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The Role of ICOS in Directing T Cell Responses: ICOS-Dependent Induction of T Cell Anergy by Tolerogenic Dendritic Cells[1]

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Tolerogenic dendritic cells (DC) play an important role in maintaining peripheral T cell tolerance in steady-state conditions through induction of anergic, IL-10-producing T cells with suppressive properties. ICOS, an activation-induced member of the CD28 family on T cells, is involved in the induction of IL-10, which itself could contribute to induction of anergy and development of suppressive T cells. Therefore, we analyzed the functional role of ICOS in the differentiation process of human $CD4^+$ T cells upon their interaction with tolerogenic DC. We compared the functional properties of $CD4^+$ T cells from healthy volunteers and ICOS-deficient patients after stimulation with tolerogenic DC. We report that induction of T cell anergy and suppressive capacity is completely blocked after knockdown of ICOS expression in T cells as well as after blocking of ICOS-ICOS ligand interaction in DC/T cell cocultures. Moreover, $CD4^+$ T cells from ICOS-deficient patients were completely resistant to anergy induction and differentiation into suppressive T cells even after supplementation of IL-10. Furthermore, ICOS/ICOS ligand interaction stabilizes IL-10R expression on T cells and thus renders them sensitive to IL-10 effects. Taken together, these results indicate a crucial role for ICOS in the induction of peripheral tolerance maintained by tolerogenic DC mediated mostly via an IL-10-independent mechanism. *The Journal of Immunology,* **2009, 182: 3349–3356.**

Dendritic cells (DC)[7] not only initiate effector T cell responses but are also involved in silencing T cell-mediated immune responses. The functional activities of DC are mainly dependent on their state of activation and differentiation. While terminally differentiated mature DC (mDC) efficiently induce the development of effector T cells, immature DC (iDC) are involved in the maintenance of peripheral tolerance by induction of anergic T cells with regulatory properties (1, 2). In this system, T cell anergy is characterized by an impaired T cell proliferation as well as reduced cytokine production of the Th1 type (3, 4). In steady state, myeloid iDC lead to the induction of anergic, IL-10-producing T cells with suppressive properties (3). However, there is limited information available about the mechanism by which iDC induce anergy and suppressive function in T cells and the molecules involved in this differentiation process. IL-10 seems to play a critical role, since the induction of anergy and suppressive properties by iDC can be abrogated by administration of anti-IL10R Abs (5), but the source of IL-10 under these circumstances remains unknown. One molecule of special interest in this setting is ICOS, since costimulation through ICOS on $CD4^+$ T cell has been linked to IL-10 secretion by these lymphocytes (6–8), which then itself could contribute to the induction of anergy and thereby initiate the development of suppressive T cells. ICOS is expressed on $CD4^+$ T cells upon activation by TCR engagement (6) and specifically interacts with its ligand, ICOS ligand (ICOS-L) on APC like DC (7). In contrast to plasmacytoid DC, the expression level of ICOS-L on myeloid DC is higher on iDC than on mDC (9).

In humans, ICOS deficiency is a rare disease. Nine individuals have been described so far with a complete loss of ICOS on their T cells, as a result of a large homozygous deletion eliminating any protein expression, and thus leading to a "knockout" phenotype (10). The clinical phenotype is that of common variable immunodeficiency (CVID), an Ab deficiency syndrome. In patients with CVID, and hence in patients with ICOS deficiency, the production of IgG, IgA, and IgM following Ag exposure is severely impaired, leading to a profound hypogammaglobulinemia and the susceptibility to recurrent infections, mostly to encapsulated bacteria. This B cell phenotype is explained by the lack of T cell help in the germinal center reaction and the lack of $CXCR5^+$ follicular helper T cells in patients with an ICOS deficiency (11). Even though the main clinical focus lays on Ab deficiency and treatment of the recurrent severe infections, the fact that ICOS usually is expressed on T cells renders the T cells of these patients an important model for studying the role of ICOS, especially in its interaction with DC. The downside of this knockout model system is the extremely small number of patients available for studies.

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[7] Abbreviations used in this paper: DC, dendritic cell; CVID, common variable immunodeficiency; EAE, experimental allergic encephalomyelitis; iDC, immature DC; iTreg, induced Treg; mDC, mature DC; siRNA, small interfering RNA; Treg, regulatory T cell.

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There have been several reports indicating a role for ICOS in the induction and function of regulatory T cells (Tregs) (12–17). In distinct mouse models such as experimental allergic encephalomyelitis (EAE) and autoimmune type 1 diabetes, blockade of ICOS signaling by anti-ICOS Abs interrupted the balance between regulatory and effector T cells and worsened disease, indicating a role for ICOS in promoting tolerance and restraining autoimmunity (12, 18). The finding that ICOS-knockout mice show enhanced susceptibility to EAE further stresses a potential role for ICOS in limiting autoimmune pathology (19).

Therefore, we analyzed the role of ICOS in the induction of T cells with regulatory properties by tolerogenic DC.

