARP and FOXP3 mRNA was assessed at day 6 by real-time RT-PCR as in Fig. 1. (B) TGF-β1-induced T\(_{reg}\) cells as in (A) were tested for proliferation and suppressor function (upper panel) and proliferation with exogenous IL-2 (lower panel); bkg. = background proliferation, stim. = T-cell proliferation induced by irradiated allogeneic EBV B cells. Proliferation was assessed at day 3 by measuring incorporation of H\(^{3}\)-thymidin (cpm).

Fig. S6 Anergy and suppressor function in alloantigen-specific T\(_{h}\) cells transduced with GARP following cryopreservation. (A) T\(_{reg}\) cells (T\(_{reg}\) cell lines MPO and HG [15]) and T\(_{h}\) cells as in Figs 1–4 were stimulated for proliferation using irradiated EBV B cells in the absence (stim.) or presence of IL-2 (stim. + IL-2) 1 week after thawing cryopreserved cells; bkg. = background proliferation. Proliferation was assessed at day 3 by measuring incorporation of H\(^{3}\)-thymidin (cpm). T\(_{reg}\) and T\(_{h}\) cells as in (A) were tested for suppressor function of alloantigen-stimulated parental T\(_{h}\) cells at a ratio of 1:1. Percent inhibition of T\(_{h}\) cell proliferation by the addition of retrovirally engineered T\(_{reg}\) cells is indicated, setting addition of wild-type T\(_{h}\) cells (T\(_{h}/T_{reg}\)) as 100%. Proliferation was assessed at day 3.

Fig. S7 Effects of S6A-mutant LGALS3 on gene expression. (A) T\(_{h}\) cells transduced with either wild-type (Th/LGALS3) or mutant (Th/LGALS3\(_{S6A}\)) were analysed for FOXP3 and CD25 expression at day 3 following stimulation with anti-CD3 and 100 U/ml IL-2 compared to T\(_{reg}\) GFP cells. (B) Quantitative real-time RT-PCR analysis of GARP, LGALS3, LGMN and KLF-2 expression of the same cells as in (A). T\(_{h}\) cells were tested under resting conditions and 3 days after stimulation with anti-CD3 and 100 U/ml IL-2. Relative mRNA expression of T\(_{h}\) GFP cells was arbitrarily set as 1.

Table S1 Differential gene expression of resting and activated CD4\(^{+}\)CD25\(^{-}\) T\(_{reg}\) versus CD4\(^{+}\)CD25\(^{-}\) T\(_{h}\) cells analysed ex vivo as detected by array analysis

Table S2 Differential gene expression T\(_{reg}\) cells, T\(_{h}\) GARP and T\(_{h}\) FOXP3 compared to T\(_{h}\) GFP cells

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