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Rapamycin Combined with TGF-β Converts Human Invariant NKT Cells into Suppressive Foxp3+ Regulatory Cells

Lúcia Moreira-Teixeira,*† Mariana Resende,*† Odile Devergne,* Jean-Philippe Herbeuval,* Olivier Hermine,* Elke Schneider,* Michel Dy,* Anabela Cordeiro-da-Silva, † and Maria C. Leite-de-Moraes*

Invariant NKT (iNKT) cells constitute a versatile T cell subset with important regulatory functions, which are thought to result essentially from their capacity to promptly produce cytokines that influence the Th1/Th2 balance. In this study, we report that these cells can also express Foxp3, an important transcriptional regulator associated with suppressive activity, once they have been exposed to TGF-β. Foxp3 was expressed by iNKT cells from both peripheral and cord blood. CD4+ INKT cells acquired Foxp3 expression preferentially, although a lower proportion of their CD4− counterpart also became positive. All Foxp3+ iNKT cells displayed CD25 but not necessarily CTLA4 or GITR, regardless of the upregulation of these markers in the presence of TGF-β. Exposure to TGF-β decreased IL-4 and IFN-γ production while increasing IL-10, independently from Foxp3 expression. IL-17 was not detected. TGF-β induced high levels of Foxp3, but no suppressor activity, which emerged only in the presence of rapamycin. Peripheral and cord blood Foxp3+ iNKT cells suppressed the proliferation of conventional autologous and heterologous CD4+ T cells equally, in a cell contact-dependent and Ag-independent manner. Our findings demonstrate that human INKT cells become suppressive in the presence of TGF-β plus rapamycin, thus adding a new facet to their complex functional properties. The Journal of Immunology, 2012, 188: 000–000.

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ariant NKT (iNKT) cells have elicited considerable interest in recent years because of their implication in several immune responses and their great potential for therapeutic modulation (1–4). They constitute a distinct subpopulation of T lymphocytes that is positively selected by CD1d molecules and coexpresses a highly restricted TCR repertoire, composed of a single invariant Vα14Jα18 chain in mice and a Vα24Jα18 chain in humans, preferentially paired with a limited repertoire of TCR Vβ-chains. Another typical feature of this population is the expression of receptors such as CD161 (homolog of mice NK1.1) and NKG2D (5, 6), which it shares with NK cells.

In contrast with their conventional T cell counterpart, iNKT cells recognize CD1d-bound glycolipids rather than peptides (7–9). In response to these ligands, iNKT cells promptly produce large amounts of various cytokines (1, 2, 7–12), enabling them to regulate autoimmune diseases, inflammation, anti-tumor responses, allergic asthma, and antimicrobial host response (10–19). iNKT cells are therefore commonly acknowledged as one of the immunoregulatory T cell populations. So far, these regulatory functions have been mainly ascribed to their capacity to shift the Th1/Th2 balance, which enables them to exacerbate asthma symptoms or to take part in the protection against tumor metastasis by supporting a Th2 or a Th1 response, respectively (13–19). iNKT cells can also act as effector cells, namely via their IL-17–producing subset, which enhances lung inflammatory responses (10, 20). In addition to their ability to amplify the immune response in various ways, iNKT cells can likewise contribute to the negative control of the immune system by promoting regulatory T (Treg) cell expansion through their secretion of IL-2 (21). Foxp3 expression at protein or mRNA levels by mouse and human iNKT cells was already described (22, 23). However, in contrast to murine cells, Foxp3 expression by conventional human T cells could merely represent an activation state rather than the ability to exert a suppressive activity (24, 25). In this study, to our knowledge we demonstrate for the first time that, in the presence of TGF-β and rapamycin, human iNKT cells express Foxp3 and become functionally capable to suppress the proliferation of conventional CD4+ T cells.

