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Identification of Regulatory Foxp3$^+$ Invariant NKT Cells Induced by TGF-β

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Invariant NKT (iNKT) cells were shown to prevent the onset of experimental autoimmune encephalomyelitis in mice following administration of their specific TCR agonist α-galactosylceramide. We found that this protection was associated with the emergence of a Foxp3$^+$ iNKT cell population in cervical lymph nodes. We demonstrate that the differentiation of these cells is critically dependent on TGF-β in both mice and humans. Moreover, in vivo generation of Foxp3$^+$ iNKT cells was observed in the TGF-β–rich environment of the murine gut. Foxp3$^+$ iNKT cells displayed a phenotype similar to that of Foxp3$^+$ regulatory T cells, and they suppress through a contact-dependent, glucocorticoid-induced TNFR-mediated mechanism. Nevertheless, Foxp3$^+$ iNKT cells retain distinctive NKT cell characteristics, such as promyelocytic leukemia zinc finger protein expression and preferential homing to the liver following adoptive transfer, where they stably maintained Foxp3 expression. Our data thus unveil an unexpected capacity of iNKT cells to acquire regulatory functions that may contribute to the establishment of immunological tolerance. ***The Journal of Immunology*****, 2010, 185: 2157–2163.**

Natural killer T cells are innate-like lymphocytes capable of producing cytokines characteristic of Th1, Th2, or Th17 responses (1–3). They were shown to influence adaptive immunity by exacerbating or suppressing a diversity of immune disorders, such as allergy, autoimmunity, or transplantation (4–7). For instance, NKT cell activation following administration of α-galactosylceramide (α-GalCer), an NKT cell-specific agonist, can protect mice from experimental autoimmune encephalomyelitis (EAE) manifestations (5, 8).

NKT cells express a TCR and receptors typical of the NK lineage, including NK1.1 and NKG2D (7). The best-studied NKT cell subset, known as type I, classical, or invariant NKT (iNKT) cells, has a semi-invariant TCR comprising an invariant α-chain and a restricted TCR β-chain repertoire. These cells recognize glycolipids presented by CD1d (7) and develop in the thymus, where they undergo a positive selection process mediated by double positive thymocytes acting as CD1d$^+$ APCs, instead of classical MHC-expressing thymic epithelial cells (9). Thus, iNKT cells are generally considered a lineage separate from T lymphocytes, characterized molecularly by expression of promyelocytic leukemia zinc finger protein (PLZF) (10). Of note, their unique TCR remains the only distinctive feature enabling iNKT cell unambiguous detection, because they share many surface molecules with T cells, namely, CD3 and CD4 in mice, as well as CD8 in humans (7, 11).

Thus far, iNKT cell subsets identified in vivo can recapitulate in the innate immunity context some of the functions characteristic of conventional CD4 T lymphocytes, such as secretion of Th1, Th2, and Th17-type cytokines (1–3, 12, 13). However, a parallel between regulatory CD4 T cells (regulatory T [Treg] cells) and iNKT lymphocytes has not yet been described. Treg cells are characterized by expression of the transcription factor Foxp3 and potent immunosuppressive properties. They are crucial for the maintenance of an immunological self-tolerance state, modulating the activation, proliferation, and function of effector T cells, thereby preventing pathological immune responses, including allergy and autoimmunity (14). Treg cells develop in the thymus or in particular contexts at the periphery, when activated in the presence of TGF-β (15–18).

We have now identified a population of Foxp3$^+$ iNKT cells in cervical lymph nodes (CLNs) of mice protected from EAE following α-GalCer administration. We further demonstrate that murine and human Foxp3$^+$ iNKT cells can be induced in vitro following activation in the presence of TGF-β. Foxp3$^+$ iNKT cells display Treg cell phenotypic hallmarks, including CD25, glucocorticoid-induced TNFR (GITR), and CTLA-4, while retaining NKT cell characteristics, namely, PLZF expression. Moreover, Foxp3$^+$ iNKT cells can occur in vivo, following activation in the TGF-β–rich mucosal environment. Because of the strong suppressive properties displayed upon Foxp3 upregulation, we termed this new NKT cell subset "Foxp3$^+$ NKTreg cell".

Materials and Methods

Mice

C57BL/6J (H-2^b, B6), B6.Cg-*Igh*^{a}*Thy1*^{a}*Gpi1*^{a}/J (H-2^b, Thy1.1), BALB/cByJ (H-2^d, BALB/c), and B6.Cg-Tg(Cd4-TGFBR2)16Flv/J (H-2^b, dominant negative [dn] TGFβRII) obtained from The Jackson Laboratory (Bar Harbor, ME); B6.129S6-Rag2^{tm1Fwa}N12 (H-2^b, RAG2$^{-/-}$) obtained from Taconic Farms (Germantown, NY); and *FoxP3*^{gfp} knockin mice (H-2^b) obtained from University of Washington (Seattle, WA) were bred and maintained in specific pathogen-free conditions at the Instituto Gulbenkian de Ciência, in Oeiras, Portugal. EAE was induced with 200 μg myelin

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CLN, cervical lymph node; dn, dominant negative; EAE, experimental autoimmune encephalomyelitis; α-GalCer, α-galactosylceramide; GITR, glucocorticoid-induced TNFR; iNKT, invariant NKT; iNKTreg, invariant regulatory NKT; iTreg, induced regulatory T; MLN, mesenteric lymph node; MOG, myelin oligodendrocyte glycoprotein; nTreg, natural regulatory T; pLN, pooled lymph node; PLZF, promyelocytic leukemia zinc finger protein; Treg, regulatory T.

oligodendrocyte glycoprotein (MOG) peptide (Biopolymers Laboratory, Harvard Medical School, Boston, MA) in 200 μg CFA (Difco/BD Diagnostics, Franklin Lakes, NJ) s.c. and 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) i.v. on days 0 and 2. Some mice received 4 μg α-GalCer (Alexis Biochemicals/Enzo Life Sciences, Plymouth Meeting, PA) on days 0 and 4. Disease severity was monitored daily and graded as follows: 1, limp tail; 2, partial hind-leg paralysis; 3, complete hind-leg paralysis; 4, front-leg weakness; and 5, moribund. In gavage experiments, 30 μg α-GalCer was delivered three times every other day.

