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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 $CD4^+CD25^{hi}$ T (T_{reg}) cells. Based on transcriptional profiling of *ex vivo* activated T_{reg} and helper $CD4^+CD25^-$ T (T_h) cells we have identified GARP (glycoprotein-A repetitions predominant), LGALS3 (lectin, galactoside-binding, soluble, 3) and LGMN (legumain) as novel genes implicated in human T_{reg} cell function, which are induced upon T-cell receptor stimulation. Retroviral overexpression of GARP in antigen-specific T_h 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 T_{reg}-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 T_{reg} 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 T_{reg} 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 T_{reg} 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 $CD4^+CD25^{hi}$ T (T_{reg}) cells are able to actively suppress immune responses and play an essential role in the homeostasis of immune reactions and tolerance [1]. T_{reg} 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

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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 T_{reg} 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 factor 1 (RUNX1) [6] and NFAT [7]. Besides this well-described negative impact on transcription, recent evidence accumulated that FOXP3 also positively modulates transcription. Many FOXP3-binding promoter regions in mice include FOXP3-induced genes, *e.g.* Nrp1, CTLA4, GITR and CD25 [8–10].

Although FOXP3 expression in murine $CD4^+$ T cells specifically marks regulatory T cells, an important difference has become apparent recently between $CD4^+$ T cells of man and mouse. Following T-cell receptor (TCR) activation, human effector $CD4^+CD25^-$ T (T_h) cells up-regulate FOXP3 protein similar to other markers of T-cell activation including CD25 and CTLA4, and produce effector cytokines like IL-2 and interferon-γ despite the presence of FOXP3 [11–13]. It follows that specific high level of FOXP3 expression in human T_{reg} cells requires exclusive control mechanisms to explain the qualitative and quantitative differences of FOXP3 expression between human activated T_h and T_{reg} cells or as suggested recently, a higher-order regulation [14].

To gain new insights into the differential regulation of FOXP3, we used whole genome transcriptom analysis of resting and activated $CD4^+CD25^{hi}$ T_{reg} cells and their respective $CD4^+CD25^-$ T_h cell counterparts to identify T_{reg}-specific genes that might control the regulatory phenotype.

Materials and methods

Cell isolation

T_{reg} cell line derived from sorted $CD4^+CD25^{hi}$ T_{reg} cells and alloantigen-specific T_h cell lines derived from sorted $CD4^+CD25^-$ T 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 $CD4^+CD8^+CD25^-$, $CD4^+CD8^+CD25^+$, $CD4^+CD8^-CD25^-$ and $CD4^+CD8^-CD25^+$ 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 $CD4^+CD25^{hi}$ 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.

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 8 to 10 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. Tegge, 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 either anti-tubulin mAb 3A2 (Synaptic Systems, Goettingen, 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].

T-cell functional assays

T-cell proliferation and suppressor activity were assessed by stimulating 3×10^4 T cells in triplicates with irradiated LG2-EBV B cells with or without IL-2 in 96 flat-bottom microtiter plates (Nunc, Wiesbaden, Germany). For transwell experiments T_h 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 [^{3}H]-thymidine after 72 hrs for the final 16 hrs.

Retroviral transduction of human alloantigen-specific T_h 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_{reg} 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^+) 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_{reg} cells

GARP and FOXP3 specific targeting sequences (siGARP[4]: 5′-GCC TGC ATA CCC TCT CAC T-3′; siFOXP3[4]: 5′-GCC TGC ATA CCC TCT CAC 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 SnaB 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_{reg} cells were infected by spin-infection with lentiviral supernatants and 8 μg/ml polybrene. Successfully transduced T_{reg} cells (GFP^+) were sorted and kept in culture by antigen-specific re-stimulation using irradiated LG2-EBV B cells and IL-2.

Semi-quantitative and quantitative RT-PCR

Total RNA was isolated from $CD4^+$ T cells using RNAeasy (Qiagen, Hilden, Germany) or nucleospin RNA-II (Macherey Nagel, Düren, Germany). cDNA synthesis was done using oligo-dT primers and superscript II reverse transcriptase (Invitrogen). For semi-quantitative RT-PCR, threefold dilutions of cDNA samples, starting with the first dilution were normalized to the expression of RPS9 and tested with specific primers (see supplemental Experimental Procedures). Quantitative real-time PCR was performed on an ABI PRISM 7000 cycler (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR kit (Stratagene, La Jolla, CA, USA) and primers specific for primers (see supplemental Experimental Procedures) as described [15].

Expression of GARP, LGALS3 and LGMN mRNA in thymic T-cell subsets were quantified as described above. For quantitative analysis of FOXP3 mRNA in thymic T-cell subsets TaqMan Universal PCR master mix and commercially available pre-designed intron-spanning primer-probe assay were used (Hs00203958_m1; Applied Biosystems). Residual genomic DNA was removed by DNAse I (Sigma-Aldrich, St. Louis, MO, USA) before cDNA synthesis. Results represent the relative expression normalized to β-actin (Hs99999903_m1, Applied Biosystems). The samples were analysed by using the iCycler iQ RealTime PCR instrument (Bio-Rad, Hercules, CA, USA).

