Regulation of the Expression of GARP/Latent TGF-β1 Complexes on Mouse T Cells and Their Role in Regulatory T Cell and Th17 Differentiation

Justin P. Edwards, Hodaka Fujii, Angela X. Zhou, John Creemers, Derya Unutmaz and Ethan M. Shevach

*J Immunol* published online 3 May 2013
http://www.jimmunol.org/content/early/2013/05/03/jimmunol.1300199

Supplementary Material

http://www.jimmunol.org/content/suppl/2013/05/03/jimmunol.1300199.DC1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Regulation of the Expression of GARP/Latent TGF-β1 Complexes on Mouse T Cells and Their Role in Regulatory T Cell and Th17 Differentiation

Justin P. Edwards,* Hodaka Fujii, † Angela X. Zhou,‡ John Creemers,§ Derya Unutmaz,‡ and Ethan M. Shevach*

GARP/LRRC32 was defined as a marker of activated human regulatory T cells (Tregs) that is responsible for surface localization of latent TGF-β1. We find that GARP and latent TGF-β1 are also found on mouse Tregs activated via TCR stimulation; however, in contrast to human Tregs, GARP is also expressed at a low level on resting Tregs. The expression of GARP can be upregulated on mouse Tregs by IL-2 or IL-4 exposure in the absence of TCR signaling. GARP is expressed at a low level on Tregs within the thymus, and Treg precursors from the thymus concomitantly express GARP and Foxp3 upon exposure to IL-2. The expression of GARP is independent of TGF-β1 and TGF-β1 loading into GARP and is independent of furin-mediated processing of pro–TGF-β1 to latent TGF-β1. Specific deletion of GARP in CD4+ T cells results in lack of expression of latent TGF-β1 on activated Tregs. GARP-deficient Tregs develop normally, are present in normal numbers in peripheral tissues, and are fully competent suppressors of the activation of conventional T cells in vitro. Activated Tregs expressing GARP/latent TGF-β1 complexes are potent inducers of Th17 differentiation in the presence of exogenous IL-6 and inducers of Treg in the presence of IL-2. Induction of both Th17-producing cells and Tregs is caused preferentially by Tregs expressing the latent TGF-β1/GARP complex on their cell surface rather than by secreted latent TGF-β1. The Journal of Immunology, 2013, 190: 000–000.

The three mammalian TGF-β genes encode a translation product consisting of an N-terminal propeptide (termed latency-associated peptide [LAP]) and bioactive TGF-β. This product (referred to in this article as pro–TGF-β) is cleaved intracellularly by furin, and LAP remains noncovalently associated with TGF-β to form the small latent complex. In most cells, the small latent complex is covalently attached to latent TGF-β–binding proteins (LTBPs) prior to secretion. Activated Foxp3+ T regulatory cells (Tregs) express a distinct LTBP termed GARP/LRRC32 (1) that is required for surface expression of latent TGF-β1 on human Tregs, as well as platelets (2–4). Recombinant latent TGF-β1 was found to bind directly to GARP by both covalent and noncovalent interactions, and GARP was critical for tethering latent TGF-β1 to the cell surface. GARP was also shown to outcompete LTBP for binding to latent TGF-β1 (5). Latent TGF-β does not have biological activity, and the release of active TGF-β from LAP is a critical regulatory step for TGF-β function and signaling. Active TGF-β can be released from the latent TGF-β/LTBP complex by the action of aV integrins, and it was reported recently that TGF-β is released from the latent TGF-β/GARP complex through similar mechanisms (5).

The contribution of the GARP/latent TGF-β1 complex to the suppressor function of Tregs remains unclear. It was originally proposed that ectopic expression of GARP in non-Tregs induced the expression of Foxp3 and endowed the cells with partial suppressive function (1). Other studies claimed that GARP was required for the stability of the human Treg, because lentiviral-mediated downregulation of GARP expression resulted in reduced suppressor function and was associated with downregulation of Foxp3 (6). Downregulation of Foxp3 resulted in a concomitant downregulation of GARP. However, more recent studies demonstrated that Foxp3 was not essential for the expression of GARP and LAP on human Tregs, because the expression of GARP and LAP was completely normal following small interfering RNA (siRNA)-mediated knock down of Foxp3. Furthermore, transduction of GARP into Foxp3− T cells allowed for the surface expression of LAP but no expression of Foxp3 (2). The in vitro suppressive function of Tregs with complete siRNA-mediated knock down of either GARP or TGF-β1 was reduced only modestly.

The role of GARP in Treg function has been analyzed using human Tregs. In this study, we describe the expression of the GARP/latent TGF-β1 complex by mouse Tregs. We find that GARP is expressed at low levels on resting Tregs, and its expression is upregulated rapidly via TCR stimulation. Surface expression of GARP is followed by the surface expression of latent TGF-β1. Upregulation of GARP expression can also be induced by culture of Tregs in the presence of IL-2 and IL-4. Expression of GARP is not dependent upon the expression of TGF-β1, because it is retained in TGF-β1−deficient Tregs. In contrast to some of the
early studies on GARP and its potential role in Treg suppressor function, GARP-deficient Tregs developed normally and were competent suppressors of T cell proliferation in vitro. Lastly, we found that active mouse Tregs that express the GARP-laten TGF-β1 complex on their cell surface are potent inducers of both Th17 differentiation in the presence of IL-6 and Treg differentiation in the presence of IL-2. Induction of Th17-producing cells and Foxp3Tregs is caused preferentially by Tregs expressing the latent TGF-β1/GARP complex on their cell surface, rather than by secreted latent TGF-β1.