Human subjects

Peripheral blood samples were obtained from healthy volunteers of both sexes after informed consent. The procedures were reviewed and approved by the Ethical Board of the Faculty of Medicine, University of Lisbon, Lisbon, Portugal.

Flow cytometry and cell sorting

CD1d-PBS57 tetramers were supplied by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). mAbs against mouse CD4, CD25, CD62L, CD103, CD127, CTLA-4, Foxp3, IFN-γ, IL-4, GITR, NK1.1, NKG2D, TCR-β, Thy1.1, Thy1.2; and human CD4, CD25, CD127, CD161, Foxp3, GITR, and TCR Vβ11 were purchased from eBioscience (San Diego, CA), BD Biosciences (San Jose, CA), Beckman Coulter (Fullerton, CA), or BioLegend (San Diego, CA). For murine NKT cell enrichment, cells were incubated with unconjugated anti-CD16/32 Ab (in-house production) to block nonspecific binding to FcR and labeled with PE-conjugated CD1d-PBS57 tetramers without washing. Anti-PE magnetic beads were added, and the magnetically labeled fraction was isolated in an autoMACS Cell Separator (Miltenyi Biotec, Auburn, CA). For human NKT cell enrichment, cells were labeled with biotinylated Abs against CD14, CD19, and CD123, bound to anti-biotin magnetic beads and enriched on an autoMACS Cell Separator, the magnetically labeled fraction being discarded. Intracellular flow cytometry stainings were performed using the Foxp3 Staining Buffer Set (eBioscience) for permeabilization and fixation. Samples were analyzed on a FACSCanto I (BD Biosciences) or sorted on a FACSAria (BD Biosciences), with doublet exclusion in all experiments. Data were analyzed by FlowJo (Tree Star, Ashland, OR).

Cell cultures

Following magnetic enrichment, iNKT cells isolated from mouse spleens were sorted by FACS, and 50,000 cells per well were stimulated with 3 μg/ml plate-bound anti-CD3 (eBioscience). In some experiments, cultures were supplemented with TGF-β (5 ng/ml; R&D Systems, Minneapolis, MN), IL-2 (5 ng/ml; eBiocience), IL-15 (100 ng/ml; eBioscience), and IL-7 (5 ng/ml; R&D Systems). Human cells were stimulated with 1 μg/ml plate-bound anti-CD3 (BD Biosciences). In some conditions, cultures were supplemented with TGF-β (10 ng/ml; R&D Systems), IL-2 (20 U/ml; Roche Diagnostic Systems, Indianapolis, IN), anti–IL-12 and anti–IFN-γ (5 μg/ml; eBioscience), anti–IL-4 (5 μg/ml; R&D Systems), and anti-CD28 (2 μg/ml; eBioscience). Culture medium was RPMI 1640 with GlutaMAX, supplemented with 10% FBS, 1% HEPES, 1% penicillin/streptomycin, 1% sodium pyruvate, and 0.1% 2−ME (Invitrogen, Carlsbad, CA).

In vitro proliferation and suppression assays

Regulatory cells isolated by FACS from the spleen of naive animals (natural Treg [nTreg] cells) or from polarizing cultures (Foxp3$^+$ and Foxp3$^-$ iNKT or CD4 T cells) were cocultured in Terasaki plates (Greiner Bio-One, Frickenhausen, Germany) with mitomycin C (Sigma-Aldrich, St. Louis, MO)-treated splenocytes and responder cells (4000 naive CD4 T cells isolated from spleen; 1:1 ratio with splenocytes) stimulated with 2.5 μg/ml soluble anti-CD3 for 4 d, with addition of 1 μCi [^{3}H]thymidine (Amersham Biosciences/GE Healthcare, Sunnyvale, CA) in the last 12 h. In some experiments, 200 μg/ml anti-IL10R or 100 μg/ml anti-GITR (19) was added. In transwell assays, 100,000 naive CD4 T responder cells labeled with 5 μM CFSE (Invitrogen) were stimulated with mitomycin C-treated splenocytes (250,000) and 1 μg/ml soluble anti-CD3.

Confocal microscopy

Sorted Foxp3-GFP$^+$ cells were plated on coverslips precoated with poly-L-lysine (Sigma-Aldrich) and incubated for 1 h at 37°C. Slides were then incubated with CD1d/PBS57-PE tetramer for 1 h at 4°C, washed with ice-cold PBS, and fixed in PBS 3% paraformaldehyde (Sigma-Aldrich) for 15 min at 4°C. Slides were mounted in DAPI Fluoromount G (Southern Biotech, Birmingham, AL) and analyzed with a laser scanning confocal microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany).

RNA extraction, RT, and PCR

RNA was extracted from 1,000–50,000 cells directly sorted into Buffer RLT with RNeasy Micro Kit (Qiagen, Valencia, CA), and cDNA synthesis was performed using random primers (Invitrogen) and Superscript III RT (Invitrogen). Primers (Bonsai Technologies, Orissa, India) were as follows: PLZF fwd: 5′-cagtttgcgactgagaatgc-3′, rev: 5′-ttcccacacagcagacagaa-3′; Foxp3 fwd: 5′-cccaggaaagacagcaacctt-3′, rev: 5′-ttctcacaaccaggccacttg-3′; EF1A fwd: 5′-acacgtagattccggcaagt-3′, rev: 5′-aggagccctttcccatctc-3′. PCRs were performed using the Power SYBR Green PCR Master Mix and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). All PCR products were run in agarose gel.

Statistical analysis

The p values were calculated by nonparametric unpaired t test with Welch's correction.