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 $CD4^+CD25^{hi}$-derived T_{reg} cells and $CD4^+CD25^-$ 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 $CD4^+CD25^{hi}$ T_{reg} 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_{reg} cells, and GARP-, FOXP3- or GFP-transduced T_h 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_hGFP 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 $CD4^+CD25^{hi}$ T_{reg} cells

We isolated $CD4^+CD25^{hi}$ T_{reg} cells and $CD4^+CD25^-$ T_h 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 $CD4^+CD25^{hi}$ T_{reg} cells upon stimulation (Fig. 1A, Table S1). To confirm these results $CD4^+CD25^{hi}$ T_{reg} cells and $CD4^+CD25^-$ T_h 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 $CD4^+CD25^{hi}$ T_{reg} cells over 12 to 48 hrs following TCR stimulation and sustained mRNA expression thereafter, accompanied by up-regulation of FOXP3 (Fig. 1B). These $GARP^{hi}$ T_{reg} cells were functionally confirmed to be suppressor cells (Fig. 1C).

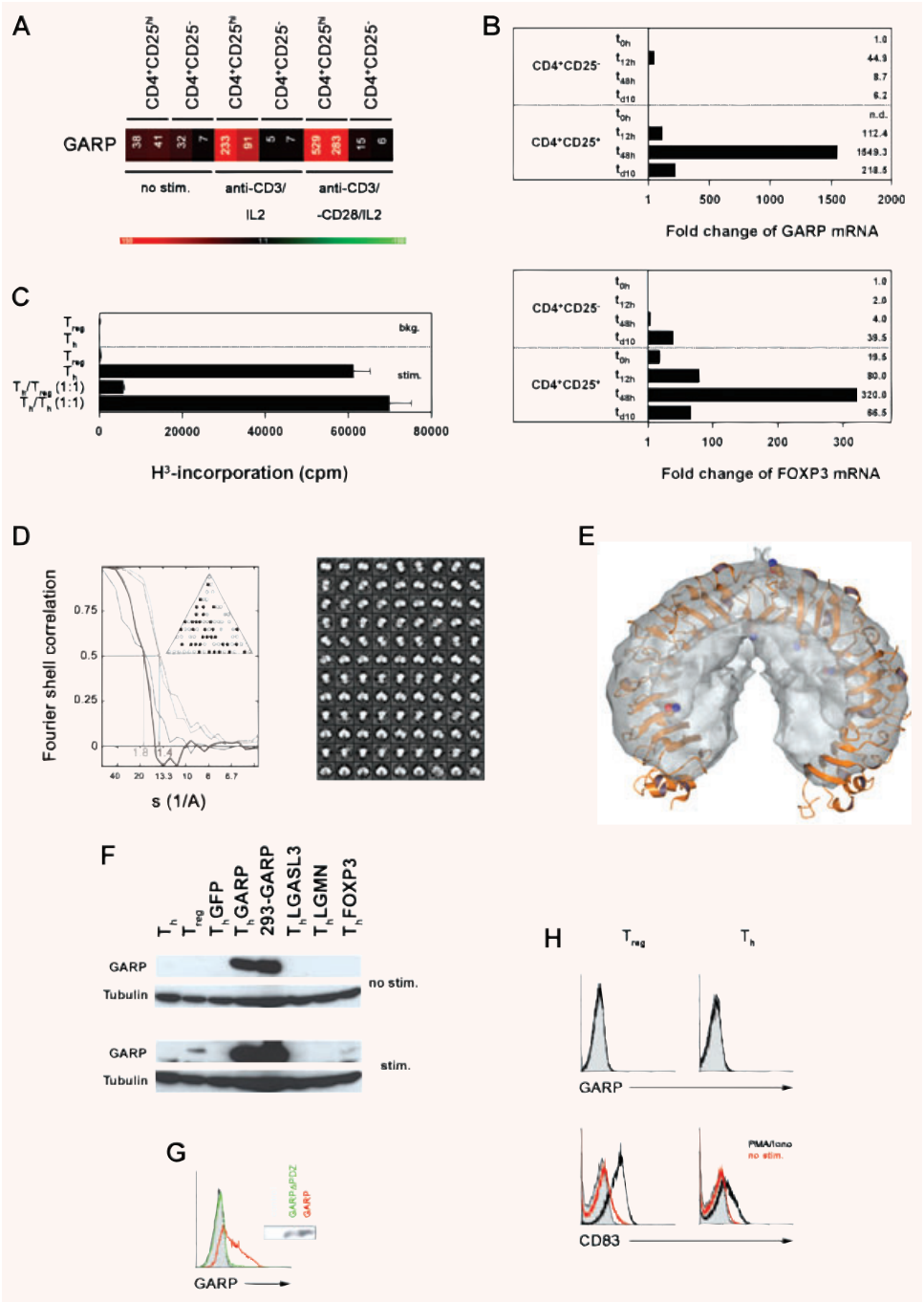

Fig. 1 GARP is exclusively induced in $CD4^+CD25^{hi}$-derived T_{reg} cells. (**A**) Human $CD4^+CD25^{hi}$ T_{reg} and $CD4^+CD25^-$ T cells were sorted to a purity of >99% and analysed *ex vivo* (no stim.) and 24 hrs after stimulation using anti-CD3/IL-2 and anti-CD3/anti-CD28/IL-2. GARP signal intensity as determined by GenChip analysis and is represented normalized to the signal of RPS9. (**B**) Confirmation by real-time RT-PCR analysis of GARP (upper panel) and FOXP3 (lower panel) mRNA expression in independently sorted $CD4^+CD25^{hi}$ T_{reg} and $CD4^+CD25^-$ T_h cells analysed *ex vivo* (t_{0h}), 12, 48 hrs and day 9 after stimulation with anti-CD3/anti-CD28 Dynalbeads at a ratio of 1:1 and IL-2. Relative mRNA expression of $CD4^+CD25^-$ T_h cells at time t_0 was arbitrarily set as 1. (**C**) The same $CD4^+CD25^{hi}$ T_{reg} cells as in (**B**) were tested for suppressor function of alloantigen-stimulated $CD4^+CD25^-$ T_h cells at a ratio of 1 to 1. Proliferation was assessed at day 3 by measuring incorporation of H^3-thymidin (cpm). (**D**) Fourier shell correlation of refinement process and EOTEST are shown (left part). The values 1.4 and 1.8 correspond to the estimated resolution of 14 and 18 Å. The insert is an asymmetric triangular showing the distribution of the particles' orientations. Representative gallery of projections of the single particles alternating with the corresponding class averages is shown on the right. (**E**) Surface display of the 3D 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_{reg} and T_h 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 GARPΔPDZ expressed in 293 cells (left panel) using mAb 272G6 compared to WB detection in the same cells (insert) using mAb 50G10. (**H**) T_{reg} and T_h cells as in (**F**) were treated for 4 hrs with PMA (40 ng/ml) and ionomycine (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 glycolysation-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 T_{reg} cells, we immunized mice with