Materials and Methods

Mice
C57BL/6 and B10.A mice were purchased from Dax, Foxp3-GFP, OVA-specific TCR-transgenic OT-II (CD45.1, Rag2−/−), and IgE-specific TCR-transgenic 5C7 (CD45.1, Rag2−/−) mice were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) and were maintained by Taconic Farms (Germantown, NY) under contract to NIAID. OT-II mice were obtained from Taconic Farms and bred to Foxp3-GFP mice. TGF-β1−/− mice (7) were generously provided by D. Ming Li (Slam-Kettering Memorial Institute, New York, NY). Furinfl/fl mice (8) were generously provided by Dr. John O'Shea (National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institutes of Health, Bethesda, MD). GARP (LRRC32)−/− mice, which have not been described previously, were generated in the laboratory of H.F. in collaboration with D.U. and are described below. CD4CRE mice were purchased from Taconic Farms. All animal protocols used in this study were approved by the NIAID Animal Care and Use Committee.

Generation of GARP (LRRC32) floxed mice

To generate mice harboring a GARP allele flanked by loxp sites, a targeting vector was constructed with a 4.94-kb 5′ homology region, a 2.2-kb 3′ homology region, a neo cassette (3′−5′ orientation) flanked with FRT and loxp sites, and a fragment in which exon 1 was flanked by loxp sequences (Supplemental Fig. 1A–E). A total of 10 μg the targeting vector was linearized by NotI and electroporated into iTIL BA1 (C57BL/6 × 129/SvEv) hybrid embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for PCR and Southern blot analysis. DNA was digested with BamHI and electrophoretically separated on a 0.8% agarose gel. After transfer to a nylon membrane, the digested DNA was hybridized with a probe targeted against the 3′ external region. DNA from C57BL/6 (B6), 129/SvEv (129), and B1A (C57BL/6 × 129/SvEv) (hybrid) mouse strains were used as wild type (WT) controls (Supplemental Fig. 1F). The expected sizes were 5.1 and 4.6 kbp. The probe used for Southern blot analysis was amplified by genomic PCR. The sequence of the probe (527 bp) is listed in Supplemental Fig. 1G. After karyotyping, the positive clones were injected into C57BL/6 blastocysts to generate chimeric mice.

Cell isolation and flow cytometry

For purification of dendritic cells (DCs), mouse spleens were fragmented and digested for 30 min at 37°C in the presence of liberase blendzyme II (Roche) and DNase (2 μg/ml; Roche) in complete medium (modified RPMI 1640 supplemented by 10% FBS [HyClone], 50 μM 2-ME [Sigma-Aldrich], 1% sodium pyruvate, 1% nonessential amino acids, 1% HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine). They were labeled with anti-CD11c beads and purified on the AutoMACS (Miltenyi Biotec). T cells from pooled lymph nodes and/or spleens were isolated using CD4 beads. T cells from pooled lymph nodes and/or spleens were stained using the following Abs (BioLegend): anti-CD45.2 (104), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-Foxp3 (FLK-16s), anti-GARP (AFKJS-9), and anti-IL-17A (eBio17B7). CD25 (PC61.5), RORγt (AFKJS-9), and Tbet (eBio4B10) (all from BioeScience, San Diego, CA). Purified mouse anti-mouse LAP clone TW7-16B4 (9) was generously provided by Howard Weiner (Harvard Medical School, Boston, MA). The LAP Ab was labeled with SureLight-APC at Columbia Biosciences (Columbia, MD). Unlabeled cell-culture grade anti-LAP clone TW7-20B9 was also obtained from BioLegend (San Diego, CA). For staining of Foxp3, Tbet, or RORγt, cells were fixed and permeabilized using the FixP3 Fixation/Permeabilization Staining kit (eBioscience). For staining of cytokines, cells were fixed and permeabilized using the Cytofix/Cytoperm kit from BD Biosciences.

Cytokines and other reagents

IL-4, IL-6, IL-7, IL-9, IL-15, IL-21, and TNF-α were all purchased from PeproTech and used at 10 ng/ml. Recombinant human active TGF-β1, latent TGF-β1, and recombinant LAP were all purchased from R&D Systems.

In vitro T cell stimulation and differentiation

To induce Th17 differentiation, sorted Tregs were activated and expanded using plate-bound anti-CD3 (1 μg/well; 24-well plate) with IL-2 (100 U/ml) for 2–3 d, followed by overnight culture with IL-2 alone; they were generally >90–95% Foxp3+. Tregs were washed and mixed 1:1 with naive CD45.1+ OT-II cells (RAG1−/−) or CD45.1+ Marilyn cells (RAG2−/−) in the presence of recombinant mouse IL-6 (10 ng/ml) and stimulated with plate-bound anti-CD3 for 4 d. In some experiments, T cells were stimulated with DCs plus soluble anti-CD3 (1 μg/ml), OVA peptide (323–333), or Dby HY peptide (Marilyn specific; AnaSpec). Control Th17 differentiation was performed by stimulating naive OT-II T cells in the presence of IL-6 (10 ng/ml) and recombinant human TGF-β1 (5 ng/ml), in the presence or absence of the neutralizing anti-IL-2 mAb (Clone S4B6). Differentiated T cells were stimulated with the Cell Stimulation Cocktail and Protein Transport Inhibitors (eBioscience).

To induce Foxp3 expression in vitro, CD4+ T cells from 5C7 (CD45.1, Rag2−/−) mice were stimulated with soluble anti-CD3 (1 μg/ml), IL-2, TGF-β1 (5 ng/ml), and irradiated T-depleted (Thy1.1) spleen cells. In other experiments, naive cells (CD4+CD25−CD62L−CD44−) from GARP T cell–conditional KO mice were stimulated with plate-bound anti-CD3, IL-2, and TGF-β1 for 4 d.

In some experiments, preactivated Tregs were used to induce Foxp3+ T cells from naive CD4+ T cells. In these experiments, preactivated Tregs were washed and mixed 1:1 with naive CD45.1+ OT-II cells (RAG1−/−) in the presence of recombinant human IL-2 (100 U/ml) and stimulated with soluble anti-CD3 (1 μg/ml) and DCs for 4 d.

In vitro suppression assay

In vitro suppression assays were performed as previously described (10).

In vivo inducible Treg differentiation

In vivo inducible Treg (iTreg) differentiation was performed as previously described (11). Briefly, CD4+ cells (105) from 5C7 (CD45.1 Rag2−/−) mice were transferred i.v. to normal B10.A recipients, which were injected i.v. 24 h later with 0.5 μg moth cytochrome c (MCC) peptide. GARP and Foxp3 expression were assessed 12–14 d posttransfer.