Materials and Methods

Patients

Blood samples were taken from patients with the molecular diagnosis of ICOS deficiency (P2 (Family A), P3 (Family B), and P4 (Family B)). Case reports and screening for ICOS deficiency in these patients have been described previously (11). According to the principles expressed in the Declaration of Helsinki and to the ethics committee-approved protocol BG239/99, informed written consent was obtained from all patients studied. Cord-blood samples were obtained after informed written consent from the obstetric department of the University Hospital Mainz (protocol 837.029.05 (4687) approved by the local ethics committee, Landesaerztekammer Rheinland-Pfalz).

Culture medium and Abs

X-VIVO-15 (Cambrex) was used for culture of DC and T cells. DC were stained with PE-conjugated anti-CD80 and -CD86 mAbs (BD Pharmingen). T cells were stained with FITC- or PE-conjugated anti-CD3, -CD4, -CD8, and -CD25 mAbs (BD Pharmingen). Unconjugated and PE-conjugated anti-ICOS mAb (F44) and anti-ICOS-L mAb (HIL131) were provided by R. Kroczek (RKI, Berlin, Germany) (6). Anti-human IL-10 mAb was purified from hybridoma supernatant (ATCC clone JES19F1), and IL-10 was purchased from Schering-Plough. IL-10R expression was determined with the Fluorokine recombinant human IL-10 Biotin kit (R&D Systems) according to the manufacturer's instructions. Cells were analyzed by flow cytometry (FACSCalibur and CellQuest software; BD Pharmingen).

Cytokine assays

T cells (10^6) were stimulated with 10^5 allogeneic DC in 24-well plates. Cytokines were determined by analysis of supernatants 48 h after stimulation. Cytometric bead array kit for human cytokines (Human Inflammation Kit I; BD Pharmingen) was used as indicated by the manufacturer. Commercially available ELISA specific for the human cytokines IFN-γ, IL-4, and IL-10 (BD Pharmingen) was used as recommended by the manufacturer. Detection limits were 15 pg/ml. For intracellular analysis, anti-IL-2-PE, anti-IL-4-PE, anti-IL-10-PE, anti-IFN-γ-FITC mAb (BD Pharmingen) were used. Six days after stimulation, T cells were reactivated with 2.4 μg/ml PHA plus 1 ng/ml PMA for 6 h. Monensin (1.3 μM/ml) was added for the last 5 h; cells were collected, washed, permeabilized (perm/fix solution; BD Pharningen), and stained with cytokine-specific mAb.

Generation of DC

DC were generated from human leukapheresis products of healthy volunteers as described previously (3, 20). Briefly, PBMC were isolated by Ficoll density gradient centrifugation and stored frozen in aliquots. For each DC preparation, frozen PBMC were thawed, and monocytes were isolated by plastic adherence and cultured in X-VIVO-15, including 800 U/ml GM-CSF (Leukomax) and 1000 U/ml IL-4 (Strathmann Biotech). Cells were fed on day 3 (X-VIVO-15, 1600 U/ml GM-CSF, 1000 U/ml IL-4). At day 5, nonadherent cells were rinsed off, washed once in PBS, and transferred to fresh 6-well plates (Corning Costar) at 7×10^5 cells in 3 ml/well. For differentiation into mDC, iDC were additionally stimulated on day 6 with 10 ng/ml IL-1β, 10 ng/ml TNF-α, 1000 U/ml IL-6 (all from Strathmann Biotech), and 1 μg/ml PGE_2 (Minprostin; Pharmacia-Upjohn). At day 7, nonadherent iDC and mDC were used for T cell stimulation.

Induction of alloreactive T cell lines

$CD4^+$ T cells from PBMC of healthy volunteers as well as from cord blood were isolated by CD4-Microbeads (Miltenyi Biotec) as described earlier (3). The average purity was >98%. Cells were depleted of $CD25^+$ cells using anti-CD25-Dynabeads (Dynal Biotech). $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T cells were isolated using CD4-multisort Microbeads (Milteny Biotec) as described before followed by positive selection of $CD45RA^+$ T cells using CD45RA Microbeads, resulting in $CD4^+$ $CD45RA^+$ and $CD4^+CD45RO^+$ T cell preparations (purity >95%). T cells (10^6) and 10^5 allogeneic iDC/mDC were cocultured in 24-well plates. Where indicated, anti-ICOS-L mAb was added at a final concentration of 10 μg/ml during primary stimulation. In some experiments anti-human IL-10 mAb (10 μg/ml) or IL-10 (20 ng/ml) was added to the cocultures. Alloreactive T cells were expanded from day 6 in the presence of 50 U/ml IL-2. Two weeks after priming, T cells were harvested and restimulated with iDC/mDC from the same donor as in primary culture and expanded from day 3 on with IL-2, followed by weekly restimulations. Proliferation assays were conducted with 10^5 T cells/well and different numbers of allogeneic iDC/mDC or stimulated with soluble anti-CD3 (0.5 μg/ml) and anti-CD28 mAb (1 μg/ml). In some experiments, additional stimulation with an agonistic anti-ICOS mAb (10 μg/ml) was performed. Where indicated, T cells (60 Gy) or PBMC (90 Gy) were irradiated. T cell proliferation was measured after 3 or 4 days, and an additional 16-h pulse with [^{3}H]Tdr (37 kBq/well). To exclude that reduced T cell proliferation after priming and restimulation with iDC was related to a delayed onset of proliferation, we additionally assessed absolute T cell numbers shown as fold expansion at indicated time points.