Materials and Methods

Cell preparation

PBMCs and cord blood mononuclear cells (CBMCs) were collected from healthy donors at the Etablissement Français du Sang or the Necker Enfants Malades Hospital, Paris. Experiments were performed in accordance with the Helsinki Declaration, with informed consent received from each donor or the donor’s family. PBMCs and CBMCs were prepared by Ficoll-Hypaque centrifugation (GE Healthcare) and further used for in vitro culture or cell sorting.
**iNKT cell expansion**

PBMCs and CBMCs were cultured in 24-well plates (Falcon) at a density of 10^6 cells per well in RPMI 1640 medium containing antibiotics, 10% FBS, 200 mM glutamine, and 10 mM HEPEs (all from Invitrogen) with α-galactosylceramide (α-GalCer; 100 ng/ml or 10 and 1 ng/ml when indicated) or OCH (500 ng/ml; both from Alexis Biochemicals) in the presence or absence of recombinant human (rh) TGF-β (5 ng/ml; R&D Systems). When indicated, 20 mM rapamycin (Sigma-Aldrich) was added at the onset of culture followed 24 h later by rhIL-2 (50 ng/ml; Immunotools). After 2 wk, cells were collected, washed extensively, and assayed for their viability by trypan blue exclusion.

**Cell purification and sorting**

Fresh PBMCs or CBMCs were labeled with CD1d-tetramer-PE (provided by National Institutes of Health Tetramer Facility), and iNKT cells were magnetically enriched with anti-PE magnetic beads (Miltenyi Biotec). CD1d-tetramer+ cells were further purified by electronic cell sorting on a FACSaria (BD Biosciences). CD1d-tetramer+ cells were cultured in 96-well plates (Falcon) with anti-CD3 plus anti-CD28 and anti-CD2–coated beads (Miltenyi Biotec) at a cell/bead ratio of 1:1 in the presence of rhTGF-β. Cultured cells were analyzed for Foxp3 expression. In some experiments, the positive fraction was labeled with anti-CD4 (eBioscience). CD1d-tetramer+CD4+ and CD1d-tetramer-CD4− cell subsets were then sorted. CD1d-tetramer− subsets were cultured with autologous mononuclear cells from the iNKT cell-depleted fraction used as feeder and expanded with α-GalCer in the presence of rhTGF-β. After 2 wk, cells were analyzed for Foxp3 expression. For the T cell proliferation assay, CD25+ T cells were sorted from PBMCs or CBMCs after staining with anti-CD4 and anti-CD25 (eBioscience). Expanded iNKT cells were stained with CD1d-tetramer and anti-CD25, and the following populations were sorted: CD1d-tetramer+CD25+ and CD1d-tetramer-CD25−.

**Surface and intracellular staining**

Fresh or cultured PBMCs or CBMCs were analyzed by flow cytometry using the CD1d-tetramer and the following Abs: anti-CD3 (ImmunoTools), anti-CD4, anti-CD25, and anti-GITR (eBioscience). Intracellular analysis of Foxp3 (clone 206A/E7) and CTLA4 (both from eBioscience) was performed after fixation and permeabilization using Foxp3 staining buffers (eBioscience). Isotype-matched Abs were used to define marker settings. For intracellular cytokine staining, expanded iNKT cells were incubated for 5 h with PMA (25 ng/ml), ionomycin (1 µg/ml), and brefeldin A (10 µg/ml; all from Sigma-Aldrich). Cells were stained with CD1d-tetramer and then fixed, washed, and permeabilized using Foxp3 staining buffer and incubated with anti-IFN-γ, anti–IL-4, anti–IL-10, anti–IL-17, and anti–IL-2 (all from eBioscience). Cells were acquired on a FACScanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**ELISA**

IL-2 was measured by DuoSet ELISA (R&D Systems), according to a standard protocol. Data were analyzed with the SoftMax program.