IL-2 immune complex treatment

C57BL/6 mice were treated with IL-2 immune complexes, as previously described (11). Briefly, IL-2−/−anti–IL-2 mAb complexes were prepared by mixing recombinant murine IL-2 (1 μg; PeproTech) with JES6-1 (5 μg) at the optimal 1:2 molar ratio and incubated for 10 min at room temperature. Immune complexes were then diluted in PBS and injected i.p. on days 0, 1, and 2. Expansion of Tregs was ~3-fold in the spleen (~45% of CD4+ T cells) by day 4.

mRNA isolation, cDNA production, and real-time PCR

mRNA isolation was performed with various γc-chain–associated cytokines and then subjected to RNA extraction using TRIzol reagent. The contaminating DNA was removed by DNase I treatment. ThermoScript RT-PCR system (InVitrogen Life Technologies) was used to generate cDNA from RNA using oligo(dT)15 primers. Real-time PCR was conducted with the ABI Prism 7900HT, using the Kapa Probe Fast Universal qPCR Kit and TaqMan Probes for GAPDH and LRRC32 (GARP).

Results

Expression of GARP and latent TGF-β1 by mouse Tregs

GARP (LRRC32) was described to be a marker of activated human Tregs and serves as a receptor for latent TGF-β1 in vitro and in vivo, we initially performed a detailed study of its expression on mouse T cells. We first analyzed un-
separated CD4+ T cells from the lymph nodes of C57BL/6 mice for expression of the LAP portion of latent TGF-β1 and GARP. Fresh CD4+Foxp3+ cells expressed a low level of GARP that increased upon stimulation with anti-CD3 and IL-2 (Fig. 1A). A small percentage of the GARP+ Tregs coexpressed LAP under resting conditions. Although nearly all Foxp3+ Tregs maximally upregulated GARP expression after 24 h of stimulation, coexpression of latent TGF-β1 (LAP) was not fully gained until 48–72 h of stimulation. GARP was not detected on CD4+Foxp3− cells under resting conditions, but it was detectable on a small subpopulation after long periods of stimulation in vitro. Importantly, LAP was almost exclusively expressed on GARP-expressing Foxp3+ Tregs, but it was also detected on the minor population of Foxp3− T cells that expressed GARP after 72 h of stimulation. LAP could also be detected on the minor Foxp3+ (~0.5%) population of CD4+CD8+ T cells.

In preliminary studies, we noted that culturing Tregs overnight in media alone resulted in upregulation of GARP, and GARP expression was further enhanced in media supplemented with IL-2 alone (Fig. 1B). Therefore, we investigated whether other γc-chain–signaling cytokines or TNF-α could also enhance the expression of GARP. Sorted CD4+Foxp3+ (GFP+) T cells were cultured overnight in media supplemented with either IL-2 (human), IL-4, IL-7, IL-9, IL-15, IL-21, or TNF-α in the presence of neutralizing anti-mouse IL-2. IL-2 and IL-4 were equally capable of enhancing GARP expression, whereas IL-7 and IL-15 enhanced it to a lesser extent (Fig. 1B). All others cytokines had minimal, if any, effect on GARP expression. Furthermore, IL-2 and IL-4 stimulation enhanced the expression on GARP mRNA (Supplemental Fig. 2A), indicating that the enhanced cell surface expression of GARP/LAP also involved de novo synthesis. To investigate whether IL-2 was capable of enhancing GARP expression on Tregs in vivo, IL-2 immune complexes (JES6-1:IL-2) were administered i.p. for 3 d, and GARP/LAP was measured on day 5. These complexes were described to direct IL-2 to IL-2Rα-expressing cells (CD25), resulting in considerable expansion of Tregs (12). We found that these complexes not only expanded the Treg population, they also resulted in enhanced GARP/LAP expression in vivo (Fig. 1C).

Because we found that culturing Tregs overnight in media alone or in IL-2/IL-4 without TCR stimulation resulted in a measurable increase in the surface expression of GARP, we investigated whether Tregs have intracellular stores of GARP. We found that staining of fresh Tregs prior to and after fixation/permeabilization resulted in significant increases in both the percent positive and mean fluorescence intensity (MFI) of both GARP (MFI, 152 to 327) and LAP (MFI, 87.8 to 177), as well as increased the percentage of LAP/GARP coexpressers, indicating that there are intracellular stores of GARP and LAP prior to activation (Fig. 1D). Preblocking the cell surface with unlabeled GARP and LAP Abs

**FIGURE 1.** Expression of GARP and latent TGF-β1 by Tregs. (A) GARP and LAP staining of freshly isolated lymph node cells gated on CD4+Foxp3− and CD4+Foxp3+ or CD4+enriched cells stimulated for 24, 48, or 72 h with plate-bound anti-CD3+IL-2. Quadrants are set based on background staining from isotype controls throughout. (B) Sorted CD4+Foxp3+GFP+ cells were immediately stained for GARP (Fresh) or cultured in the presence of anti-IL-2 alone or anti-IL-2 plus IL-2 (human), IL-4, IL-7, IL-9, IL-15, IL-21, or TNF-α, and GARP was measured by FACS. (C) IL-2/anti-IL-2 mAb complexes were injected i.p. on days 1–3. Splenocytes were stained for GARP/LAP on day 5. Gated on CD4+Foxp3+ cells. (D) Freshly isolated lymph node cells gated on CD4+Foxp3− cells were stained with isootype controls, surface stained for GARP/LAP, stained for GARP/LAP before and after fixation/permeabilization, or blocking of GARP/LAP with 10× unlabeled Ab prior to fixation/permeabilization, followed by intracellular staining for GARP/LAP.

Downloaded from http://www.jimmunol.org/ by guest on April 17, 2017
prior to fixation and staining allowed us to selectively visualize the considerable contribution of intracellular GARP/LAP.