Small interfering RNA (siRNA) preparation and nucleofection

Six 25-bp stealth RNA interference sequences were selected from the Invitrogen database (Invitrogen). Sequences that displayed the highest suppressive activity to knockdown ICOS expression were used: ICOS-sense, UCA GCU GGC AAC AAA GUU GUG AUU C; and ICOS-antisense, GAA UCA CAA CUU UGU UGC CAG CUG A. Nucleofection was performed according to Amaxa's optimized protocol using the primary Human T Cell Nucleofector kit (Amaxa). ICOS knockdown was controlled by FACS staining following up to 6 days after nucleofection.

Statistics

The paired Student's *t* test (two-tailed) was used for the comparison of group values. The number of individuals studied in each experiment is given in the corresponding figure legend. Significance was analyzed using Prism (GraphPad Software), and values of $p < 0.05$ were considered significant and indicated in the corresponding figures (*, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$).

Results

ICOS knockdown in human $CD4^+$ T cells abrogates anergy induction by iDC

An essential first step in the conversion of resting $CD4^+$ T cells into IL-10-producing Tregs by iDC is the induction of T cell anergy, characterized by an impaired T cell proliferation and function. Blockade of anergy induction (i.e., by addition of exogenous IL-2) completely prevents the differentiation of $CD4^+$ T cells into Tregs (3, 5). Signals exchanged during the initial interactions of T cells and iDC are critical in this differentiation process, because the state of T cell anergy is irreversible. Resting $CD4^+CD25^-$ T cells primed with iDC remained anergic, as shown in the first restimulation (Fig. 1*a*, *left panel*). This effect was even more pronounced after further repetitive stimulations with iDC in agreement with published data (3) (Fig. 1*a*, *right panel*) and correlated with the cytokine profile induced using mDC or iDC for priming and restimulation (Fig. 1*b*). Furthermore, even after optimal restimulation with immunostimulatory mDC from the same allogeneic donor or by polyclonal restimulation using anti-CD3 and anti-CD28 mAb, T cells remained hyporesponsive (Fig. 1*c*). It has been reported that IL-10 plays an essential role in this context (5). Since ICOS signaling has been linked to IL-10 secretion in T cells (8), we investigated the role of ICOS in the process of anergy induction. Resting $CD4^+CD25^-$ T cells from healthy volunteers were transduced with ICOS-specific siRNA, which prevented ICOS expression on these T cells after activation for up to 3 days (Fig. 2*a*). As a control, an unspecific scrambled siRNA (SC) with comparable amounts of cytosine and guanine was used (data not shown).

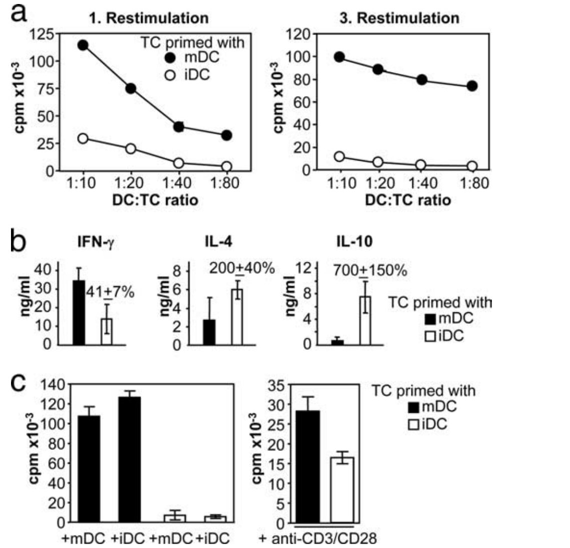

FIGURE 1. Repetitive stimulation with tolerogenic DC results in the induction of T cell anergy. *a*, $CD4^+CD25^-$ T cells from healthy donors (TC) were primed with allogeneic mDC or iDC at a DC/TC ratio of 1:10. After 2 wk, equal numbers of recovered viable cells were restimulated with the same type of DC as in the primary stimulation at different DC/TC ratios. One typical experiment of the first (*left panel*) and the third restimulation (*right panel*) out of five is shown. Proliferation is presented as mean ± SD of triplicates. *b*, Cytokine levels in DC:TC cocultures were determined after the first restimulation using ELISA technique. Cytokine production of three independent experiments is shown as mean ± SD. In addition, percetage of cytokine production of TC primed and restimulated with iDC compared with mDC set to 100% is shown. *c*, $CD4^+CD25^-$ TC primed with either allogeneic mDC or iDC were restimulated with allogeneic mDC and iDC at a DC/TC ratio of 1:10 (*left panel*) or with soluble anti-CD3/anti-CD28, respectively (*right panel*). One typical experiment out of five is shown. Proliferation in the first restimulation is presented as mean ± SD of triplicates.