bacterially expressed human GARP protein or a synthetic peptide $GARP_{296–308}$, respectively, to generate anti-GARP specific mAbs. Immunoblot analysis using anti-GARP mAb 50G10 revealed GARP protein expression in activated T_{reg} cells but not in alloantigen-specific T_h cells (Fig. 1F). But unlike cell surface expression in epithelial 293 cells transfected with GARP using with the epitope specific anti-$GARP_{296–308}$ mAb 272G6 (Fig. 1G), detection on Jurkat T cell tranduced with GARP nor on T_{reg} 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 T_{reg} *versus* T_h cells [22], did not let to detectable surface expression of GARP using mAb 272G6 on T_{reg} 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 $CD4^+CD25^{hi}$-derived T_{reg} cells following TCR activation suggesting a potential contribution of GARP to the regulatory phenotype.

Ectopic expression of GARP in human antigen-specific T_h 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 T_h 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 T_h cells (T_hGARP) 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 T_h cells retrovirally transduced in parallel with GFP alone (T_hGFP) served as negative control. Transduction with FOXP3 (T_hFOXP3) and an established and well-characterized T_{reg} cell line [15] were included for comparison. Immunoblot analysis confirmed the ectopic expression of GARP in T_hGARP cells (Fig. 1E).

Flow-cytometry of T_hGARP cells under resting conditions revealed sustained high levels of FOXP3 protein expression comparable to FOXP3-transduced allo-reactive T_h cells and T_{reg} cells (Fig. 2A). This was accompanied by up-regulation of the T_{reg}-markers CD25, CTLA4, β-galactoside binding protein LGALS3 [15] and CD27 [24] in T_hGARP and T_hFOXP3 cells, whereas CD33 (see below), GITR and CD83 [22] were not similarly affected (Fig. 2B).

We further wanted to test whether TCR activation of T_hGARP 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 T_h cells that ectopically expressed GARP or FOXP3 but not T_hGFP 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 T_h 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 T_{reg} markers CD25, CTLA4, LGALS3 and CD27. Transduction of FOXP3, in contrast, did not result in a similar T_{reg}-like expression of GARP while CD25, CTLA4, LGALS3 and CD27 were similarly induced.

GARP induces a T_{reg}-signature of transcriptional control

T_hGARP cells exhibited a phenotype that resembled activated T_{reg} cells. We, therefore, wanted to establish whether such cells would show also impaired IL-2 transcription like T_{reg} cells. Following stimulation of T_hGFP cells with anti-CD3/IL-2, transcription of IL-2 was effectively induced (Fig. 3A). In contrast, T_hGARP cells revealed severe repression of IL-2 transcription similar to T_{reg} and T_hFOXP3 cells (Fig. 3A).

We further tested genes known to be up-regulated in T_{reg} cells and alloantigen-specific T_h 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 T_h 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).