The expression of GARP was investigated in various lymphoid tissues, including the peripheral lymph nodes, mesenteric lymph nodes, spleen, Peyer’s patches, and thymus. We found that GARP was expressed at similar levels on resting Tregs in the peripheral lymph nodes, mesenteric lymph nodes, and spleen (Supplemental Fig. 2B, 2C). Similarly, the coexpression of LAP and GARP was similar in these locations (Supplemental Fig. 2C). In contrast, Tregs in the thymus and Peyer’s patches expressed much lower levels of GARP and GARP/LAP complexes (Fig. 2A, Supplemental Fig. 2B, 2C).

Expression of GARP is acquired in the thymus simultaneously with Foxp3

Although GARP and the GARP/LAP complex were expressed at very low levels on thymic Tregs, we determined at what developmental stage thymic Tregs acquired GARP expression. We sorted CD4⁺CD8⁻Foxp3⁻CD25hi Treg precursors from thymi of Foxp3-GFP–knockin mice. As reported previously (13), culture of these precursors with IL-2 resulted in induction of Foxp3 expression in a substantial proportion of these cells. GARP expression was also induced by culture in IL-2 and was selectively expressed on the Foxp3⁺ subpopulation (Fig. 2B, 2C).

GARP is expressed by in vitro– and in vivo–generated iTregs

iTregs were induced in vitro from TCR-transgenic 5CC7 cells on a Rag2⁻/⁻ background by activation with soluble anti-CD3 and anti-CD28, and exposure to IL-2 (100 U/ml) and TGF-β1. The cells were then stained for CD4, Foxp3, GARP, and LAP. As shown in Figure 3A, IFN-γ (left panel) and IL-17A (right panel) expression were not detected in these cells. Figure 3B shows that 88% of the transferred Foxp3⁺ cells expressed GARP⁺. Figure 3C shows the average percentage of GARP⁺ cells of transferred CD4⁺CD45.1⁺ cells from five mice. Data are mean ± SD (n = 5 mice).
irradiated T cell–depleted spleen cells in the presence of TGF-β1 and IL-2. A high percentage of the cells expressed Foxp3 (Fig. 3A). Surprisingly, Foxp3+ and Foxp3− cells expressed similar levels of GARP and LAP. This result differs markedly from our previous studies on human T cells induced to express Foxp3 in vitro under similar culture conditions that were GARP and LAP negative (2).

To examine the expression of GARP/LAP on Tregs in vivo, we used a previously established system in which pigeon cytochrome c–specific-transgenic (5CC7) T cells on a RAG2−/− background are transferred into B10.A mice, followed by a low dose of MCC peptide (11). Approximately 2 wk posttransfer, 5CC7 cells were assessed for their expression of Foxp3 and GARP. A total of 10–30% of 5CC7 cells gained expression of Foxp3, and the majority of the Foxp3+ population expressed GARP (Fig. 3B, 3C). This result differs from the studies on in vitro–generated iTregs in which both the Foxp3+ and Foxp3− populations expressed GARP/LAP. It remains possible that activation in the presence of IL-2 and TGF-β1 may be sufficient to induce the expression of GARP in vitro, whereas the expression of Foxp3 seems to be required to stably express GARP in vivo.

Expression of GARP is independent of TGF-β1, and loading of TGF-β1 is independent of furin processing

Because GARP is expressed concomitantly with latent TGF-β1, we investigated the dependence of GARP expression on the expression of TGF-β1. We crossed TGF-β1fl/fl mice to CD4-CRE mice (7). Unstimulated CD4+Foxp3+ cells from homozygous floxed CRE+ mice expressed GARP at levels similar to CRE− littermates, but they did not express latent TGF-β1 (Fig. 4A, left panels). Furthermore, stimulation of the TGF-β1−/− CD4+ T cells with anti-CD3 and IL-2 in serum-free media resulted in upregulation of GARP to levels similar to those of the CRE− littermates but with no expression of latent TGF-β1 (Fig. 4A, right panels). To determine whether TGF-β1−/− CD4+ T cells could bind free latent TGF-β1, unfractionated CD4+ T cells were activated for 48 h, incubated with human latent TGF-β1, washed, and stained for human LAP (Fig. 4B). Expression of LAP could readily be detected on Foxp3+ T cells but not on Foxp3− T cells.

TGF-β1 is produced as an inactive precursor polypeptide (pro–TGF-β) that is proteolytically processed by the preprotein convertase, furin, to produce latent TGF-β1, which must be processed further to produce biologically active TGF-β1 (14). To determine whether furin processing of pro–TGF-β1 is required for binding to GARP, furin−/− mice were crossed to CD4-CRE mice (8, 15), CRE recombinase–mediated deletion of furin exon 1 in CD4+ T cells was confirmed by PCR (data not shown). Unstimulated CD4+ Foxp3+ cells from the homozygous furin−/−CRE+ mice coexpressed GARP and latent TGF-β1 at levels similar to the CRE− littermates (Fig. 4C, left panels). Furthermore, stimulation of furin−/− T cells from CRE+ mice with anti-CD3 and IL-2 in serum-free media resulted in upregulation of the GARP/latent TGF-β1 complex to levels similar to those seen in the CRE− littermates (Fig. 4C, right panels). Therefore, furin processing of pro–TGF-β1 to latent TGF-β1 is not required for loading onto GARP.