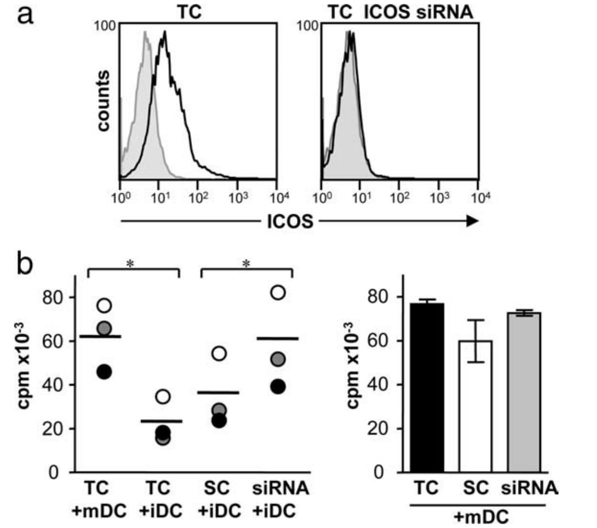

FIGURE 2. siRNA-mediated ICOS knockdown prevents T cell anergy induction by tolerogenic DC. *a*, $CD4^+CD25^-$ TC from a healthy donor were either left untreated or nucleofected with siRNA against ICOS (ICOS siRNA) and stimulated with allogeneic mDC. After 3 days, ICOS expression (open histogram) was analyzed by flow cytometry and plotted against isotype control staining (gray histogram). *b*, After nucleofection with either ICOS siRNA or scrambled control siRNA (SC), $CD4^+CD25^-$ TC were primed with allogeneic iDC (*left panel*) or mDC (*right panel*). Untreated $CD4^+CD25^-$ TC primed with either mDC or iDC served as controls (*left panel*). After 2 wk, TC were restimulated with mDC at a DC/TC ratio of 1:10, and proliferation was determined. Results of three independent experiments are shown for iDC (*left panel*). One typical experiment is shown as mean ± SD of triplicates for mDC (*right panel*).

Only ICOS siRNA, but not control treatment, significantly enhanced the proliferative capacity of $CD4^+$ T cells in the presence of allogeneic iDC (Fig. 2*b*, *left panel*). Reduced ICOS expression on T cells during their initial stimulation decreased the induction of T cell anergy. These T cells showed strong proliferation after priming and restimulation with iDC, comparable to T cells primed with mDC (Fig. 2*b*, *left panel*). In contrast, ICOS knockdown in T cells did not have any effects on their response to stimulation with mature DC (Fig. 2*b*, *right panel*).

Blockade of ICOS/ICOS-L interaction prevents anergy induction in resting $CD4^+$ T cells

To test our hypothesis that ICOS/ICOS-L interaction between T cells and iDC during the priming phase is crucial for anergy induction, we used a blocking Ab to ICOS-L. In the presence of blocking anti-ICOS-L mAb, resting $CD4^+CD25^-$ T cells primed with iDC showed a stronger proliferative capacity than T cells primed with iDC alone (Fig. 3*a*). Since the differences seen in unseparated $CD4^+CD25^-$ Th cells were already striking and the induction of anergy and suppressive capacity by tolerogenic DC is dependent on the T cell differentiation state (3), we consequently compared the relevance of ICOS expression on resting $CD4^+CD45RA^+$ T cells and memory $CD4^+CD45RO^+$ T cells from healthy volunteers. While $CD4^+CD45RA^+$ T cells were anergic after priming with iDC, blockade of ICOS/ICOS-L interaction during initial coculture with allogeneic iDC prevented this anergy induction (Fig. 3*b*, *left panel*). Anergy induction was more pronounced in $CD4^+CD45RA^+$ T cells than in $CD4^+CD45RO^+$ T cells (Fig. 3*b*). Furthermore, blockade of ICOS/ICOS-L interaction in cultures of $CD4^+CD45RO^+$ T cells and iDC did not significantly enhance the proliferative capacity of these T cells after restimulation.

To confirm the results seen in resting $CD4^+CD45RA^+$ T cells, we isolated naive $CD4^+$ T cells from human cord blood and used these T cells for repetitive stimulation with allogeneic iDC/mDC in the presence or absence of blocking ICOS-L mAb. Blockade of ICOS/ICOS-L interaction abolished anergy induction in naive $CD4^+$ T cells (Fig. 3*c*) and enhanced their expansion (Fig. 3*d*). These results strengthen the view that ICOS/ICOS-L interaction is mandatory for anergy induction by tolerogenic DC in naive T cells.