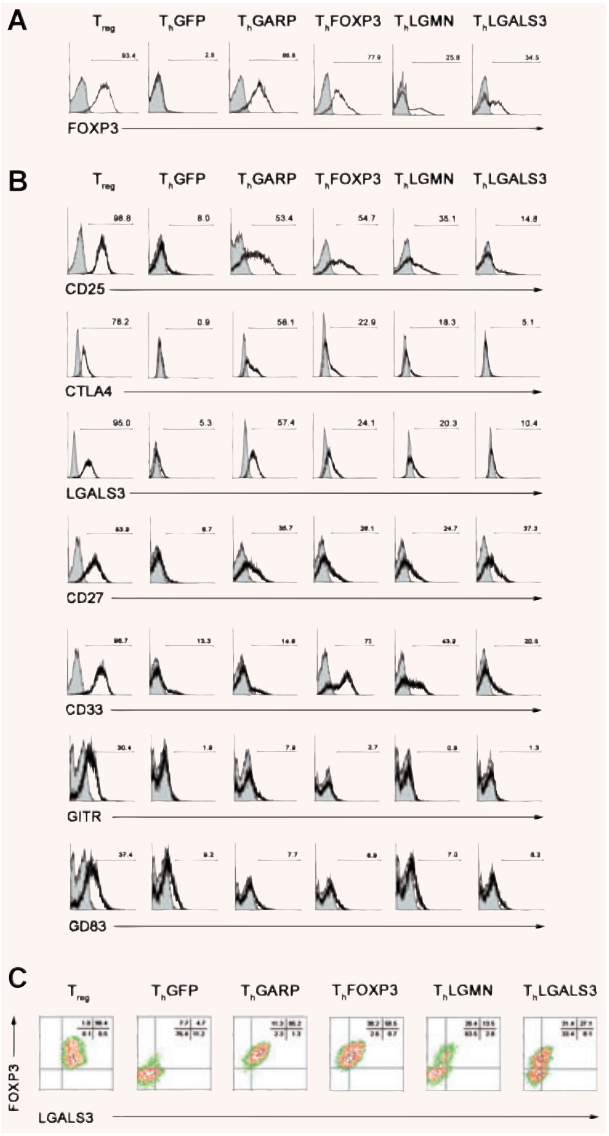

Fig. 2 Ectopic expression of GARP in human alloantigen-specific T_h cells induces sustained expression of FOXP3. (**A**) T_h cells as in Fig. 1F were analysed for FOXP3 and (**B**) CD25, CTLA4, LGALS3, CD27, CD33, GITR and CD83 expression by flow cytometry under resting conditions. (**C**) The same cells as in (**A**) were stimulated for 3 days with cognate antigen and 50 U/ml IL-2 and analysed for FOXP3 and LGALS3 protein expression. Gates for figures (**A**–**C**) were set according to non-stained control (CD25) or isotype control (CTLA4, LGALS3, FOXP3, CD33, CD83, GITR) represented as shaded histogram (**A**, **B**) or set as quadrant (**C**); percentage of positive cells is indicated. T_{reg} cells were included for comparison (**A**–**C**).

Because ectopic expression of GARP in alloantigen-specific T_h cells redirected TCR signalling towards transcriptional regulation normally associated with FOXP3 in $CD4^+CD25^{hi}$-derived T_{reg} cells, we extended this analysis by gene expression profiling. Cluster analysis of expression profiles derived from anti-CD3/IL-2 stimulated T_{reg} cells compared to T_h cells transduced with GARP, FOXP3 or GFP revealed that 1286 out of 8973 differentially expressed transcripts were similarly up-regulated in T_hGARP, T_hFOXP3 and T_{reg} cells compared to T_hGFP cells (cluster 5 in Fig. 3B, Table S2). These genes represent a T_{reg}-signature, because many of these genes are known to be expressed at high levels in T_{reg} cells or induced by FOXP3 in alloantigen-specific T_h cells, *i.e.* FOXP3, GARP, LGALS3 [15, 25], CTLA4, LAG3 [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 T_{reg}-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 T_{reg}, T_hFOXP3 and T_hGARP cells compared to the T_hGFP 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 T_{reg}-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 alloantigen-specific T_h cells indeed induced higher levels of KLF-2 expression compared to T_hGFP cells (Fig. 3C). Thus, a potential role of KLF-2 in the regulation of T_{reg} 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 T_{reg} cells (Fig. 2B), a minor portion of antigen-specific T_h cells reveal similar high levels. Thus, we separated antigen-specific T_h cells according to their different levels of CD33 expressing by cell sorting. Such $CD33^{hi}$ T_h cells express higher levels of FOXP3 compared to their $CD33^{lo}$ T_h 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, $CD33^{hi}$ T_h cells up-regulate higher levels of CD83 following activation (Fig. 3E) although not reaching T_{reg} 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 T_{reg} 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 T_h cells is not totally equivalent to T_{reg} cells.