Results

Protection from EAE upon α-GalCer treatment leads to the emergence of Foxp3$^+$ iNKT cells in CLNs

Immunization with α-GalCer had been shown to prevent the onset of EAE in wild-type mice (5, 8) (Fig. 1*A*). Because this protection is known to be iNKT cell dependent (20) (Supplemental Fig. 1), we evaluated the number and phenotype of iNKT cells at the peak of disease. Surprisingly, we found a population of Foxp3$^+$ iNKT cells in CLNs (draining the CNS) of mice protected from EAE, which was absent from other anatomical locations and in CLNs of naive animals (Fig. 1*B*, 1*C*, Supplemental Fig. 2). In contrast to Foxp3$^+$ iNKT cells, the number and frequency of Foxp3$^+$ Treg cells remained unchanged in α-GalCer–treated mice, when compared with animals with EAE or naive controls (Fig. 1*D*).

Foxp3 expression has thus far been noted to be confined to conventional αβ T cells (21–23). Consistent with this, we found no evidence for Foxp3 expression by iNKT cells from liver, spleen, pooled lymph nodes, Peyer's patches, or thymus of naive BALB/c or C57BL/6 mice (Supplemental Fig. 3). This finding strongly suggests that Foxp3 expression is not imprinted during thymic iNKT cell development. Instead, our data indicate that peripheral induction of Foxp3$^+$ iNKT cells can occur under particular pathophysiological settings, such as EAE.

TGF-β induces de novo expression of Foxp3 in iNKT cells

We investigated the capacity of iNKT cells to express Foxp3 when activated in the presence of TGF-β—a condition known to convert conventional CD4 T cells into Foxp3$^+$ "inducible" Treg cells (14, 16). Sorted iNKT and CD4$^+$CD25$^-$ T cells were stimulated with plate-bound anti-CD3 in the presence of IL-2 and TGF-β. After 3 d, Foxp3 expression was detectable in a significant proportion of both iNKT (29.35% ± 11.80) and CD4 (53.21% ± 12.03) T cell cultures (Fig. 2*A*, 2*B*). Similar results were obtained with iNKT cells from mice harboring a Foxp3-GFP knockin allele (*Foxp3^{gfp}* mice) (21) and BALB/c mice (Fig. 2*C*, Supplemental Fig. 4), and with iNKT cells sorted from the thymus, albeit the latter exhibited a lower conversion efficiency (Fig. 2*D*). Foxp3^{gfp} iNKT cells were sorted after conversion and individual cells analyzed by confocal microscopy. As shown in Fig. 2*E*, staining with CD1d tetramer loaded with the PBS57 ligand confirms that these Foxp3-expressing cells bear in their surface the invariant TCR that recognizes glycolipid Ags, a feature exclusively attributed to iNKT cells. Therefore, bona fide iNKT cells were similar to conventional CD4 T cells in their ability to upregulate the Foxp3 transcription factor when stimulated under appropriate conditions. Of note, this property was not shared by other unconventional

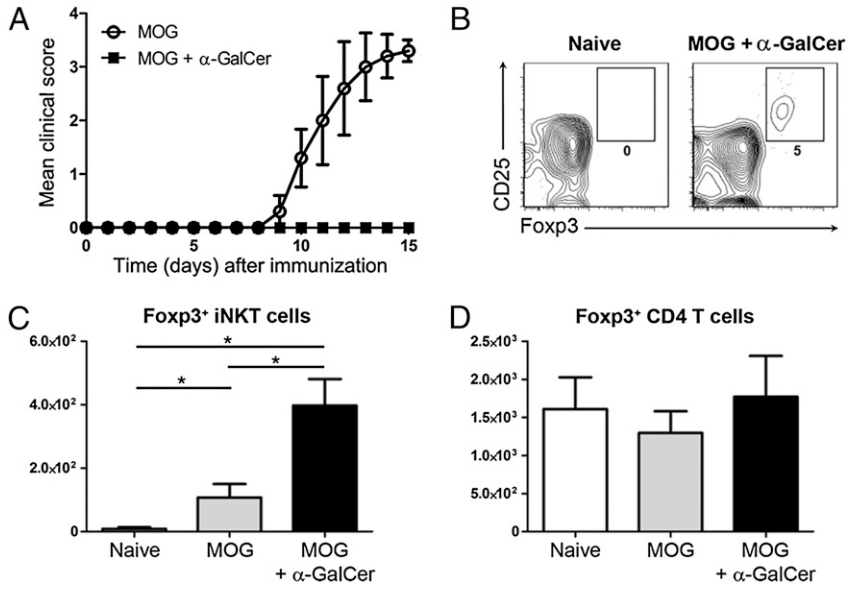

FIGURE 1. Foxp3$^+$ iNKT cells in α-GalCer–treated mice. EAE was induced in C57BL/6 mice (MOG), some of which were treated with α-GalCer (MOG + α-GalCer). *A*, Mice treated with α-GalCer were protected from EAE. $n = 5$; $p < 0.01$. *B*, Representative data from CLNs of naive and α-GalCer–treated mice (gated on iNKT cells, identified as CD1d/PBS57$^+$TCRβ$^+$ cells). *C*, Number of Foxp3$^+$ iNKT cells in CLNs, showing increased Foxp3$^+$ iNKT cell numbers in α-GalCer–treated mice. $n = 5$; $*p < 0.05$. *D*, The number of Foxp3$^+$ Treg cells in CLN remained constant. Data are representative of three independent experiments.

(non–MHC-restricted) T cells, such as γδ T cells, which failed to upregulate Foxp3 upon activation in the presence of TGF-β (M. Monteiro and J. Ribot, unpublished observations).