To confirm previously published results on human T cells that GARP is required for the surface expression of latent TGF-β1 in the murine system, we crossed GARP−/− mice to CD4-CRE mice. Because these mice have not been described previously, we examined Treg and Tconv development. The percentages of thymocyte subpopulations (Supplemental Fig. 3A, upper panels), including the percentages of Foxp3+ cells within the CD4 single-positive population (Supplemental Fig. 3A, lower panels), were identical in CRE+ and CRE− littermates. The percentages of

FIGURE 4. Expression of GARP/LAP on TGF-β1−, furin−, and GARP-deficient Tregs. Staining of GARP/LAP CD4+Foxp3+–gated cells from lymph nodes or on CD4-enriched cells after activation with plate-bound anti-CD3 and IL-2 in serum-free media. Cells are from rgeb100 × CD4-CRE (A), furin000 × CD4-CRE (C), and Irec3500 × CD4-CRE (GARP deficient) (D) mice. (B) CD4+ T cells from rgeb100 × CD4-CRE mice were stimulated as in (A) for 48 h, incubated in fresh media with (black lines) or without (gray lines) recombinant human latent TGF-β1 for 30 min at 37°C, washed thoroughly, and stained with anti-human LAP. Gating on CD4+ Foxp3+ cells (left panel) or CD4+Foxp3− cells (right panel).
CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ T cells in lymph nodes and spleen were also identical in CRE⁺ and CRE⁻ littermates (Supplemental Fig. 3B). In addition, the level of CD25 expression was similar on Foxp3⁺ T cells from CRE⁺ and CRE⁻ littermates (Supplemental Fig. 3C). Therefore, it does not seem that expression of GARP is required for the normal development of Tregs in the thymus or for their maintenance in the periphery. Fresh CD4⁺Foxp3⁺ cells from GARPfl/fl-CRE⁺ mice did not express GARP, nor did they have any surface latent TGF-β1 expression, compared with CRE⁻ littermates (Fig. 4D, left panels). Furthermore, stimulation with anti-CD3 and IL-2 in serum-free media resulted in the upregulation of GARP and latent TGF-β1 only in CRE⁻ littermates (Fig. 4D, right panels), thus confirming previous reports (2–4) that GARP is required for surface expression of TGF-β1 on Tregs.

To determine whether GARP is required for in vitro differentiation of iTregs, naive CD4⁺CD25⁻CD62LhiCD44low T cells from GARPfl/fl-CRE⁺ and CRE⁻ littermates were stimulated with plate-bound anti-CD3 for 4 d in the presence of TGF-β1 and IL-2. No difference was observed in the induction of Foxp3⁺ cells in the presence and absence of GARP expression (Supplemental Fig. 3D).

Expression of GARP or TGF-β1 is not required for Treg suppression in vitro

To determine the ability of GARP⁻/⁻ Tregs to act as suppressor cells in vitro, we sorted CD4⁺CD25⁺ cells from GARPfl/fl-CRE⁺ and CD4⁻CRE⁻ mice. WT and GARP⁻/⁻ Tregs were equally effective in suppressing the proliferation of CD4⁺ responder cells (Fig. 5A). Similarly, Tregs from TGF-β1⁻/⁻ mice were as effective as Tregs from WT mice in suppressing responder cell proliferation (Fig. 5B), as reported previously (9). Furthermore, Tregs from GARP⁻⁻ mice were similar to Tregs from WT mice in that they were nonresponsive to TCR stimulation alone, but they proliferated vigorously in response to stimulation with anti-CD3 and IL-2 (Fig. 5C). Thus, neither cell surface–associated nor secreted TGF-β1 plays a major role in the standard in vitro assays for Treg function.

**Treg-mediated Th17 differentiation and Treg-mediated Foxp3 induction are primarily mediated by GARP-associated TGF-β1**

It was reported that, in the presence of IL-6, activated Tregs are capable of driving differentiation of naive CD4⁺ T cells to Th17 cells (16). To directly determine the contributions of secreted versus cell-associated TGF-β1 to the induction of Th17 cells, we activated sorted Tregs from Foxp3-GFP mice for 72 h so that they would express high levels of the GARP/LAP complex on the cell surface. The activated Tregs were then cocultured with congenically marked naive T cells in the presence of IL-6, and the cocultures were restimulated with plate-bound anti-CD3 for 4 d. Approximately 40% of the naive cells expressed IL-17A upon restimulation with PMA and ionomycin (Fig. 6A). Surprisingly, this level of IL-17 induction was similar to that seen when the naive responder cells were stimulated in the presence of TGF-β1, IL-6, and anti-IL-2, conditions that we previously showed to be optimal for induction of Th17 cells (17). Furthermore, these cells lacked the ability to produce IL-10, similar to those stimulated in the presence of TGF-β1, IL-6, and anti-IL-2 and in contrast to those stimulated in the presence of TGF-β1 and IL-2 (Supplemental Fig. 4A). Additionally, these cells expressed RORγt while lacking the expression of Foxp3 and T-bet. This is in contrast to those stimulated in the presence of TGF-β1 and IL-6, which not only expressed RORγt, but also expressed considerable Foxp3 (Supplemental Fig. 4B).

**Induction of Th17-producing cells in the presence of activated Tregs was completely inhibited by the pan-neutralizing TGF-β Ab 11D1 (Fig. 6B) and substantially by recombinant human LAP. Th17 induction was also inhibited by 60–70% by anti-mouse LAP (clone TW7-16B4) but not by a second anti-LAP mAb (TW7-2089) that recognizes an epitope distinct from that recognized by TW7-16B4 (9) (Fig. 6C). Anti-GARP (clone YGIC86) (Fig. 6C) or two different anti-GARP mAbs generated in-house had no inhibitory effects on Treg-mediated Th17 induction (data not shown).

These studies suggest that Treg-mediated Th17 differentiation requires their production of TGF-β1 and likely involves conversion of latent TGF-β1 to the active form. It remains possible that inhibitory effects of anti-LAP mAbs are mediated by interfering with the conversion of latent TGF-β1 to active TGF-β1. To determine whether the source of the active TGF-β1 needed for Treg-mediated Th17 differentiation is the cell surface–associated GARP/latent TGF-β1 complex or secreted latent TGF-β1, we compared the capacity of activated CD4⁺CD25⁺ cells from T cell conditional (CD4⁻CRE⁺) TGF-β1⁻⁻, Furin⁻⁻, and GARP⁻⁻ mice to drive Th17 differentiation with that of their WT counterparts (CD4⁻CRE⁻). Induction of Th17 cells was completely dependent on T cell–produced TGF-β1, because Tregs from TGF-β1⁻⁻ mice were unable to drive Th17 differentiation (Fig. 7A). Furthermore, Tregs from Furin⁻⁻ mice were also completely un-