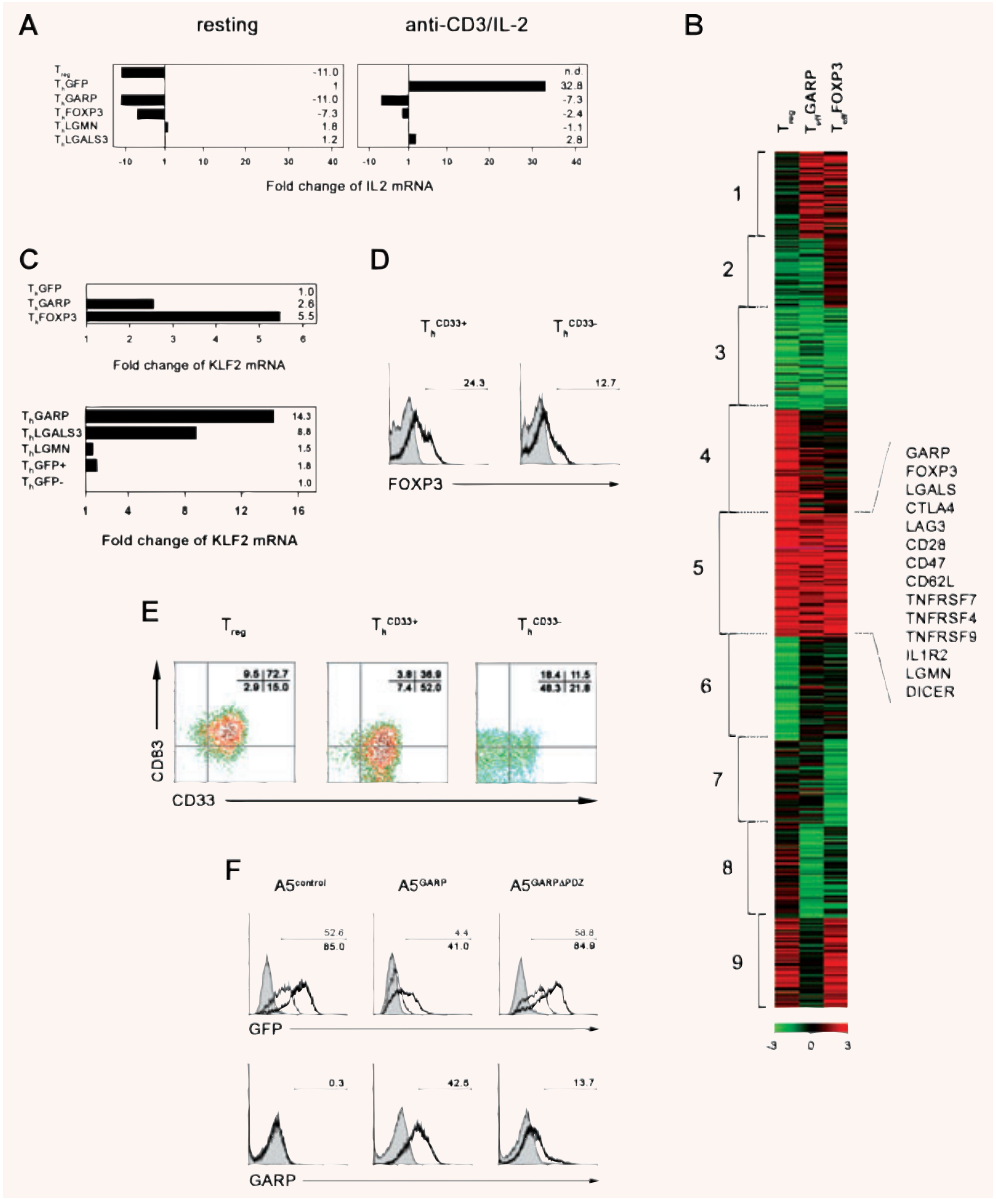

Fig. 3 GARP induces T_{reg}-signature of transcriptional control. (**A**) Resting and anti-CD3/IL-2 stimulated T cells as in Figs 1 and 2 were analysed for mRNA expression of IL-2 by real-time RT-PCR. The individual fold change of relative mRNA expression of indicated T_h and T_{reg} cells were compared to T_hGFP cells, arbitrarily set as 1, is indicated.; n.d. = not detected. (**B**) K-Means clusterization of significantly regulated genes in T_{reg}, T_hGARP and T_hFOXP3 cells compared to T_hGFP cells, all stimulated for 3 days with anti-CD3/IL-2. The heat map represent signal log ratios. Numbers correspond to clusters of Table S2. (**C**) Real-time RT-PCR analysis of KLF-2 expression in anti-CD3/IL-2 stimulated T_h cells transduced with GARP, FOXP3 or GFP. Relative mRNA expression of T_hGFP cells was arbitrarily set as 1 (upper panel). Real-time RT-PCR analysis of KLF-2 mRNA in T_h cells transduced with LGALS3 and LGMN; T_h cells transduced with GFP, GARP, and parental cells without transduction (T_hGFP-) served as control (cell line T_hB). Relative mRNA expression of T_hGFP- cells was arbitrarily set as 1 (lower panel). (**D**) FOXP3 expressio5n in antigen-specific T_h cells separated for different levels of CD33 expression (T_h^{CD33+} and T_h^{CD33-} cells, respectively) by cell sorting following stimulation for 3 days using cognate antigen and IL-2. Isotype control = grey filled, FOXP3 = bold black. (**E**) The same cells as in (D) were analysed for cell surface CD83 and CD33 expression following stimulation with cognate antigen and IL-2 for 3 days. (**F**) A5 cells, transduced with GARP, mutant GARPΔPDZ or control lentiviral vector were analysed for GFP expression without (grey filled) and with 1 μg/ml Ionomycine stimulation for 2 (thin line) and 4 (thick line) hrs (upper panel);% GFP is represented. Lower panel represents the corresponding cell surface expression of GARP, detected with mAb 272G6 as in Fig. 1.

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_hGARP cells show sustained high levels of FOXP3, potential FOXP3-independent effects of GARP cannot be assessed. Thus,