Anergy induction by iDC is completely abolished in $CD4^+$ T cells from ICOS-deficient patients

A small subgroup of patients diagnosed with CVID is characterized by a homozygous genomic deletion of ICOS in exons 2 and 3 (21). The phenotype of these ICOS-deficient patients involves an increased susceptibility to bacterial infections, which has been mainly attributed to the loss of Ab production and memory B cells due to impaired T cell help. Despite the loss of $CXCR5^+$ follicular Th cells, $CD4^+$ T cells from these patients do not show dramatic phenotypical or functional alterations (21). However, compared with healthy controls, peripheral blood from ICOS-deficient patients contains drastically reduced numbers of total PBMC (2–6 $\times 10^5$ total PBMC compared with 1–2 $\times 10^6$ total PBMC/ml blood in normal donors) and of $CD4^+$ T cells. Flow cytometric analysis did not reveal phenotypical differences in T cells from ICOS-deficient patients compared with healthy controls (data not shown). Blood from three distinct ICOS-deficient patients was available for

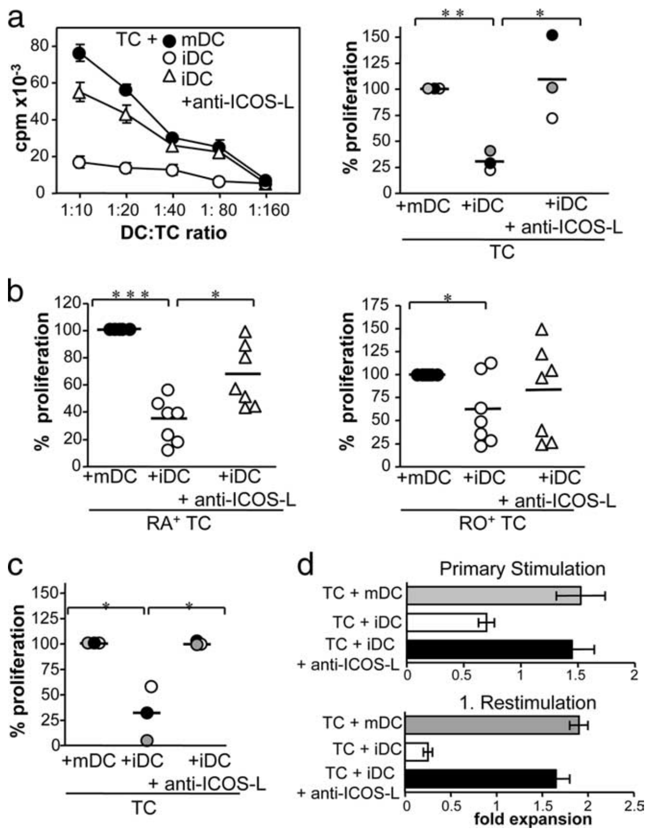

FIGURE 3. Anergy induction in naive CD4$^+$ T cells is completely abolished after blocking of ICOS/ICOS-L interaction. *a*, Proliferation in the first restimulation of CD4$^+$CD25$^-$ TC primed with allogeneic iDC in the presence of blocking anti-ICOS-L mAb was determined. TC primed with mDC or iDC served as controls. Proliferation (mean ± SD of triplicates) of one typical experiment out of three is shown (*left panel*). Proliferation of three independent experiments is presented as percentage of proliferation of TC primed and restimulated with mDC at a ratio of 1:10 set to 100% (*right panel*). *b*, Proliferation in the first restimulation of CD4$^+$CD45RA$^+$ (RA$^+$ TC, *left panel*) or CD4$^+$CD45RO$^+$ TC (RO$^+$ TC, *right panel*) primed with iDC in the presence of anti-ICOS-L mAb was determined after restimulation with allogeneic mDC. TC either primed with mDC or iDC served as controls. Proliferation of seven independent experiments is shown as percentage of proliferation of TC primed and restimulated with mDC set to 100%. *c*, Naive cord-blood-derived CD4$^+$ TC were primed with mDC, iDC, or iDC in the presence of anti-ICOS-L mAb. Proliferation 5 days after the first restimulation with allogeneic mDC is presented as percentage of proliferation of TC primed and restimulated with mDC set to 100% ($n = 3$). *d*, In parallel, expansion rates of the primary culture (2 wk after priming) and the first restimulation (1 wk after restimulation) were determined by dividing the cell numbers counted at the end of a culture period. Proliferation rates are shown as mean expansion ± SD of independent experiments in the primary stimulation ($n = 4$) and in the first restimulation ($n = 2$).

repetitive analysis. CD4$^+$CD25$^-$ T cells were isolated and stimulated with allogeneic DC as indicated. The proliferation of ICOS-deficient T cells primed with iDC was comparable to T cells primed with mDC and did not result in anergy induction (Fig. 4*a*). Compared with CD4$^+$ T cells from healthy volunteers, alloreactive CD4$^+$ T cells from ICOS-deficient patients expanded more vigorously during primary culture with iDC, and no anergy induction was observed after restimulation (Fig. 4*b*). No increased cell death was detectable in T cell cultures from healthy volunteers in comparison to ICOS-deficient patients as determined by trypan blue exclusion and propidium iodide staining (data not shown). In conclusion, these results show again that ICOS expression is mandatory for anergy induction by tolerogenic DC.