We analyzed the kinetics of Foxp3 induction and evaluated the stability of Foxp3 expression by iNKT cells in culture for 14 d (Supplemental Fig. 5). We observed that, despite a lower conver-

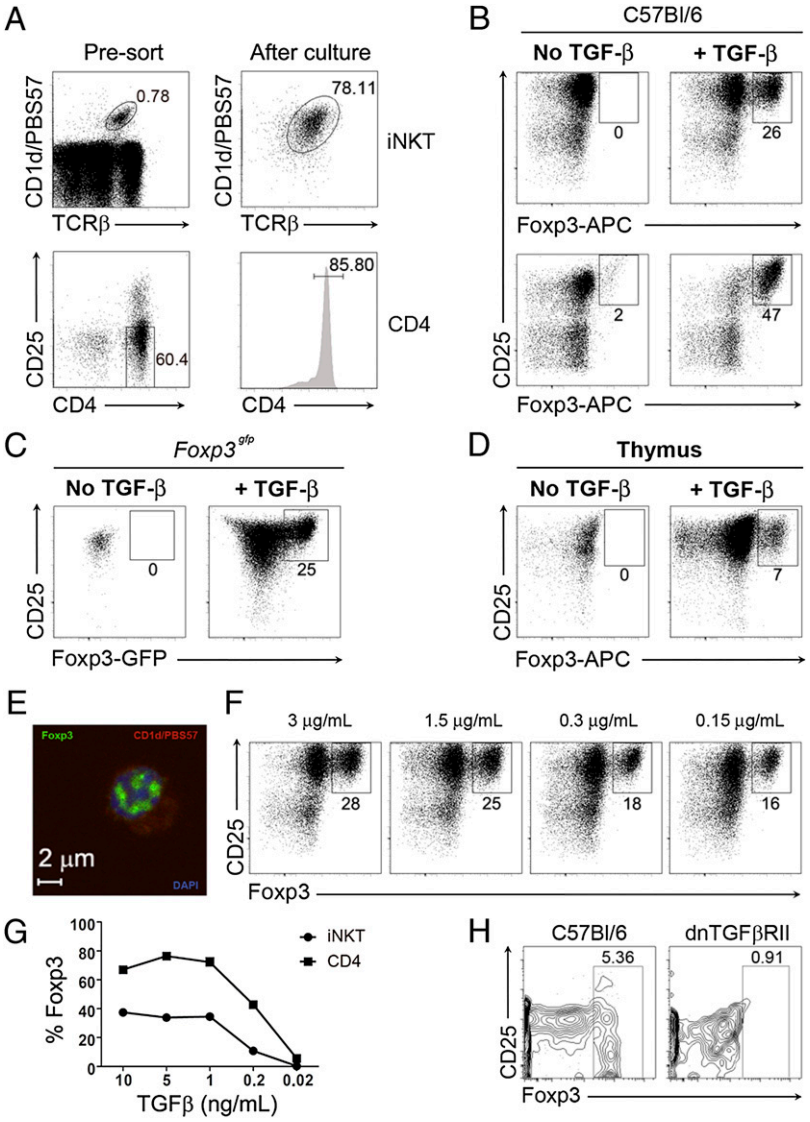

FIGURE 2. iNKT cells upregulate Foxp3 expression in the presence of TGF-β. *A–C*, Splenic iNKT cells and CD4$^+$CD25$^-$ T cells were sorted and stimulated with or without added TGF-β. *A*, Gating strategy used for cell sorting of iNKT cells (*upper panels*) and naive CD4 T cells (*lower panels*). The gating strategy used for analysis after 3 d of culture is also depicted for iNKT cells and CD4 T cells. *B*, Foxp3 expression in iNKT cells (*upper panels*) and control CD4 T cell cultures (*lower panels*) from C57BL/6 mice. *C*, Foxp3 expression in splenic iNKT cells from *Foxp3^{gfp}* knockin mice. *D*, Foxp3 expression in iNKT cells isolated from thymi of C57BL/6 mice. *E*, Foxp3 expression by iNKT cells was confirmed at single-cell level by confocal microscopy. Foxp3^{gfp}cells were FACS sorted after 3 d of culture, their invariant TCR was restained with PE-labeled CD1d/PBS57 tetramer (red), and the nucleus was counterstained with DAPI (blue). Foxp3 expression fluoresces in green (original magnification ×630). *F* and *G*, Titration of anti-CD3 or TGF-β concentration to address the impact on Foxp3 induction in splenic iNKT cells. *F*, iNKT cells cultured for 3 d in the presence of 5 ng/ml IL-2 and TGF-β, and stimulated with the indicated concentrations of anti-CD3. *G*, iNKT cells and CD4$^+$ CD25$^-$ T cells stimulated with 3 μg/ml anti-CD3 in the presence of 5 ng/ml of IL-2 and different concentrations of TGF-β after 3 d of culture. *H*, Foxp3 expression in iNKT cells from MLNs of C57BL/6 or dn TGFβRII mice following intragastric delivery of α-GalCer over 1 wk. Results are representative of three independent experiments with three mice per group.

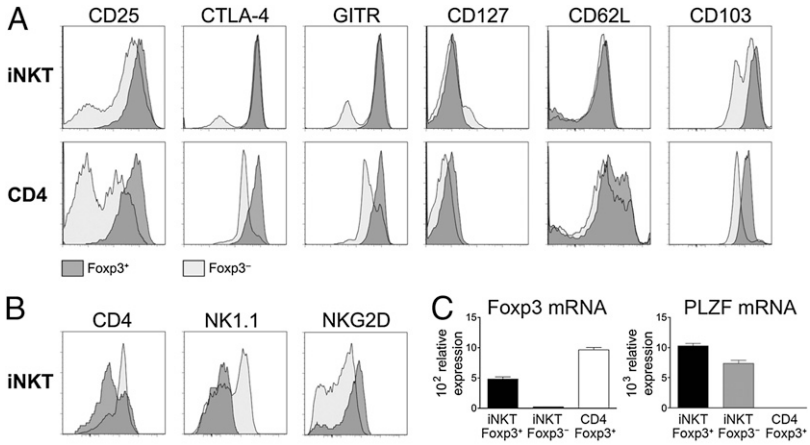

FIGURE 3. Phenotype of Foxp3$^+$ iNKT cells. *A* and *B*, iNKT cells and CD4$^+$CD25$^-$ T cells from the spleen of BALB/c (*A*) or C57BL/6 (*B*) mice were FACS sorted and cultured for 3 d in the presence of IL-2 and TGF-β. Histograms represent Foxp3$^+$ (gray) and Foxp3$^-$ (white) cells within iNKT or CD4 T cell subpopulations after conversion. Results are representative of two independent experiments. *C*, Quantification of Foxp3 and PLZF transcripts, relative to EF1A expression, from Foxp3-GFP$^+$ iNKT cells or CD4 T cells sorted after culture.

sion efficiency of iNKT cells when compared with CD4 T cell controls, iNKT cell numbers increased continuously over 14 d in culture.