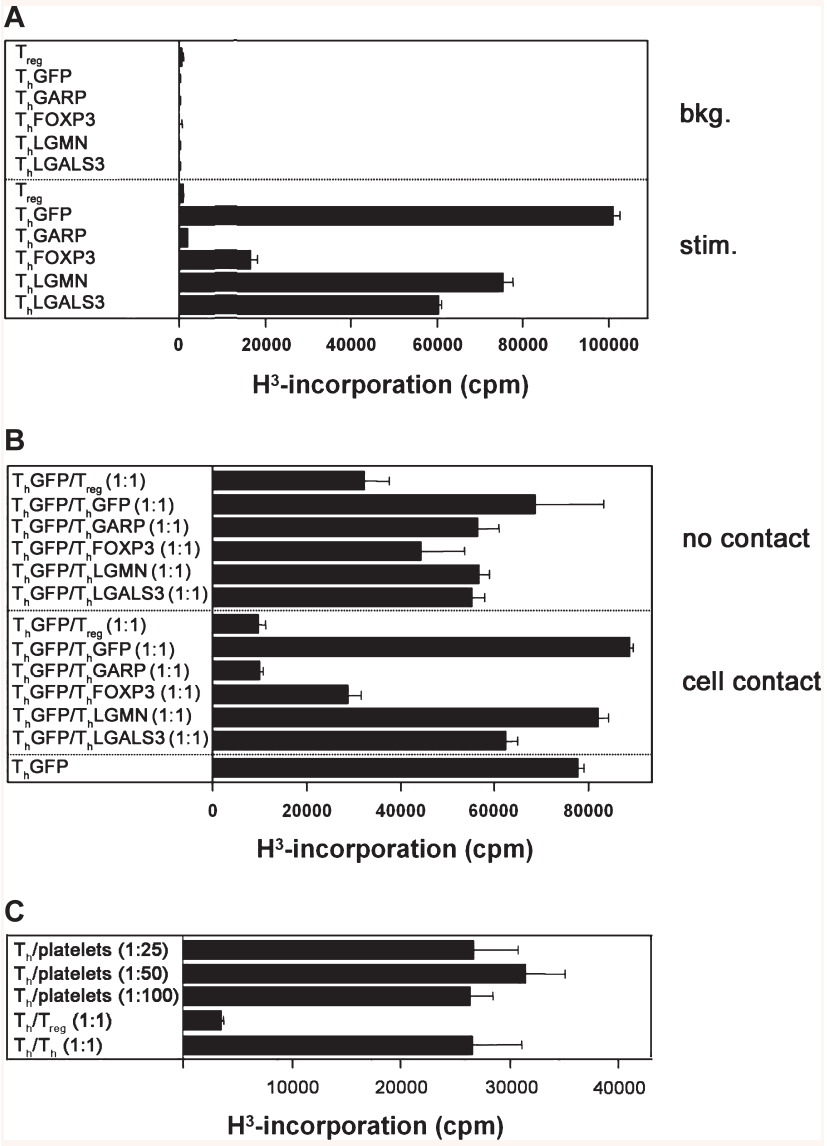

Fig. 4 Anergy and suppressor function induced by overexpression of GARP in human alloantigen-specific T_h cells. (**A**) T_{reg} cells and T_h cells as in Figs 1–3 were stimulated for proliferation using irradiated allogeneic EBV B cells (stim.); bkg. = background proliferation. Proliferation was assessed at day 3 by measuring incorporation of H^3-thymidin (cpm). (**B**) T_{reg} and T_h cells as in (**A**) were tested for suppressor function of alloantigen-stimulated T_hGFP 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 T_hGFP 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 T_h cells as responder cells instead of T_hGFP cells (Fig. S6B). (**C**) Single donor platelets as natural source of $GARP^+$ cells was tested for suppressor function of alloantigen-stimulated T_h cells at indicated ratios as in (**B**); addition of T_h and T_{reg} cells as in (**B**) at a ratio of 1:1 were included as negative and positive control of suppressor function, respectively.

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 ionomycine for 2 and 4 hrs, flow-cytometry revealed a sever 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 $A5^{GARP}$ cells (data not shown).

GARP confers regulatory function to human antigen-specific T_h cells

The above results indicate that GARP is sufficient to cause T_{reg}-like phenotypic and transcriptional changes in antigen-specific T_h cells. Because the hallmark of T_{reg} cells is their anergic proliferative response and suppressor function, we stimulated T_hGARP cells to assess their functional properties. Ectopic expression of GARP in alloantigen-specific T_h 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 T_hFOXP3 cells but not in T_hGFP control cells (Fig. 4A).

Moreover, T_hGARP 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_hGARP cells compared to T_hFOXP3 cells.

To investigate a potential direct role of GARP in the suppression of T_h cell proliferation, we used platelets as natural source of $GARP^+$ cells [23] in a suppressor assay as in Fig. 4. No suppression of T_h 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_h cells was sufficient to reprogram TCR signalling to induce anergy and suppressor functions similar to T_{reg} cells *via* induction of T_{reg}-signature of transcriptional control and induction and maintenance of a regulatory program.

Positive feedback loop between GARP and FOXP3 in human T_{reg} cells

The above results indicate that overexpression of GARP controls FOXP3 in T_h 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 $CD4^+CD25^{hi}$-derived T_{reg} 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_{reg} cell line is presented Fig. S4. T_{reg} 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_{reg} cells (data not shown). In contrast, GARP-specific siRNA mediated down-regulation of GARP in T_{reg} cells (T_{reg}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_{reg} cells, and down-regulation of CD25 (Fig. 5B). More importantly, T_{reg}siGARP and T_{reg}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_{reg} 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 $CD4^+CD25^{hi}$-derived T_{reg} cells

Natural T_{reg} cells and TGF-β-induced T_{reg} 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_{reg} cells would lead to an up-regulation of GARP. Sorted $CD4^+CD25^-$ T_h cells were stimulated with anti-CD3/anti-CD28/IL-2 in the presence of TGF-β_1. Quantitative real-time RT-PCR analysis revealed that GARP was not up-regulated in such TGF-β_1-induced T_h cells although FOXP3 was present at high levels (Fig. S5A) and the cells displayed suppressor function (Fig. S5B). Thus, GARP is not required for the function of induced T_{reg} cells arguing that natural and induced T_{reg} 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_h cells [38].

GARP and FOXP3 are co-regulated during thymic T_{reg} cell development

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

Together, these results show that overexpression of GARP reprograms alloantigen-specific T_h 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_h 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_h 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.

LGMN 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_h 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_{reg} cells similar to GARP and FOXP3, a potential contribution to the GARP-FOXP3 feedback loop could be suggested. Therefore, we retrovirally transduced LGMN (T_hLGMN) and LGALS3 (T_hLGALS3) in antigen-specific T_h cells as described above.

Flow-cytometry and immunoblot analysis of T_hLGMN cells revealed that LGMN protein expression was comparable between