**Suppression assay**

Suppressor activity of expanded iNKT cells was assessed in a coculture assay set up with autologous or heterologous CD25−CD4+ T cells labeled with 5 µM CFSE (Invitrogen), used as responder T cells. Cells were incubated at a concentration of 2–5 × 10^7/well in serum-free X-VIVO 15 medium (Lonza) supplemented with 5% human normal serum (Dynal Biotech) and stimulated with anti-CD3 plus anti-CD28 and anti-CD2–coated beads (Miltenyi Biotec) at a cell/bead ratio of 1:1, with or without increasing numbers of sorted CD1d-tetramer+CD25+ cells. In some experiments, anti–GIFTR (10 µg/ml; R&D Systems) was added in the beginning of the coculture, whereas in others, IL-2 was measured in the supernatants obtained after 4-d culture. Supernatants were also collected from CD1d-tetramer+CD25+ cells stimulated for 24 or 48 h with anti-CD3 plus anti-CD28 and anti-CD2–coated beads in the presence or absence of responder T cells. Proliferation was assessed after a 4-d stimulation by cytometric analysis on a FACScanto II using FlowJo software. Relative suppression was calculated according to the following equation: % suppression = [100 − (% responder T cells that proliferated more than once when cultured in the presence of sorted CD1d-tetramer+CD25+ cells/% responder T cells that proliferated more than once when cultured alone)] × 100.

**Real-time RT-PCR**

Total RNA was extracted from sorted CD1d-tetramer+CD25+ cells with the RNeasy Micro kit (Qiagen), and first-strand cDNAs were prepared using the RT First Strand kit (SABiosciences). Template cDNAs were characterized using the Human T-cell Anergy and Immune Tolerance PCR Array with the TGF-β SYBR Green/ROX qPCR Master Mix (all from SABiosciences on the StepOnePlus real-time PCR system (Applied Biosystems). Fold changes in gene expression between the RNA from iNKT cells cultured in the presence of TGF-β and rapamycin or in the presence of medium were calculated using the ∆∆Ct method in the PCR array data analysis (SABiosciences).

**Statistical analysis**

Statistical significance of differences between data sets was established by the Mann–Whitney U test.

**Results**

**TGF-β induces Foxp3 expression in human iNKT cells**

We set out to evaluate Foxp3 expression in two freshly isolated human iNKT cell populations (Fig. 1A) considered functionally immature or mature, according to their origin from CBMCs or PBMCs, respectively (26, 27). As shown in Fig. 1B and 1C, both populations were negative for the transcription factor ex vivo, in contrast with conventional CD4+CD25+ Treg cells used as a positive control.

Given the critical role ascribed to TGF-β in the differentiation of conventional human CD4+ T cells into Treg cells (28), we verified whether this cytokine had a similar effect on iNKT cells. To this end, we cultured PBMCs or CBMCs with α-GalCer, the iNKT cell-specific Ag, and IL-2, with or without TGF-β. Two weeks later, the proportion of iNKT cells among total mononuclear cells increased at least 100-fold (from ~0.1% ex vivo to 10–40% after culture) and Foxp3 expression was consistently induced among iNKT cells from both CBMCs and PBMCs when they were incubated with TGF-β (Fig. 1D, 1E, right), whereas no signal was detected without TGF-β (Fig. 1D, 1E, left). TGF-β–induced Foxp3 expression also occurred following less strong TCR sig-

**Figure 1.** Foxp3 expression by human iNKT cells. A, Representative FACS profile of iNKT cells gated ex vivo as a CD1d-tetramer−CD3+ subset. B and C, Representative FACS profile showing the expression of intracellular Foxp3 (solid line histograms) in iNKT cells (left) or CD4+CD25+ cells (right) from freshly isolated cord (B) or peripheral blood (C). The shaded histogram represents the isotype control. Data are representative of three to five independent donors. D and E, Representative FACS profile of Foxp3 expression among gated iNKT cells from cord (D) or peripheral blood (E) expanded with α-GalCer plus IL-2 in the presence of medium (left) or TGF-β (right). The shaded histogram represents the isotype control and percentages are indicated. Data are from 10 CBMC and 20 PBMC donors.
mental Fig. 1). Additionally, ex vivo-sorted iNKT cells activated in the presence of TGF-β also expressed Foxp3 (Supplemental Fig. 1B), showing that these molecules suffice to induce the expression of Foxp3. Taken together, these findings indicate that iNKT cells could respond to more physiological and less potent stimuli than α-GalCer to express Foxp3.