We next tested the impact of TCR signal strength, costimulation, and cytokine addition on the conversion of iNKT cells into Foxp3 expressers. Maximal induction of Foxp3 was achieved with 3 μg/ml plate-bound anti-CD3 and 5 ng/ml TGF-β and IL-2, whereas further addition of IL-15 or IL-7 had little impact (Fig. 2*F*, Supplemental Fig. 4). Of note, titration of TGF-β concentration clearly revealed that this cytokine is essential for the induction of Foxp3 expression in iNKT cells (Fig. 2*G*).

TGF-β is required for expression of Foxp3 in iNKT cells in vivo

Th17 cell differentiation is critical for EAE development and requires a combination of cytokines, including TGF-β (24, 25). Accordingly, TGF-β neutralization at the time of EAE induction was shown to prevent the disease onset (26). Thus, to confirm TGF-β requirement for in vivo generation of Foxp3$^+$ iNKT cells, we adopted an alternative approach, taking advantage of the TGF-β–rich environment of the gut mucosa. We investigated whether intragastric delivery of α-GalCer could lead to the emergence of Foxp3$^+$ iNKT cells in mesenteric lymph nodes (MLNs), in the same way oral tolerance leads to de novo induction of Foxp3$^+$ Treg cells (27). We observed that α-GalCer delivery led to an accumulation of Foxp3$^+$ iNKT cells in MLNs (Fig. 2*H*). Of note, most Foxp3$^+$ iNKT cells from MLNs expressed low levels of CD25, something observed with the in vivo peripheral conversion of Treg cells in some conditions. To address whether in vivo generation of Foxp3$^+$ iNKT cells required TGF-β, we used dn TGFβRII mice (28), whose T and NKT cells are unable to transduce TGF-β signals, and observed no induction of Foxp3 expression in iNKT cells (Fig. 2*H*). Taken together, our observations suggest Foxp3$^+$ iNKT cells do not naturally arise during their development in the thymus, but can be induced in the periphery in environments where TGF-β is present.

Foxp3$^+$ iNKT cells display phenotypic characteristics of Treg cells and NKT cells

Once we established that iNKT lymphocytes could express Foxp3, we examined the phenotype of the converted cells. Many of the phenotypic characteristics of Foxp3$^+$ iNKT cells were shared with in vitro converted Foxp3$^+$ CD4 Treg cells. Both populations were predominantly CD25$^+$, CTLA-4$^+$, GITR$^+$, CD103$^+$, and IL-7Rα$^-$ (Fig. 3*A*). However, we also observed some differences between the two populations: Whereas Foxp3$^+$ CD4 T cells were heterogeneous for CD62L expression, Foxp3$^+$ iNKT cells were homogeneously CD62L^{low}. The absence of CD62L, in association with the high expression of CD103, suggests that in vivo Foxp3$^+$iNKT cells are excluded from lymph nodes and preferentially migrate to peripheral tissues. Interestingly, iNKT cells that had upregulated Foxp3 expression failed to secrete IL-4 and IFN-γ upon restimulation (Supplemental Fig. 6).

We also found that the capacity to induce Foxp3 expression was shared by CD4$^+$ and CD4$^-$ iNKT cells, as sorted CD4$^-$ and CD4$^+$ iNKT cells exhibited similar conversion efficiency (Fig. 3*B*) (M. Monteiro and C.F. Almeida, unpublished data). In addition, Foxp3$^+$ iNKT lymphocytes were NK1.1$^-$, and the majority expressed NKG2D. We also could detect the expression of PLZF, a transcription factor reported to distinguish NKT cells from conventional αβ T cells (10), in both sorted Foxp3$^+$ and Foxp3$^-$ iNKT cell subsets (Fig. 3*C*). Together, these observations indicate that induction of Foxp3 expression in iNKT lymphocytes does not corrupt their NKT cell nature.

Foxp3$^+$ iNKT cells migrate to the liver and maintain Foxp3 expression in vivo

To investigate the in vivo stability of Foxp3 expression in iNKT cells, we injected converted Foxp3-GFP$^+$ iNKT cells or control induced Foxp3-GFP$^+$ CD4 T (iTreg) cells into RAG2$^{-/-}$ mice (Fig. 4) or congenic Thy1.1 hosts (Supplemental Fig. 7). By contrast to iTreg cells, which migrated preferentially to the lymph nodes, but could also be found in the spleen and liver, 21 d after adoptive transfer iNKT cells could be detected only in the latter organ, where 50–80% (in empty hosts) or up to 10% (in wild-type congenic hosts) of iNKT cells maintained Foxp3 expression

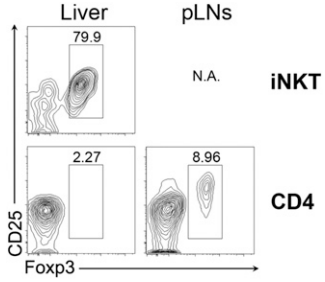

FIGURE 4. Foxp3$^+$ iNKT cells migrate to the liver. A total of 5×10^4 Foxp3-GFP$^+$ iNKT cells or CD4 T cells sorted from polarizing in vitro cultures were injected i.v. into RAG2$^{-/-}$ recipients. Data show Foxp3 and CD25 expression of iNKT cells and CD4 T cells, identified respectively as TCRβ intermediate, tetramer positive, and TCRβ$^+$CD4$^+$ cells inside the lymphocyte gate, in the liver, and in pLNs 21 d after adoptive transfer. iNKT cells were detected only in the liver. Data are representative of two independent experiments. pLNs, pooled lymph nodes.