**FIGURE 5.** Neither GARP nor TGF-β1 contributes to Treg suppression. (A) WT T effector cells (CD4⁺CD25⁻, 5 × 10⁵) were activated with soluble anti-CD3 and irradiated T cell–depleted splenocytes in the presence or absence of WT or GARP⁻⁻ Tregs from lrrc32floflo × CD4-CRE mice at an effector/Treg ratio of 16:1–1:2 for 3 d. Cultures were pulsed with [³H]thymidine for the last 6 h of culture. (B) As in (A), except that WT or TGF-β1⁻⁻ Tregs from lrrc32floflo × CD4-CRE mice were used. (C) WT or GARP⁻⁻ Tregs from lrrc32floflo × CD4-CRE mice were stimulated with soluble anti-CD3 and irradiated T cell–depleted splenocytes in the presence or absence of IL-2. Cultures were pulsed as in (A) after 3 d.
able to drive Th17 differentiation (Fig. 7B), most likely because the TGF-β1 produced by furin+/−/− Tregs remains in the proprotein form. The induction of Th17 cells in the presence of GARP+/−/− Tregs was reduced by ∼75%, demonstrating that the majority of the activated TGF-β1 mediating Th17 induction was derived from the cell surface–associated GARP/latent TGF-β1 complex (Fig. 7C).

Previously, our group and other investigators (18) described the ability of activated Tregs to drive the differentiation of naive T cells to express Foxp3 when activated in the presence of IL-2 and reported that this was dependent upon TGF-β1 produced by Tregs. To determine whether the induction of Foxp3 expression was dependent upon the GARP/latent TGF-β1 complex, naive T cells were activated with anti-CD3 in the presence of activated polyclonal Tregs from CD4-conditional KOs of GARP or their WT counterparts (CD4-CRE2) and IL-2. GARP+/−/− Tregs were unable to induce Foxp3 expression efficiently in naive T cells in comparison with GARP-sufficient Tregs (Fig. 7D).

Treg-mediated Th17 differentiation requires TCR reactivation

Thus far, both the activated Tregs and the naive responder cells were simultaneously activated via the TCR for induction of IL-17–producing cells. To determine whether TCR reactivation of the activated Tregs was required for induction of Th17 cells, activated polyclonal Tregs were cultured with naive OT-II CD4+ T cells and OVA. No induction of Th17 cells was seen in the presence of OVA323–329 and polyclonal Tregs (Fig. 8A). In contrast, Th17 cells were induced with activated polyclonal Tregs in the presence of IFN-γ. Thus, although activated Tregs express high levels of the GARP/latent TGF-β1 complex, they must be reactivated via the TCR to initiate release and activation of latent TGF-β1 required for induction of Th17 cells.

To determine whether activated Tregs could mediate induction of Th17 cells by a bystander pathway, we cultured activated OT-II Tregs and naive T cells from congenically marked Marilyn mice that are specific for an HY peptide (Dby) in the presence of OVA323–329, Dby (Fig. 8B). As expected, culture of Marilyn T cells with IL-6 alone did not result in Th17 differentiation, whereas culture with IL-6 and OT-II Tregs in combination drove Th17 differentiation. Therefore, Tregs responding to different Ags presented by the same APCs can drive Th17 differentiation from naive T cells.

Discussion

We report an analysis of the expression of GARP and the GARP/latent TGF-β1 complex on mouse Tregs and highlight potential functions of the complex on Tregs. Resting mouse Tregs express low levels of GARP, and much of this constitutive expression is not complexed with latent TGF-β1. The expression of GARP is upregulated by culture of Tregs in media alone in the absence of cytokine, and it can be upregulated further by the addition of IL-2 or IL-4. TCR stimulation induces an initial upregulation of GARP.
expression that is followed rapidly by detection of the GARP/latent TGF-β1 complex. A large proportion of this initial “burst” in surface expression of GARP may be due to its release from intracellular stores. We observed that the baseline surface expression of GARP/latent TGF-β1 varies in different tissues tested, which may be a reflection of the cytokine milieu in different sites or the activation status of the Tregs (Fig. 2, Supplemental Fig. 2). Thymic Tregs, in general, expressed a low, but reproducible, level of GARP, consistent with their resting state. When Treg precursors were induced to express Foxp3 by culture in IL-2, the induction of Foxp3 expression was accompanied by GARP expression.

Both in vivo– and in vitro–generated iTregs expressed the GARP/LAP complex. This result should be contrasted with results of studies on human T cells, which failed to express GARP or the GARP/latent TGF-β1 complex (2) following in vitro induction of Foxp3 expression by TCR stimulation in the presence of TGF-β1. However, it should be noted that the human Foxp3+ T cells induced in vitro lacked Treg suppressor function and produced IL-2. We also observed that a substantial proportion of mouse T cells in the iTreg generation cultures that did not express Foxp3 expressed the GARP/latent TGF-β1 complex. In contrast, in our in vivo Treg-induction model, GARP/latent TGF-β1 complexes could only be detected on cells that expressed Foxp3. It is likely that expression of Foxp3 is required for stable expression of GARP/LAP; however, in the artificial in vitro situation, the presence of TGF-β1 and IL-2 is sufficient to induce their expression. We could only detect very low percentages of Foxp3+GARP/latent TGF-β1–expressing cells in any lymphoid population studied, consistent with other studies. Thus, expression of GARP represents an excellent marker of bona fide Foxp3+ Tregs in the mouse, although it does not distinguish thymic-derived Tregs from peripherally induced Tregs.

Expression of GARP was not dependent upon the expression of latent TGF-β1, because cells from T cell–conditional TGF-β1−/− mice expressed levels of GARP equivalent to those detected on
WT Tregs both before and after T cell activation. This result confirms studies in which activated human T cells continued to express GARP after siRNA-mediated knock down of TGF-β1 (2). As was observed with human Tregs, we could reconstitute LAP expression on activated mouse Tregs from TGF-β1−/− mice by incubation with human recombinant latent TGF-β1. We could not detect binding of latent TGF-β1 to activated Foxp3+ T cells. Thus, the GARP/latent TGF-β1 complex can be generated intracellularly, as well as extracellularly. It remains to be determined what fraction of the GARP/latent TGF-β1 complex is created by these two mechanisms during T cell activation. It is also possible that free GARP molecules on the cell surface may compete with LTBP for binding of secreted latent TGF-β1. We could readily detect the GARP/latent TGF-β1 complex on the surface of activated Tregs from T cell−conditioned furin−deficient mice, indicating that both prolatent TGF-β1 and furin−processed latent TGF-β1 could interact with GARP.