Knowing that human iNKT cells are heterogeneous as to their expression of CD4 (6, 29), we examined whether the ability to become Foxp3+ was associated preferentially with either subset. It turned out that both CD4+ and CD4- iNKT cells from peripheral blood shared this potential (Fig. 2A). However, Foxp3+ cells were more frequent among gated CD4+ iNKT cells than among their CD4- counterpart (Fig. 2B). Similarly, higher levels of Foxp3+ were expressed among CD4+ iNKT cells sorted ex vivo and activated with α-GalCer in the presence of TGF-β (Supplemental Fig. 1C).

**Foxp3 expression by iNKT cells is not associated with all Treg cell surface markers**

Because Treg cells are characterized by constitutive surface expression of the IL-2 receptor α-chain (CD25), we verified whether this applied also to Foxp3+ iNKT cells. We found that TGF-β significantly upregulated CD25 expression among the expanded iNKT cell population and that its Foxp3+ subset was mainly CD25+ whether it originated from cord or peripheral blood (Fig. 3). However, Foxp3+ iNKT cells were not necessarily CTLA4+ or GITR+, even though the expression of these markers increased in TGF-β-expanded iNKT cells (Fig. 3).

Taken together, these results indicate that TGF-β can induce Foxp3 expression by iNKT cells following Ag stimulation, without conferring a typical Treg cell phenotype.

**TGF-β inhibits IL-4 and IFN-γ, but increases IL-10 production by iNKT cells**

A fundamental prerequisite for the immunoregulatory functions of iNKT cells in autoimmune diseases, tumor surveillance, allergic asthma, and infectious diseases is their ability to produce cytokines that determine the orientation of the immune response, such as IL-4 and IFN-γ. This characteristic, together with the implication of TGF-β in the peripheral differentiation of the specific cytokine-producing T cell subset (30), led us to assess its effect on the cytokine profile of expanded iNKT cells. We found that IL-4 and IFN-γ production was decreased when iNKT cells had been exposed to TGF-β (Fig. 4). Our data also revealed that cytokines were predominantly generated by the Foxp3+ iNKT cell fraction (Fig. 4A, 4B).

With regard to IL-10, a cytokine naturally produced by Treg cells (28), we found that its production was increased in Foxp3+ iNKT cells generated in the presence of TGF-β from cord and peripheral blood (Fig. 4A, 4B, respectively). However, IL-10+ cells were also detected among the Foxp3- subset (Fig. 4). Foxp3+ iNKT cells produced no IL-17 (Fig. 4), contrary to what has recently been reported for their Treg cell counterpart (31).

**TGF-β alone does not confer suppressor functions to Foxp3+ iNKT cells**

Foxp3 has been identified as a key transcription factor initiating the regulatory program that confers the Treg cell phenotype and suppressor functions to conventional CD4+CD25- T cells (32–34). We assessed whether suppressor activity was likewise associated with Foxp3 expression by iNKT cells expanded from cord or peripheral blood in the presence of α-GalCer plus IL-2 with or without TGF-β and sorted 2 wk later as a CD25+ fraction. We chose this procedure of enrichment (Fig. 3) because intracellular staining of Foxp3 excluded electronic sorting of viable cells. We assessed the suppressor activity of CD25+ iNKT cells by tracing cell divisions of labeled CD4+CD25- T cells (responder T cells)

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**FIGURE 2.** Foxp3 expression by human CD4+ and CD4- iNKT cell subsets. A. Intracellular staining of Foxp3 in CD4+ or CD4- iNKT cell subsets (gated as shown in the left graph) from peripheral blood cultured with α-GalCer plus IL-2 in the presence of medium (shaded histograms) or TGF-β (solid line histograms). Percentages are indicated. **B.** Frequency of cells expressing Foxp3 among gated CD4+ or CD4- iNKT cell subsets from peripheral blood cultured as described in A. Each symbol represents one donor; horizontal bars indicate mean. Data are established from 20 donors.