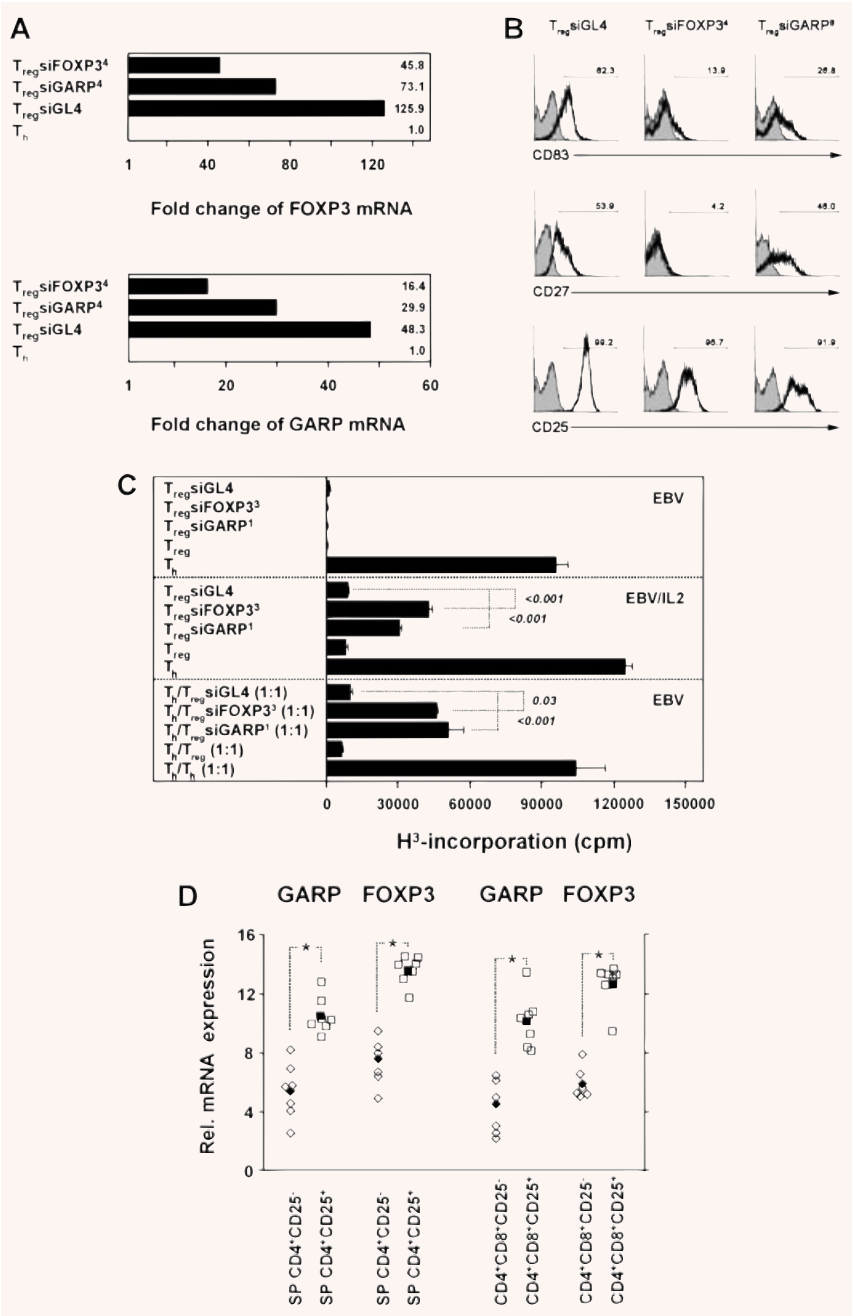

Fig. 5 Positive feedback loop between GARP and FOXP3 in human T_{reg} cells. (**A**) Real-time RT-PCR analysis of GARP and FOXP3 expression in human T_{reg} cells, lentivirally transduced with siRNA constructs specific for FOXP3 (T_{reg}siFOXP3^4), GARP (T_{reg}siGARP^4) or non-specific control (T_{reg}siGL4). Relative mRNA expression of T_h 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_{reg}siFOXP3^4 and T_{reg}siGARP^1 cells of compared to T_{reg}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 FOXP3 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.

T_hLGMN, T_hGARP and T_hFOXP3 cells but less up-regulated in T_hLGALS3 cells (Fig. S2B). LGALS3 protein expression was up-regulated in T_hLGALS3 cells. Interestingly, LGALS3 protein expression was higher in T_hGARP, T_hFOXP3 and T_{reg} cells and even T_hLGMN cells compared to T_hLGALS3 cells (Fig. 2B). This suggests that LGALS3 acts down-stream of GARP, FOXP3 and LGMN. As serine-6 phosphorylation of LGALS3 has been reported to control its function [40], we tested whether deletion of this caseine-kinase I site would affect its FOXP3-inducing capacity. Analyses of FOXP3 expression in activated T_h cells transduced with either wild-type or serine-to-alanine mutant LGALS3 revealed the importance of serine-6, because the mutant LGALS3 did not induce FOXP3 (Fig. S7A).

To understand the impact of LGMN and LGALS3 on the regulation of GARP and FOXP3 we analysed our alloantigen-specific T_h cells transduced with LGMN and LGALS3 for expression of GARP

and FOXP3. Analysis under resting conditions revealed that GARP protein expression was only minimally affected in T_hLGMN and T_hLGALS3 cells (Fig. 1D), while FOXP3 protein was obviously induced but not reaching the levels detected in T_hGARP or T_{reg} cells (Fig. 2A). Levels of CD25, CTLA4 and CD33 in T_hLGMN and T_hLGALS3 cells did also not reach that observed in T_hGARP and T_{reg} cells (Fig. 2B), whereas CD27 was obviously induced in T_hLGMN similar to T_hFOXP3 cells (Fig. 2B). Similar to T_hGARP and T_hFOXP3 cells, LGMN and LGALS3 overexpression did not affect CD83 or GITR expression (Fig. 2B).

Because activation of T_h cells up-regulates expression of FOXP3, we tested T_hLGMN and T_hLGALS3 cells for expression of FOXP3, LGALS3 and GARP after antigen-specific stimulation. Activation of T_hLGMN and T_hLGALS3 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 T_hGARP and T_{reg} cells. This suggests an involvement of LGMN and LGALS3 in the GARP-FOXP3 feedback loop mainly following T-cell activation.