Using CD4−conditional KOs of GARP, we confirm the previously published siRNA data that showed the requirement of GARP for expression of latent TGF-β1 on the cell surface of human Tregs. The contribution of cell surface−expressed latent TGF-β1 in mediating the Treg suppression of responder T cell activation has been controversial (1, 2, 4, 6). A modest reduction in suppressor capacity was observed following siRNA−mediated knock down of human GARP. However, GARP−/− mouse Tregs were as suppressive as were WT Tregs in mediating suppression in vitro, and they maintained their anergic phenotype because they failed to proliferate in response to stimulation with anti−CD3 alone. Although GARP−/− Tregs can still secrete latent TGF-β1, as measured by ELISA (data not shown), it is unlikely that this secreted latent TGF-β1 plays any role in Treg suppression in vitro, because the suppressive ability of TGF-β1−/− Tregs is unimpaired (7).

Because previous studies suggested that activated Treg could also induce Th17 cells in the presence of IL−6 (16), we explored, in depth, the role of the GARP/latent TGF-β1 complex in this in vitro assay. Activation of cocultures of activated Tregs and naive responder T cells in the presence of IL−6 resulted in a high efficiency of induction of IL−17−producing cells, approximating the number of IL−17 producers observed in cultures of naive responder cells in the presence of TGF−β1 and IL−6. The induction of IL−17−producing cells was markedly inhibited by recombinant LAP, certain mAbs to LAP, and anti−TGF−β, indicating that activated Tregs released latent TGF−β1 following reactivation and that the latent TGF−β1 was then processed to active TGF−β1. Activated Tregs from T cell−conditioned KOs of TGF−β1 or of furin failed to induce IL−17−cells, indicating that Treg−derived TGF−β1 was the major source of TGF−β1 in this system. To distinguish whether secreted latent TGF−β1 or the LAP/latent TGF−β1 complex was the source of the active TGF−β1 for induction of Th17 cells, we compared WT and GARP−/− Tregs for their capacity to induce IL−17−producing cells. Surprisingly, WT Tregs induced three to four times more IL−17−producing cells than did Tregs from T cell−conditional GARP−/− mice. This result strongly suggests that one major function of the GARP/latent TGF−β1 complex may be to act as a source of active TGF−β1 for induction of Th17 production.

We demonstrated previously that activation of cocultures of activated Tregs and naive T cells resulted in induction of Foxp3 expression and Treg function in a low percentage of responder T cells in a TGF−β1−dependent manner (18). The Tregs induced in this process of infectious tolerance were suppressive in vitro and in vivo, and this pathway may represent a mechanism by which Tregs maintain tolerance. The major source of TGF−β1 in this system also seems to be the GARP/latent TGF−β1 complex, because activated Tregs from GARP−/− mice were poor inducers of Foxp3 expression in naive responder cells. It is likely that, in vivo, Tregs and Ag−specific T cells are stimulated on the surface of the APC in the form of a three−cell interaction. Stimulation of the Treg would induce the GARP/latent TGF−β1 complex, which, depending on the local milieu (≥IL−6), would result in the induction of Treg or Th17 cells.

Lastly, one must question why Foxp3+ Tregs, the most potent suppressor T cell population, would play an important role in the induction of potentially pathogenic Th17 cells. There is little doubt that TGF−β1 is required for the induction of pathogenic
Th17 cells in vivo, because local, but not systemic, administration of anti–TGF-β inhibits Th17 cell generation (19), and mice with a T cell–specific deletion of TGF-β1 that fail to generate Th17 cells are resistant to experimental autoimmune encephalomyelitis (7). However, mice with a Treg-specific deletion of TGF-β1 generated Th17 cells and were susceptible to experimental autoimmune encephalomyelitis (20). Conditional deletion of TGF-β1 in Tregs resulted in increased numbers of Tregs in certain lymphoid tissues but not others, raising the possibility that TGF-β1 produced by Tregs is specifically required for inhibiting Treg proliferation and controlling the size of the Treg pool. One study suggested that Tregs are critically important for the induction of Th17 differentiation (21), but this study proposed that Tregs promote Th17 production by a TGF-β1–independent mechanism related to their capacity to consume IL-2. Although Th17 cells are thought to be pathogenic, some studies demonstrate the existence of nonpathogenic IL-17–producing Th17 cells (22, 23), and it remains possible that Treg-induced Th17 cells are actually nonpathogenic. Furthermore, the development of pathogenic versus nonpathogenic Th17 cells may be contingent on the type of TGF-β present during the initial stage of Th17 differentiation, and studies from the Kuchroo laboratory (24) demonstrated that TGF-β3, but not TGF-β1, is the critical TGF-β isoform required for induction of pathogenic Th17 cells. Intriguingly, latent human TGF-β1 and TGF-β2, but not TGF-β3, were capable of forming complexes with human GARP (2), raising the possibility that latent TGF-β1/GARP complexes may play a unique role in the differentiation of a subset of Th17 cells.

Acknowledgments
We thank Julie Edwards, Tom Moyer, and Elina Stregevsky (National Institute of Allergy and Infectious Diseases Flow Cytometry Section) for sorting cells. We thank Howard Weiner (Harvard Medical School) for providing the anti-mouse LAP Ab TW7-16B4 and John O’Shea (National Institute of Arthritis and Musculoskeletal and Skin Diseases/ National Institutes of Health) for providing furin floxed mice.

Disclosures
The authors have no financial conflicts of interest.