**FIGURE 3.** Phenotype of Foxp3+ iNKT cells. Expression of Foxp3 and CD25, CTLA4, or GITR by iNKT cells expanded from cord (A) or peripheral blood (B) with α-GalCer plus IL-2 in the presence of medium (top) or TGF-β (bottom). Percentages are indicated in each quadrant. Data are representative of at least three donors.

**FIGURE 4.** Intracellular staining of Foxp3 in CD4+ or CD4- iNKT cell subsets (gated as shown in the left graph) from peripheral blood cultured with α-GalCer plus IL-2 and medium (shaded histograms) or TGF-β (solid line histograms). Percentages are indicated. B. Frequency of cells expressing Foxp3 among gated CD4+ or CD4- iNKT cell subsets from peripheral blood cultured as described in A. Each symbol represents one donor; horizontal bars indicate mean. Data are established from 20 donors.
by subsequent CFSE dilution during a 4-d coculture with different ratios of CD25+ iNKT together with anti-CD3 plus anti-CD28 and anti-CD2 beads. Contrasting with freshly sorted natural Treg (CD4+CD25+) cells, which drastically reduced responder cell proliferation (Fig. 5A), CD25+ iNKT cells failed to exert suppression, regardless of whether they had been expanded in the presence of TGF-β (Fig. 5A).

We repeated these experiments with more “experienced” iNKT cells derived from peripheral blood, with similar results (Fig. 5B). The lack of suppressor function was confirmed by cocultures with the CD25+CD4+ iNKT subset, which contains a higher proportion of cells expressing Foxp3 at higher levels (Fig. 2).

The acquisition of Foxp3 expression by human iNKT cells is therefore not sufficient to confer suppressor functions, in agreement with the finding that mere TCR stimulation can induce Foxp3 expression in apparently naive human conventional Foxp3+ CD4+ T cells without rendering them suppressive (24, 25).

Rapamycin synergizes with TGF-β to confer suppressive activity to Foxp3+ iNKT cells

Knowing that the addition of the mammalian target of rapamycin (mTOR) enhances the suppressor functions of human CD4+ T cells (35), we assessed the effect of this treatment on Foxp3+ iNKT cells. We found that ~70% of iNKT cells from both cord and peripheral blood expressed Foxp3 when expanded in the presence of TGF-β plus rapamycin (Fig. 6A), as compared with controls cultured with TGF-β alone (Fig. 1D, 1E). Furthermore, exposure to rapamycin reduced the number of iNKT cells recovered by at least one half, but maintained the expression of Foxp3 over time, whereas it was lost in the presence of TGF-β alone (Fig. 6B). The cells treated with the mTOR inhibitor were CD25high but once again were not necessarily CTLA4+ and GITR+ (Supplemental Fig. 2A, 2B) and failed to produce IL-4 and IFN-γ while secreting IL-10 (Supplemental Fig. 3A, 3B).

Regardless of their phenotypic similarity with TGF-β–expanded iNKT cells, rapamycin-treated cells acquired suppressive activity, as assessed by their ability to inhibit proliferation of autologous or heterologous CD25+CD4+ T cells (Fig. 6C, 6E). Peripheral and cord blood iNKT cells were equally suppressive and expressed similar levels of Foxp3 (Fig. 6D), although the latter are considered less experienced, and we have previously reported that they display a distinct cytokine profile (12).