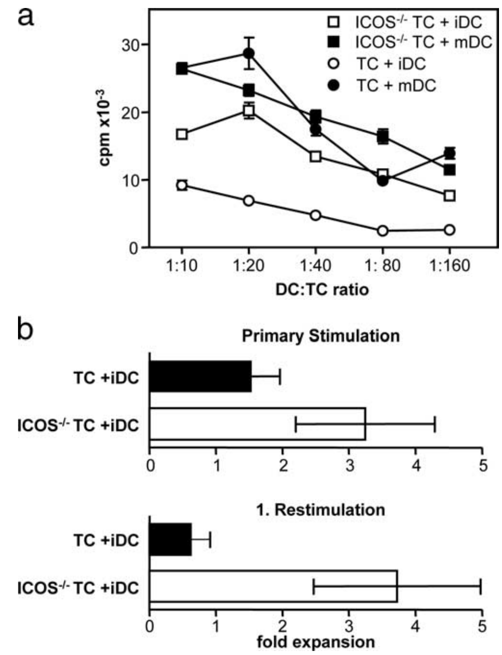

FIGURE 4. No anergy induction by tolerogenic DC in ICOS-deficient T cells. *a*, CD4$^+$CD25$^-$ TC from ICOS-deficient patients (ICOS$^{-/-}$) were primed with either allogeneic mDC or iDC at a DC/TC ratio of 1:10. CD4$^+$CD25$^-$ TC from healthy donors primed with iDC or mDC served as control. After 2 wk, equal numbers of recovered viable cells were restimulated with the same allogeneic DC as in the primary culture at different DC/TC ratios. One typical experiment out of three is shown. Proliferation in the first restimulation is presented as mean ± SD of triplicates. *b*, CD4$^+$CD25$^-$ T cells from either ICOS-deficient patients (ICOS$^{-/-}$ TC) or healthy donors (TC) were primed and restimulated with allogeneic iDC at a DC/TC ratio of 1:10. The expansion rates of the primary cultures and the first restimulation (1.RS) were determined by dividing the cell numbers counted at the end of a culture period by the cell numbers at the beginning. The proliferation rates of four independent experiments are shown as mean expansion ± SD.

Absence of ICOS leads to decreased IL-10 sensitivity through down-regulation of the IL-10R expression on T cells

Since ICOS seems to be involved in IL-10 production, which in itself could drive anergy induction and development of T cells with a suppressive phenotype, we analyzed the cytokine profile of ICOS-deficient T cells differentiated in the presence of iDC. Anergy induction in T cells by repetitive stimulation with allogeneic iDC is accompanied by a shift in the cytokine profile from a Th0 profile (intermediate amounts of IL-4 and IFN-γ) during the first restimulation toward a profile with high amounts of IL-10 and reduced secretion of IL-2, IFN-γ, or IL-4 in following restimulations (3). We primed and restimulated CD4$^+$CD25$^-$ T cells from ICOS-deficient patients and healthy donors with allogeneic iDC as described. Supernatants from ICOS-deficient T cells stimulated with iDC showed an increased production of IL-10 and IL-4 compared with the controls (Fig. 5*a*). To clarify the source of IL-10—since both T cells and DC are potential producers—intracellular staining for cytokines was performed. In contrast to the cytokine profile detected by ELISA, intracellular staining of T cells revealed only a slight decrease of IL-10 production in ICOS-deficient T cells and no major differences in the production of IFN-γ and IL-2 compared with normal T cells (Fig. 5*b*). These data suggest that

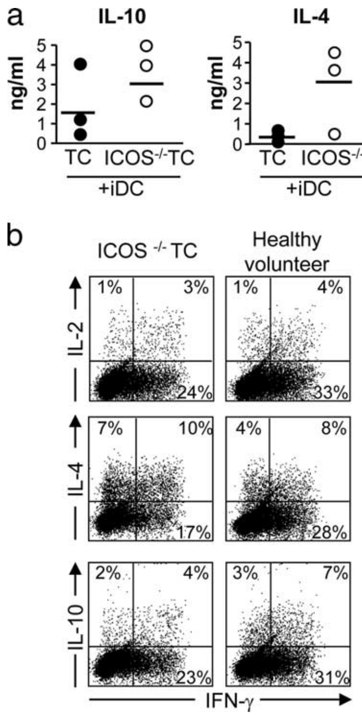

FIGURE 5. Cytokine profile in T cells primed in the absence of ICOS. *a*, $CD4^+CD25^-$ TC from either an ICOS-deficient patient ($ICOS^{-/-}$) or a healthy donor were primed and restimulated with allogeneic iDC at a DC/TC ratio of 1:10. Supernatants were collected 48 h after the first restimulation with iDC and analyzed for IL-10 (*left panel*) and IL-4 (*right panel*) using the cytometric bead array assay. The amount of IL-10 and IL-4 in three independent experiments is shown. *b*, Detection of cytokine production of TC in the fourth restimulation by intracellular flow cytometric analysis and gating on lymphocytes. Numbers represent the percentage of cells in each quadrant; quadrants were set according to isotype control. One typical experiment out of five is shown.