LGMN and LGALS3 induce a partial T_{reg}-signature

We extended our analysis to the transcriptional control of genes of the T_{reg}-signature. Quantitative RT-PCR revealed repression of IL-2 transcription in T_hLGMN and T_hLGALS3 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 T_hLGMN and T_hLGALS3 cells (Fig. S3B), the presence of such levels of specific mRNA was not sufficient to induce T_{reg}-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 T_{reg}-signature were not up-regulated in T_hLGMN and T_hLGALS3 cells. These included RYR-1, HPGD and CPE (Fig. S3A). A further difference represented the up-regulation of IL7R observed only in T_hLGMN cells (Fig. S3A). In contrast, KLF-2 induction was observed only in T_hLGALS3 but not T_hLGMN 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 interrelation 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 T_hLGMN and T_hLGALS3 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-FOXP3 feedback loop. In line with that is the fact that LGMN and LGALS3 knock-out mice do not show obvious T_{reg} cell deficiencies [41, 42].

Discussion

In this study, we demonstrate that GARP represents a key receptor that is specifically induced in human $CD4^+CD25^{hi}$ regulatory T cells upon TCR stimulation. Unlike other T_{reg} markers like FOXP3, CD25 and CTLA4 that are shared between T_{reg} and activated $CD4^+CD25^-$ T_h cells, GARP is not induced in human naïve $CD4^+CD25^-$ T_h and antigen-specific T_h cells following TCR activation, nor in TGF-β_1-induced T_{reg} cells. Thus, GARP represents a specific marker suitable to differentiate natural derived $CD4^+CD25^{hi}$ T_{reg} cells from activated effector T_h cells and induced T_{reg} cells. We further demonstrate that GARP dominantly controls FOXP3 *via* a positive feedback loop able to stably reprogram antigen-specific T_h cells towards T_{reg} cells. Thus, GARP plays a key role in peripheral and most likely thymic T_{reg} 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 T_{reg} cells represent only a minor population of human $CD4^+$ T cells and GARP protein expression is induced in T_{reg} cells only following TCR stimulation, detection of GARP expression in T_{reg} 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 exerted 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 T_h 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

represent minor and redundant constituents of the FOXP3 regulating system controlled by GARP.

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_h cells in the presence of TGF-β did not induced 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 T_h 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 $CD4^+CD25^{hi}$ T_{reg} cells following TCR activation. GARP is sufficient to reprogram human alloantigen-specific T_h 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 T_{reg} cell-intrinsic tolerance mechanism as T_{reg} 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 T_{reg} cells in chronic infection, tumour immunotherapy, autoimmune diseases and transplantation.

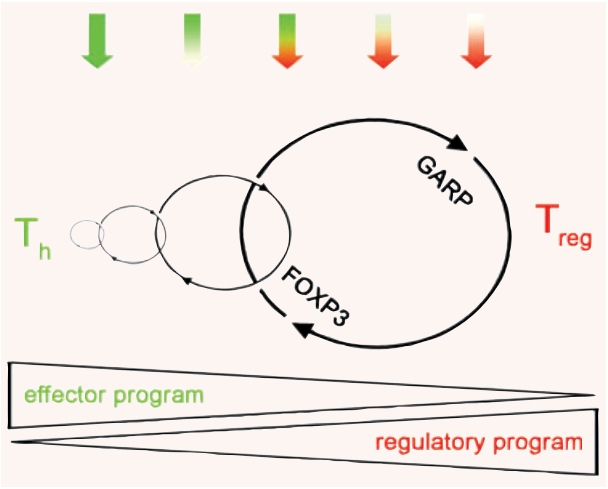

Fig. 6 Simplified model of the reprogramming or 'transdifferentation' of effector towards regulatory T cells *via* the GARP-FOXP3 positive feedback loop. The upper part illustrates the change in the 'quality' of TCR signalling outcome from effector (green) towards regulatory (green) TCR signalling. Thus, each TCR stimulation enhances the positive feedback indicated by the size of the feedback loop illustrated in the middle. For simplicity, other components of the regulatory network described, like LGALS3, LGMN, CD33, CD27 and CD83 or direct impairment of NFAT by GARP have been excluded. Identification of further components, fine tuning, timed-sequential expression and interconnectivity between the components of the regulatory network represents a major challenge for the molecular definition of the regulatory program.

Acknowledgements

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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: 2A0Z) 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 (▼) could have similar functions for dimerization as proposed for TLR3. Putative glycolysation 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 under resting conditions (no stim.) and 3 days after stimulation with anti-CD3 and 100 U/ml IL-2 as described in Fig. S1. (**B**) Quantitative real-time RT-PCR analysis of GARP and FOXP3 in T_h cells as in (**A**) tested under resting conditions and 3 days after stimulation as in (**A**). Relative mRNA expression of T_hGFP 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 alloantigen-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-β_1. 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-β_1-induced T_{reg} cells. (**A**) Sorted $CD4^+CD25^-$ T_h cells were stimulated with anti-CD3/-CD28/IL-2 without (T_h), in the presence of solvent ($T_h^{vehicle}$), and 10 ng/ml human TGF-β_1 ($T_h^{TGF\text{-}\beta1}$) 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-β_1-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_h cells is indicated, setting addition of wiltype T_h cells (T_h/T_h) 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 (T_hLGALS3) or mutant (T_hLGALS3^{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_hGFP 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_hGFP cells was arbitrarily set as 1.

Table S1 Differential gene expression of resting and activated $CD4^+CD25^{hi}$ 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_hGARP and T_hFOXP3 compared to T_hGFP cells

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