To learn more about the mechanisms accounting for the suppressive activity of Foxp3+ iNKT cells induced by mTOR inhibition, we sorted CD25+ iNKT cells cultured with rapamycin plus TGF-β, stimulated them, and further tested the effect of their cell culture supernatants on the proliferation of conventional CD25−CD4+ T cells. CD25+ iNKT cell supernatants did not inhibit T cell proliferation (Fig. 7A), indicating the requirement of a cell contact-dependent mode of action. Nevertheless, the suppressive effect of human iNKT cells was not affected by GITR blockage (Fig. 7B), and apoptosis of conventional CD25−CD4+ T cells was not observed during coculture. We analyzed some potential mediators of suppression by RT-PCR and observed enhanced transcription of Foxp3, IRF-4, IL-2RA, CCR4, and galec tin-3 (LGALS3) mRNAs when rapamycin and TGF-β were present (Supplemental Fig. 4). However, FasL or granzyme B transcripts were not amplified in both cord and peripheral blood (Supplemental Fig. 4), suggesting the involvement of other molecules. A possible candidate is CD25, the IL-2Ra-chain, that is highly expressed by suppressive iNKT cells. In this context we observed that IL-2 concentrations were reduced in supernatants of responder T cells cultured with suppressive iNKT cells compared with supernatants of responder T cells cultured alone (Fig. 7C). These results suggest that Foxp3+CD25high iNKT cells exert their suppressive activity by consumption of IL-2.

Discussion

We show in this study that human iNKT cells upon stimulation with TGF-β in combination with rapamycin express Foxp3 and suppress conventional CD4+ T cell proliferation. In agreement with these data, it has recently been reported that activated murine and human iNKT cells can express Foxp3 at mRNA or protein levels (22, 23). These studies did not demonstrate that human Foxp3+ iNKT cells could inhibit the expansion of other T cells. In this
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A

Representative FACS profile of Foxp3 expression in gated iNKT cells from cord (CBMCs) or peripheral blood (PBMCs) expanded with α-GalCer plus IL-2 in the presence of TGF-β plus rapamycin. The shaded histogram represents the isotype control. Percentages are indicated. Data are from 15 CBMC donors and 20 PBMC donors. B, Frequency of Foxp3-expressing cells among gated iNKT cells expanded with α-GalCer plus IL-2 in the presence of TGF-β alone or TGF-β plus rapamycin at different times of culture. Data are representative of three donors. C, CD25+ cells were sorted from iNKT cells cultured with α-GalCer plus IL-2 in the presence of TGF-β alone or TGF-β plus rapamycin. The cytokine profile differs likewise between iNKT cells from cord and peripheral blood with the former being more pro-Th2 than the latter (12, 26). Globally, cord blood iNKT cells produce less cytokines and require additional priming/differentiation to become fully functional immune cells (26, 27). It could therefore be expected that iNKT cells from cord and peripheral blood behave differently in terms of Foxp3 expression and suppressive activity. This was actually not the case, as they do exactly the opposite and become highly sensitive to apoptosis of rapamycin, being resistant to apoptosis, whereas effector T cells are apoptotic and downregulate proapoptotic proteins in the presence of rapamycin. Treg cells upregulate anti-apoptotic and downregulate proapoptotic proteins in the presence of rapamycin, being resistant to apoptosis, whereas effector T cells do exactly the opposite and become highly sensitive to apoptosis (42). We can speculate that, similarly, TGF-β–induced Foxp3+ iNKT cells will be more resistant to apoptosis than will Foxp3+ iNKT cells, being selectively expanded in the presence of rapamycin. Overall, the effect observed on the expression of Foxp3 by human iNKT cells in the presence of rapamycin may be explained as the sum of the two effects, that is, the promotion of Foxp3 induction by TGF-β and the selective proliferation of Foxp3+ iNKT cells, which become resistant to apoptosis in the presence of rapamycin.