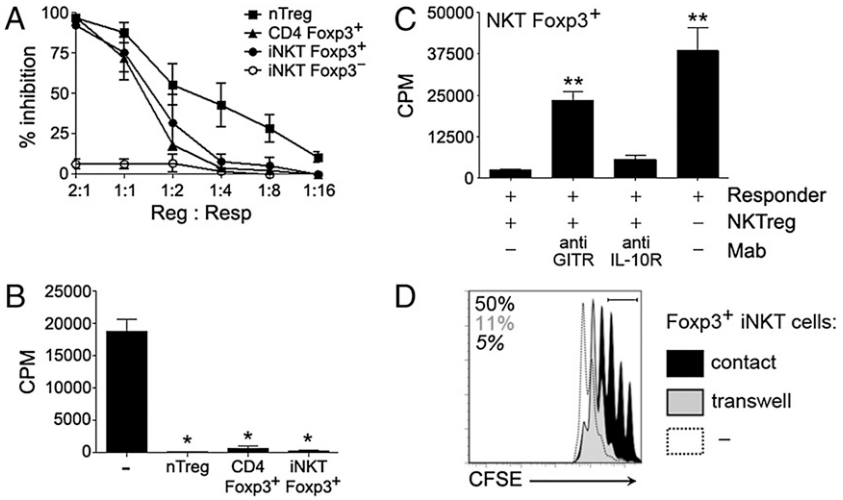

FIGURE 5. Foxp3$^+$ iNKT cells suppress T cell proliferation through a GITR-mediated contact-dependent mechanism. Foxp3$^+$ iNKT cells and Foxp3$^-$ iNKT cells sorted from the same cultures, iTreg cells (CD4 Foxp3$^+$), and nTreg cells were cocultured at different ratios with responder T cells. *A*, Average inhibition of proliferation from three independent experiments (each one with triplicates) normalized to proliferation of responder cells alone, assessed by thymidine incorporation. *B*, Representative experiment at a 2:1 ratio. $n = 3$; $*p < 0.05$. *C*, Addition of anti-GITR, but not anti–IL-10R, abrogated the suppressive effect of Foxp3$^+$ iNKT cells. $n = 4$; $**p < 0.01$. *D*, Proliferation of CFSE-labeled responder cells cultured for 72 h at 1:1 ratio with Foxp3$^+$ iNKT cells in a transwell assay, in which the two populations were cultured in contact (black histogram) or separated by a transmembrane (gray histogram). Dotted histogram shows proliferation in the absence of regulatory cells. *Upper left* numbers indicate the frequency of responder cells from the three conditions within the indicated gate.

(Fig. 4, Supplemental Fig. 7). In fact, it is becoming apparent that Foxp3 expression by conventional Treg cells is less stable than initially anticipated, especially under lymphopenic conditions (29). Our data indicate that in vivo Foxp3 stability in converted iNKT cells is not inferior to that of iTreg cells, although both populations have distinct homing potentials: Whereas CD4 Treg cells migrate predominantly to secondary lymphoid organs upon adoptive transfer, Foxp3$^+$ iNKT cells preferentially home to the liver. We thus suggest that the recruitment of regulatory iNKT cells to peripheral tissues may be important to complement and/or reinforce the local action of Treg cells (30).

Invariant regulatory NKT cells display contact-dependent GITR-mediated suppressive function

To evaluate the regulatory function of Foxp3$^+$ iNKT cells, we tested their ability to suppress proliferation of CD4$^+$CD25$^-$ "responder" cells. We used iNKT cells derived from *Foxp3^{gfp}* mice converted in the presence of TGF-β and, as controls, natural (nTreg) and iTreg cells. Titration of regulatory/responder cell ratio revealed that converted Foxp3$^+$ iNKT cells can indeed inhibit the proliferation of responder cells with similar efficiency to iTreg cells, and only slightly inferior to the efficiency of nTreg cells (Fig. 5*A*, 5*B*). Addition of anti-GITR (19), but not anti-IL-10R, neutralizing Abs to the cultures reversed suppression, indicating that GITR plays a predominant role in the regulatory function of Foxp3$^+$ iNKT lymphocytes (Fig. 5*C*). In agreement with these results, Foxp3$^+$ iNKT cells showed impaired suppressive effect compared with responder cell proliferation when cultured in a separate transwell (Fig. 5*D*), which excludes a major contribution for soluble factors to regulation. These results demonstrate that, similarly to conventional Treg cells (31, 32), induction of Foxp3 in iNKT lymphocytes endows these cells with suppressive function exerted through a contact-dependent

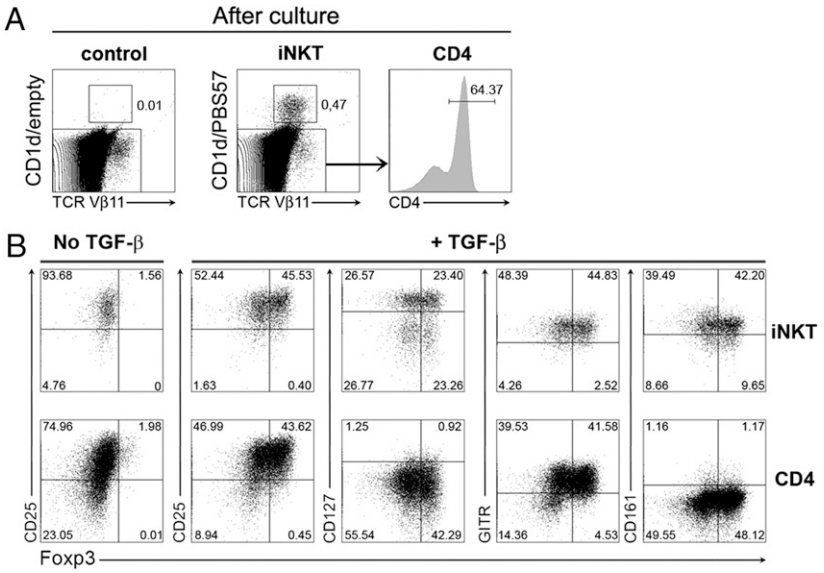

FIGURE 6. Foxp3 expression can be induced in human iNKT cells. Human iNKT cells and CD4$^+$ T cells from peripheral blood were magnetically enriched and cocultured for 5 d in the presence or absence of a conversion mixture, including or not including TGF-β. *A*, After culture, iNKT cells were identified by costaining of human CD1d/PBS57 tetramer and anti–TCR-Vβ11 Ab inside the lymphocyte gate (iNKT). Background of tetramer staining was evaluated with an empty human CD1d tetramer (control). CD4 T cells were gated inside the CD1d/PBS57-negative region (CD4). *B*, Flow cytometry data showing the coexpression of Foxp3 along with CD25, CD127, GITR, or CD161 in iNKT cell (*upper panels*) and CD4$^+$ T cell gates (*lower panels*). Results are representative of three independent experiments from different blood donors with at least three replicate cultures per condition.

mechanism mediated by GITR. This parallel with Foxp3+ Treg cells prompted us to name these Foxp3+ iNKT cells "Foxp3+ invariant regulatory NKT (iNKTreg) cells".