DC are responsible for the enhanced IL-10 production in the absence of ICOS-ICOSL interaction. However, to analyze the possibility that an altered kinetic in the production of IL-10 during the interaction of iDC with ICOS-deficient T cells might be responsible for the observed effects, we added IL-10 to the T cell/DC cocultures during primary stimulation. Whereas the addition of IL-10 to T cell/DC cocultures of normal donors resulted in a significant reduction of T cell proliferation, exogenous IL-10 did not reduce the proliferative capacity of ICOS-deficient T cells stimulated with iDC (Fig. 6*a*), indicating that IL-10 cannot influence the activation process of $CD4^+$ T cells in the absence of ICOS expression. In contrast, addition of blocking anti-IL-10 mAb to primary cultures of iDC and $CD4^+$ T cells from healthy volunteers partially prevented the anergy induction (Fig. 6*b*), suggesting that IL-10 and ICOS might cooperate in this process, but ICOS expression is mandatory for the inhibitory potential of IL-10. To analyze this concept in more detail, cord-blood-derived naive $CD4^+$ T cells were stimulated with iDC in the presence or absence of anti-ICOSL mAb, and IL-10R expression was analyzed on activated T cells while mDC served as controls. As shown in Fig. 6*c*, IL-10R expression was more pronounced on T cells stimulated with iDC compared with stimulation with mDC. Nevertheless, addition of a blocking anti-ICOS-L mAb resulted in a reduced expression of IL-10R on these T cells. These results showed a good correlation with the surface expression of the two DC populations used for stimulation. Although mDC showed a high expression of the costimulatory molecules CD80/86, iDC were characterized by