According to their origin, iNKT cells can express distinct surface markers, with the majority (>90%) of cord blood-derived cells being CD4+CD161low, whereas in peripheral blood up to 95% are CD161− and the frequency of CD4+ varies individually, but is generally <50% (12). The cytokine profile differs likewise between iNKT cells from cord and peripheral blood, with the former being more pro-Th2 than the latter (12, 26). Globally, cord blood iNKT cells produce less cytokines and require additional priming/differentiation to become fully functional immune cells (26, 27). It could therefore be expected that iNKT cells from cord and peripheral blood behaved differently in terms of Foxp3 expression and suppressive activity. This was actually not the case, as they inhibited the proliferation of conventional CD4+ T cells similarly, which suggests that the immunosuppressive potential is present at birth. Furthermore, direct mechanisms are responsible for the suppressive iNKT cell activity since it requires no APCs, as it study we report that the acquisition of this Foxp3+ phenotype was only associated with that of suppressor functions when rapamycin was present together with TGF-β, which is consistent with several reports showing that a significant number of human activated T cells express Foxp3 but do not acquire regulatory activity (24, 25). Thus, to our knowledge our results represent the first report that human Foxp3+ iNKT cells can suppress the proliferation of conventional human CD4+ T cells. This capacity was observed both in peripheral and cord blood iNKT cells, suggesting that it represents an intrinsic ability of these cells. It has been documented that sustained Foxp3 expression in Treg cells is necessary for maintenance of the Treg cell phenotype and suppressor function (36, 37), which is the case when the mTOR pathway is blocked by rapamycin, which confers suppressive activity to human Foxp3+ iNKT cells. The induction of Foxp3 by TGF-β depends on the activation of the transcription factor SMAD3 that cooperates with NFAT to induce Foxp3 gene expression (38). Interestingly, signaling through the mTOR pathway can inhibit activation of SMAD3 by TGF-β (39), providing an explanation for the observation that mTOR inhibition by rapamycin supports Foxp3 upregulation. Additionally, rapamycin blocks proliferation of conventional effector T cells, whereas it promotes selective survival and growth of highly suppressive Treg cells (40, 41). It has been suggested that this differential response of effector T cells and Treg cells is due to their divergent activation/cell death response in the presence of rapamycin. Treg cells upregulate anti-apoptotic and downregulate proapoptotic proteins in the presence of rapamycin, being resistant to apoptosis, whereas effector T cells do exactly the opposite and become highly sensitive to apoptosis (42). We can speculate that, similarly, TGF-β–induced Foxp3+ iNKT cells will be more resistant to apoptosis than will Foxp3+ iNKT cells, being selectively expanded in the presence of rapamycin. Overall, the effect observed on the expression of Foxp3 by human iNKT cells in the presence of rapamycin may be explained as the sum of the two effects, that is, the promotion of Foxp3 induction by TGF-β and the selective proliferation of Foxp3+ iNKT cells, which become resistant to apoptosis in the presence of rapamycin.
occur when sorted iNKT cells and conventional CD4+ T cells are cocultured, indicating that it is an intrinsic property of iNKT cells.

TGF-β has been identified as a major factor mediating the differentiation of naive CD4+ T cells into both Th1 and Treg cells (43). The ability of TGF-β to inhibit Th1 and Th2 cytokine production is one of the mechanisms responsible for this effect. We found that TGF-β inhibited IL-4 and IFN-γ production by activated iNKT cells and that the Foxp3+ subset did not produce these cytokines, in contrast with their Foxp3- counterpart. Despite the evidence for IL-17 by human iNKT cells in response to TGF-β, IL-1β, and IL-23 (12), as well as by a fraction of conventional Treg cells (31, 44), this cytokine was not generated by Foxp3+ iNKT cells.

Conversely, IL-17–producing iNKT cells did not express Foxp3. Results obtained in this study and in our previous report (12) suggest that human iNKT cells differ from conventional T cells inasmuch as they do not pass through a Foxp3-RORC double-positive stage (45), which could be a common transition before differentiation into functional IL-17 producers or suppressors.