TGF-β–mediated induction of Foxp3 expression in human iNKT cells

Finally, we also evaluated whether Foxp3 expression could be induced in human iNKT cells. Given the lower frequency of iNKT lymphocytes in human peripheral blood (31), we enriched total T cells by magnetic separation and cultured these bulk populations in polarizing conditions that included TGF-β and a mixture of neutralizing Abs against IL-12, IFN-γ, and IL-4 (32) (Fig. 6*A*). After 5 d of culture, up to 40% of human iNKT cells had upregulated Foxp3, an efficiency of conversion comparable to that of conventional CD4+ T cells (Fig. 6*B*). The converted human Foxp3+ NKT cells were CD25+, GITR+, and predominantly CD161+, whereas CD127 was expressed by approximately half of the Foxp3+ iNKT cells. Critically, these data establish that the induction of Foxp3 in NKT cells is a conserved phenomenon between rodents and humans.

Discussion

Among hematopoietic cells, Foxp3 expression has been claimed to be restricted to conventional T cells (24–27, 33). We now describe, for the first time, that iNKT and CD4 T lymphocyte populations share a similar competence in responding to a tolerogenic cytokine environment, namely, TGF-β, by expressing Foxp3 and undergoing functional specialization toward a regulatory phenotype. It should be noted, however, that Foxp3 induction upon activation in the presence of TGF-β is not a common feature of all lymphocytes: In particular, we were unable to convert sorted γδ T cells by CD3 stimulation in the presence of TGF-β (M. Monteiro and J. Ribot, unpublished observations). In addition, this potential appears to be a conserved feature of iNKT cells, as we were able to induce Foxp3 expression in samples from different murine strains and from human beings.

Our results clearly demonstrate that TGF-β is the critical cytokine promoting Foxp3 upregulation in iNKT cells, as described for CD4+ T cell conversion into Foxp3+ Treg cells (15–18). As a consequence, we took advantage of the TGF-β–rich environment of the gut to assess in vivo conversion of Foxp3+ iNKT cells. In fact, it has already been described that oral delivery of an Ag can lead to a state of mucosal tolerance in which Ag-specific T cells are locally converted into Treg cells (27). In this paper, we show that intragastric delivery of α-GalCer induces the emergence of Foxp3+ iNKTreg cells in MLNs. However, this process was impaired in mice with NKT cells unable to integrate the signals delivered through the TGF-βR, thus establishing a crucial role for TGF-β in the in vivo conversion of Foxp3+ NKT cells. Therefore, although oral tolerance to proteic Ags, such as OVA, can be achieved in the absence of iNKT cells, as it has been shown using TCR-transgenic RAG-deficient mice (27), our results suggest that iNKT cells in the gut, exposed to glycolipid ligands, can convert into Foxp3+ cells and synergize with local Treg cells in the promotion of tolerance to food Ags.

Most reports have shown that Foxp3+ Treg cells suppress immune responses through cell contact-dependent mechanisms (34, 35). Several surface molecules preferentially expressed by Treg cells, such as GITR, have been implicated in their suppressive function (31, 32). We found that NKTreg cells can also suppress naive T cell proliferation by a GITR-dependent, IL-10–independent mechanism. Moreover, iNKTreg cells were unable to suppress responder cell proliferation when physically separated in a transwell, which excludes the additional contribution of soluble regulatory factors.

Although iNKT cells expressing Foxp3 have never been found in naive mice (21), the presence of this population has never been investigated in the context of an immune response. For instance, iNKT cells were reported to play important regulatory roles in the development of anterior chamber-associated immune deviation, transplantation tolerance, and prevention of autoimmunity, such as EAE, as lack of iNKT cells or decreased iNKT cell numbers compromise tolerance. However, in none of these studies was Foxp3 expression assessed in iNKT cells (33). Importantly, iNKT cell surface molecules largely overlap with those expressed by conventional CD4 T lymphocytes, and Foxp3+ iNKT cells downregulate expression of NK1.1, a molecule often used in previous studies to distinguish NKT cells from T lymphocytes. Therefore, it is reasonable to assume that without an unambiguous identification with a specific tetramer, Foxp3+ iNKT cells could have been erroneously detected as conventional Treg cells. Furthermore, until the development of a conditional knockout mouse lacking Foxp3 exclusively in iNKT cells—for instance, based on a PLZF-driven Cre expression system—it will not be possible to discriminate between the contribution of NKTreg cells and that of Treg cells in immune pathology. Although this topic surely deserves further investigation, the results presented in this paper clearly establish a new type of iNKT cell characterized by Foxp3 expression and immune regulatory properties similar to those of conventional Treg cells, one that might contribute to the maintenance of immune tolerance in the periphery.

Finally, the strong immunosuppressive properties displayed by NKTreg cells raise the possibility of their therapeutic application in the control of immune-mediated diseases, in particular, liver immune-mediated inflammation. Given their invariant specificity, Foxp3+ NKTreg cells are assumed to have a general immunosuppressive action. It should be noted, however, that polyclonal Treg cell populations are likely to have, at least in part, a similar nonspecific immunosuppressive effect, possibly due to cross-reactivity with self-Ags (36). Indeed, these nonspecific effects can serve as the basis of Treg cell suppression in lymphopenia-driven proliferation and graft-versus-host disease (36–38). Notably, the invariant TCR expressed by Foxp3+ iNKT cells might provide an advantageous, universally applicable method for their isolation, conversion, and expansion, regardless of genetic background or pathology.