References
**SUPPLEMENTAL FIGURE 1.** Genetic constructs of GARP-floxed mice. Schematic representation of the wild-type GARP locus (A), the targeting vector (B), the targeted allele (C), the floxed allele (D), and the deleted allele (E). The exon 1 of mouse GARP gene encodes the signal peptide (SP) required for cell surface expression. It was shown that deletion of SP disrupts GARP function (1). (F) Southern blot analysis of BamH I-digested ES cell DNA. (G) Sequence of probe used in Southern blot analysis.

**SUPPLEMENTAL FIGURE 2.** Induction of GARP expression by γC cytokines and expression of GARP/LAP in various lymphoid organs. (A) Relative GARP mRNA from sorted fresh CD4+Foxp3, fresh CD4+Foxp3+, or CD4+Foxp3+ cells cultured overnight with anti-IL-2, anti-IL-2 + IL-2 (human), or anti-IL-2 and IL-4. Values are normalized to GAPDH and set relative to mRNA from fresh CD4+Foxp3- cells. (B) Corrected MFI of GARP staining on CD4+Foxp3+ gated cells from pLN (pooled axillary and inguinal nodes), mLN, spleen, Peyer’s Patches, and CD4+CD8-Foxp3+ cells from the thymus. Corrected MFI represents MFI with anti-GARP minus the MFI with isotype control. Each symbol represents one mouse with the horizontal line the mean for each location. (C) Same gating as in (B), measuring the percent GARP/LAP double positive cells among Tregs.

**SUPPLEMENTAL FIGURE 3.** Foxp3+ Treg develop normally in lrrc32fl/fl x CD4-CRE mice. (A) Staining of thymuses from wild type (CRE-) and CD4-conditional knockout lrrc32fl/fl mice. (Upper panels) Proportions of cells within CD4SP, CD8SP, and CD4CD8DP populations. (lower panels) Percentage of Foxp3+ cells within the CD4SP
population in the thymus. (B) Gating on CD4+ cells, each gate represents Foxp3+ in each lymph node and spleen in wild type (CRE-) and CD4-conditional knockout from \textit{lrrc32}\textsuperscript{β/β} mice. (C) CD25 expression on CD4+Foxp3+ cells and CD4+Foxp3- cells from wild type (CRE-) and CD4-conditional knockout \textit{lrrc32}\textsuperscript{β/β} mice. (D) Sorted naïve CD4+CD25-CD62L\textsuperscript{hi}CD44\textsuperscript{low} cells from wild type (CRE-) and CD4-conditional knockout \textit{lrrc32}\textsuperscript{β/β} mice were stimulated with plate-bound anti-CD3, TGF-β1, and IL-2 for 4 days, then stained for CD4, Foxp3, and GARP.

**SUPPLEMENTAL FIGURE 4.** Cytokine and transcription factor expression by Treg-Mediated Th17 Cells. Naïve CD45.1\textsuperscript{+} OT-II cells from Rag1\textsuperscript{-/-} mice were activated as in Figure 6 in the presence of IL-6, IL-6+TGF-β1, IL-6+TGF-β1+Anti-IL-2, or IL-6+Activated Tregs for 4 days. Cells were then reactivated with Cell Stimulation Cocktail and Protein Transport Inhibitors for and stained for IL-10 and IL-17A (A) or stained for RORγ\textsuperscript{t}, T-bet, and Foxp3 immediately (B).
Supplemental Figure 1:

A

WT allele

B

Targeting vector

C

Targeted allele

D

Floxed allele

E

Deleted allele

F.

ES clones

G.

AGTAGGTGCTGGGAAAGGACAAAGTTATAATTGGAGTAAAGGACACTGGAGTCTAATGGT
GATGTCTCTTTTAAATTAGTATTTTCAAGTTAAATTGTCAATTCAAGATTCCCGTTGAGTCTG
GTATCCCTTTAATTCTATTTCTGTCCTCCTACTCTAGCCCTGATGTCTGCGACCA
GAGCAGGCCACTGATATGGGTAGTTTAGCAGCTGCAAGAAAGGAACGAGTCACAGC
AGGAATGTGGCTGAGCTGCTGCTGGAGCAACAAATGCAAGGGCTGCGACAAAGCA
CTGCTGTTTTCCCTGTTGGCAACATCTTTTCTCTGATCAGCTCCTACGCTAAAGCTAGA
ATTTTATCTGGAGTTGGCAAGGCCACGGATGTGAAACTGGAGCTGTGCGGCAGCTG
TGCCCCCTCTGTCTGTGCTCATGACTGTGAATATGGCTAGTTTTCCCATAGAAGAGG
ATACTAGTCAAACATAGCTATTCTTAGAGCTCCTACTATGTCTCTGTCCTACTGCTCCC
TTCTGC
Supplemental Figure 2:

A. Relative GARP mRNA

B. Corrected MFI (GARP-IC)

C. Percent GARP/LAP DP (of CD4+Foxp3+)

- Fresh CD4+GFP Cells
- Fresh CD4+GFP Cells (human)
- +aIL-2
- +aIL-2+IL-4

- pLNs
- mLNs
- Spleen
- Peyer's
- Thymus
Supplemental Figure 3:

A. WT: GARP Fl/Fl CD4 CRE-
   CD4SP 8.51%
   CD4CD8-DP 82.5%
   CD8SP 2.05%

B. WT: GARP Fl/Fl CD4 CRE-
   Foxp3+ 14.5%
   Inguinal
   Foxp3+ 14.4%
   Axillary
   Foxp3+ 15.1%
   Cervical

C. Foxp3
   WT-Foxp3- 10.5%
   WT-Foxp3+ 15.0%
   KO-Foxp3- 4.8%
   KO-Foxp3+ 9.7%

D. WT (GARP Fl/Fl, CRE-)
   4.48% 93.8%
   KO (GARP Fl/Fl, CRE+)
   98.5% 0.310%
Supplemental Figure 4:

A. 

B. 

IL-6 IL-6 + TGFb1 IL-6 + TGFb1 + Anti-IL-2 IL-6 + Tregs

IL-10 IL-10

IL-17A IL-17A

Foxp3 Foxp3

ROR(g) T-bet