All cytokines were not downregulated in the presence of TGF-β since Foxp3+ iNKT cells continued to produce IL-10, considered a mediator of Treg cell suppression that in human cells can occur through the following mechanisms: 1) inhibitory cytokines, 2) cytolyis, 3) metabolic disruption, and 4) modulation of dendritic cell maturation and function. Despite their presence, IL-10 and other soluble factors were not responsible for the suppression of conventional CD4+ T cell proliferation by iNKT cells. Cytolysis is also an unlikely mechanism, as we detected neither apoptosis in the target cells nor enhanced transcription of FasL or granzyme B mRNAs in suppressor iNKT cells. We can also discard the fourth possibility, as APCs or dendritic cells are not required in our suppressor assay. We favor the explanation that Foxp3+CD25+ iNKT cells absorb the IL-2 produced in the system and thus hamper CD4+ T cell proliferation through direct cytokine consumption. In support of this mechanism, rapamycin, which confers the suppressive activity to iNKT cells, strongly upregulated their CD25 expression, and lower levels of IL-2 were found in the supernatants of responder T cells cultured with suppressive iNKT cells than when cultured alone.

Conventional Foxp3+ T cells bear a diverse TCR repertoire potentially capable of suppressing activation and/or effector functions of other T cells. Natural Treg cells need to be stimulated through their TCRs to exert suppression, whereas induced Treg cells exhibit non-Ag–specific bystander activity, at least in vitro (46). Foxp3+ iNKT cells inhibit the proliferation of conventional CD4+ T cells in a non-Ag–dependent manner since they recognize glycolipids instead of peptides. According to recent reports, innate signals rather than bacterial Ags account mainly for their participation in diverse anti-microbial responses (47). In this study, we suggest that Foxp3+ iNKT cells might be implicated mostly in the surveillance of autoreactive cells and that they broadly suppress autoimmunity once they have become activated by endogenous Ags presented by CD1d molecules. They could act in conjunction with conventional Treg cells to suppress the proliferation of CD4+ T cells, but we cannot exclude the possibility that Foxp3+ iNKT cells could have more specific roles in patrolling the immune system. As reported in this study, Foxp3+ iNKT cells can suppress both autologous and heterologous CD4+ T cells, suggesting that these cells could contribute to controlling exacerbated inflammatory responses and to autoimmunity, but also to the expansion of heterologous cells as in the case of graft-versus-host disease. In support of this notion, several studies have provided evidence for a role of iNKT cells in promoting or maintaining tolerance in a variety of experimental graft-versus-host disease or autoimmune disorders (18, 19, 48–52). The mechanisms accounting for their tolerogenic effects are still unclear but they were shown to be independent from IL-4 and IL-10 in autoimmune diabetes (53). The strong suppressive properties displayed by Foxp3+ iNKT cells described in this study might explain some of these observations. It has been demonstrated that iNKT cells can prevent type 1 diabetes through inhibition of autopathogenic T cell expansion and full differentiation, rendering them unable to destroy pancreatic islets. Interestingly, this immunoregulatory function of iNKT cells required cell contact (53), which mirrors Foxp3+ iNKT cell regulatory function and our findings that cytokines are not always the principal mediator of iNKT cell action during the immune responses.

In conclusion, our findings clearly demonstrate that human iNKT cells are endowed with the capacity to become suppressor cells when stimulated in the presence of TGF-β plus rapamycin. Foxp3+ iNKT cells are likely to be implicated in tolerogenic immune responses and could contribute to dampen autoimmune inflammation, as in diabetes. We provide additional proof of the plasticity of human iNKT cells and the major role of the environment in controlling their functional properties, thus offering additional means of manipulating iNKT cell functions, making the way for adoptive immunotherapy.
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Disclosures

The authors have no financial conflicts of interest.

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