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Disclosures

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References

1. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 23: 877–900.
2. Gumperz, J. E., S. Miyake, T. Yamamura, and M. B. Brenner. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J. Exp. Med.* 195: 625–636.
3. Michel, M. L., A. C. Keller, C. Paget, M. Fujio, F. Trottein, P. B. Savage, C. H. Wong, E. Schneider, M. Dy, and M. C. Leite-de-Moraes. 2007. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J. Exp. Med.* 204: 995–1001.
4. Hong, S., M. T. Wilson, I. Serizawa, L. Wu, N. Singh, O. V. Naidenko, T. Miura, T. Haba, D. C. Scherer, J. Wei, et al. 2001. The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat. Med.* 7: 1052–1056.
5. Singh, A. K., M. T. Wilson, S. Hong, D. Olivares-Villagómez, C. Du, A. K. Stanic, S. Joyce, S. Sriram, Y. Koezuka, and L. Van Kaer. 2001. Natural killer T cell

activation protects mice against experimental autoimmune encephalomyelitis. *J. Exp. Med.* 194: 1801–1811.
6. Kim, E. Y., J. T. Battaile, A. C. Patel, Y. You, E. Agapov, M. H. Grayson, L. A. Benoit, D. E. Byers, Y. Alevy, J. Tucker, et al. 2008. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nat. Med.* 14: 633–640.
7. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu. Rev. Immunol.* 25: 297–336.
8. Mars, L. T., L. Araujo, P. Kerschen, S. Diem, E. Bourgeois, L. P. Van, N. Carrié, M. Dy, R. S. Liblau, and A. Herbelin. 2009. Invariant NKT cells inhibit development of the Th17 lineage. *Proc. Natl. Acad. Sci. USA* 106: 6238–6243.
9. Godfrey, D. I., and S. P. Berzins. 2007. Control points in NKT-cell development. *Nat. Rev. Immunol.* 7: 505–518.
10. Savage, A. K., M. G. Constantinides, J. Han, D. Picard, E. Martin, B. Li, O. Lantz, and A. Bendelac. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29: 391–403.
11. Van Kaer, L. 2007. NKT cells: T lymphocytes with innate effector functions. *Curr. Opin. Immunol.* 19: 354–364.
12. Coquet, J. M., S. Chakravarti, K. Kyparissoudis, F. W. McNab, L. A. Pitt, B. S. McKenzie, S. P. Berzins, M. J. Smyth, and D. I. Godfrey. 2008. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc. Natl. Acad. Sci. USA* 105: 11287–11292.
13. Rachitskaya, A. V., A. M. Hansen, R. Horai, Z. Li, R. Villasmil, D. Luger, R. B. Nussenblatt, and R. R. Caspi. 2008. Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. *J. Immunol.* 180: 5167–5171.
14. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775–787.
15. Cobbold, S. P., R. Castejon, E. Adams, D. Zelenika, L. Graca, S. Humm, and H. Waldmann. 2004. Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J. Immunol.* 172: 6003–6010.
16. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4 +CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
17. Belghith, M., J. A. Bluestone, S. Barriot, J. Mégret, J. F. Bach, and L. Chatenoud. 2003. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat. Med.* 9: 1202–1208.
18. Ghiringhelli, F., C. Ménard, M. Terme, C. Flament, J. Taieb, N. Chaput, P. E. Puig, S. Novault, B. Escudier, E. Vivier, et al. 2005. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J. Exp. Med.* 202: 1075–1085.
19. Tone, M., Y. Tone, E. Adams, S. F. Yates, M. R. Frewin, S. P. Cobbold, and H. Waldmann. 2003. Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells. *Proc. Natl. Acad. Sci. USA* 100: 15059–15064.
20. Jahng, A. W., I. Maricic, B. Pedersen, N. Burdin, O. Naidenko, M. Kronenberg, Y. Koezuka, and V. Kumar. 2001. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J. Exp. Med.* 194: 1789–1799.
21. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22: 329–341.
22. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
23. Kapp, J. A., K. Honjo, L. M. Kapp, X. Xu, A. Cozier, and R. P. Bucy. 2006. TCR transgenic CD8+ T cells activated in the presence of TGFbeta express FoxP3 and mediate linked suppression of primary immune responses and cardiac allograft rejection. *Int. Immunol.* 18: 1549–1562.
24. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.
25. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179–189.
26. Veldhoen, M., R. J. Hocking, R. A. Flavell, and B. Stockinger. 2006. Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. *Nat. Immunol.* 7: 1151–1156.
27. Mucida, D., N. Kutchukhidze, A. Erazo, M. Russo, J. J. Lafaille, and M. A. Curotto de Lafaille. 2005. Oral tolerance in the absence of naturally occurring Treg cells. *J. Clin. Invest.* 115: 1923–1933.
28. Gorelik, L., and R. A. Flavell. 2000. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12: 171–181.
29. Komatsu, N., M. E. Mariotti-Ferrandiz, Y. Wang, B. Malissen, H. Waldmann, and S. Hori. 2009. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc. Natl. Acad. Sci. USA* 106: 1903–1908.
30. Graca, L., S. P. Cobbold, and H. Waldmann. 2002. Identification of regulatory T cells in tolerated allografts. *J. Exp. Med.* 195: 1641–1646.
31. Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 3: 135–142.
32. McHugh, R. S., M. J. Whitters, C. A. Piccirillo, D. A. Young, E. M. Shevach, M. Collins, and M. C. Byrne. 2002. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16: 311–323.
33. Nowak, M., and J. Stein-Streilein. 2007. Invariant NKT cells and tolerance. *Int. Rev. Immunol.* 26: 95–119.
34. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188: 287–296.
35. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* 10: 1969–1980.
36. Graca, L., A. Le Moine, C. Y. Lin, P. J. Fairchild, S. P. Cobbold, and H. Waldmann. 2004. Donor-specific transplantation tolerance: the paradoxical behavior of CD4+CD25+ T cells. *Proc. Natl. Acad. Sci. USA* 101: 10122–10126.
37. Annacker, O., R. Pimenta-Araujo, O. Burlen-Defranoux, T. C. Barbosa, A. Cumano, and A. Bandeira. 2001. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J. Immunol.* 166: 3008–3018.
38. Edinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. S. Negrin. 2003. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* 9: 1144–1150.