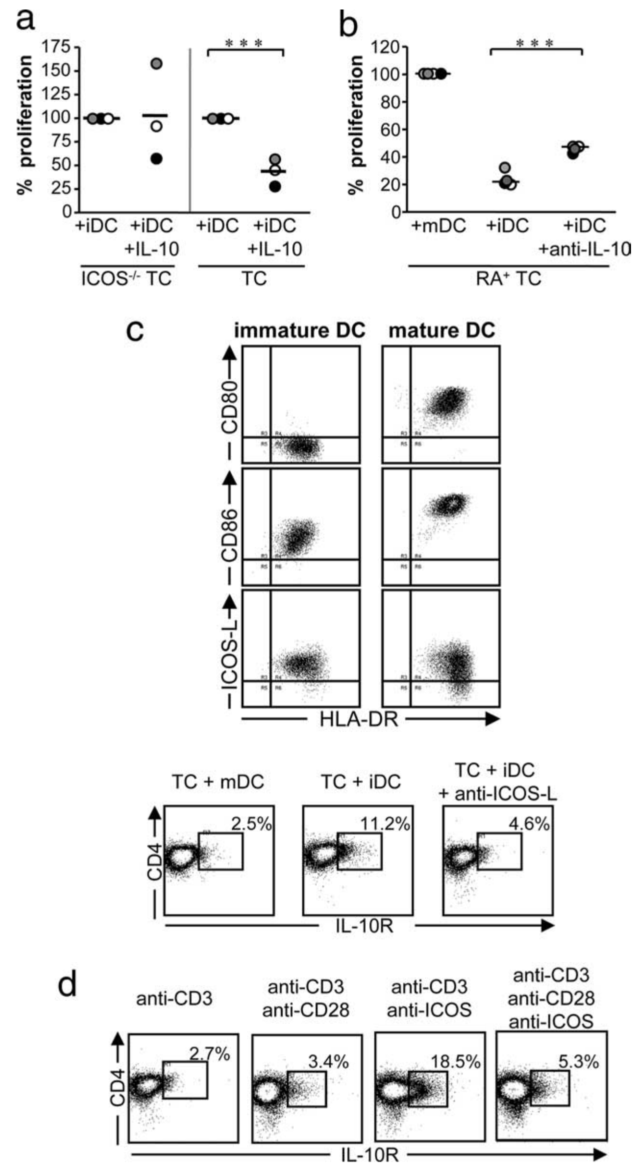

FIGURE 6. ICOS-dependent IL-10 sensitivity and IL-10R expression in T cells. *a*, $CD4^+CD25^-$ TC from an ICOS-deficient patient ($ICOS^{-/-}$) were primed with allogeneic iDC alone or with iDC in the presence of IL-10. As a control, the same experiment was performed using $CD4^+CD25^-$ TC from a normal donor. Proliferation in the first restimulation of three independent experiments is presented as percentage of proliferation of TC cells primed and restimulated with iDC alone set to 100%. *b*, Proliferation in the first restimulation of $CD4^+CD45RA^+$ TC (RA^+ TC) primed with iDC in the presence of anti-IL-10 mAb was determined. $CD4^+CD45RA^+$ TC either primed with mDC or iDC served as controls. Proliferation in the first restimulation of four independent experiments is presented as percentage of proliferation of T cells primed and restimulated with mDC set to 100%. *c*, Naive cord-blood-derived $CD4^+$ T cells were stimulated with iDC in the presence or absence of anti-ICOSL mAb as indicated; mDC served as a control. Five days after stimulation, IL-10R expression on T cells was determined. Percentage of double-positive cells is shown, and gating was set according to the isotype control. One typical experiment out of three is shown (*upper panel*). FACS staining of mDC and iDC using the indicated mAb was performed at day 7 of culture. Surface expression of CD80, CD86, and ICOS-L is shown. One of 10 typical experiments is shown. *d*, Naive cord-blood-derived $CD4^+$ T cells were stimulated with different combinations of soluble anti-CD3, anti-CD28, and anti-ICOS mAb as indicated. Five days after stimulation, IL-10R expression was determined on the T cell surface. Percentage of double-positive cells is shown, and gating was set according to the isotype control. One typical experiment out of five is shown.

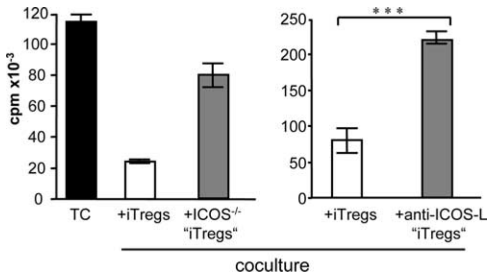

FIGURE 7. Lack of ICOS/ICOS-L interaction during priming with allogeneic iDC prevents the induction of Tregs. $CD4^+CD25^-$ TC from either an ICOS-deficient patient ($ICOS^{-/-}$"iTreg") or a healthy donor (iTreg) were primed and restimulated with allogeneic iDC at a DC/TC ratio of 1:10 (*left panel*, $n = 1$). Additionally, T cells were repetitively stimulated with iDC in the presence of anti-ICOS-L mAb (anti-ICOS-L "iTreg", *right panel*, $n = 3$). Seven days after restimulation, TC were washed, irradiated, and 1×10^5 were cocultured with 1×10^5 freshly isolated $CD4^+CD25^-$ TC from a healthy donor in the presence of 3×10^5 irradiated allogeneic PBMC and soluble anti-CD3. After 3 days of culture, proliferation was determined by additional 16 h of [^{3}H]TdR incorporation.

intermediate expression of CD86 and no expression of CD80. In contrast, iDC showed a similar or slightly higher expression of ICOSL (mean fluorescence intensity: 32) compared with mDC (mean fluorescence intensity: 27) (Fig. 6*c*). These data demonstrate that the influence of ICOS/ICOS-L interaction has a greater impact on T cell proliferation in the context of weak CD28 signaling using iDC for priming and restimulation. In contrast, using mDC for T cell stimulation, CD28 signaling through CD80/86 interaction dominates ICOS signaling.

To confirm our concept that ICOS/ICOS-L interaction influences the IL-10R expression on T cells, cord-blood-derived naive $CD4^+$ T cells were stimulated with an agonistic soluble anti-ICOS and anti-CD3 mAb. IL-10R expression on activated T cells was analyzed 5 days after initial stimulation (Fig. 6*d*). T cells stimulated with anti-CD3, anti-CD3/anti-CD28, or anti-CD3/anti-CD28/anti-ICOS served as controls. Whereas mRNA expression of IL-10R did not show any differences in the T cell populations analyzed (data not shown), flow cytometry revealed that stimulating T cells with anti-ICOS/anti-CD3 led to an up-regulation and stabilization of the IL-10R expression on the surface of the corresponding T cell population. However, combining all three stimuli, additional CD28 signaling reduced the up-regulation of the IL-10R expression on T cells, indicating that CD28 signaling dominates the signals set by stimulation through ICOS.

Taken together, these data indicate that ICOS-deficient $CD4^+$ T cells are insensitive to the tolerogenic effects of iDC, even in the presence of the suppressive cytokine IL-10. One possible explanation for this decreased IL-10 sensitivity may be a differential regulation and stabilization of the IL-10R expression on activated T cells.

Induction of suppressor function in naive $CD4^+$ T cells is abrogated in the absence of ICOS/ICOS-L interaction

Induction of T cells with a suppressive phenotype is linked to the development of anergy in these cells (3). Since we observed a loss of anergy induction in ICOS-deficient T cells, we analyzed the effect of ICOS/ICOS-L interaction on the differentiation of T cells with suppressive function as well.

The suppressive properties of ICOS-deficient T cells and T cells from healthy volunteers were compared after priming with tolerogenic iDC in cocultures with resting $CD4^+$ Th cells from a second donor at a ratio of 1:1. While anergic $CD4^+$ T cells differentiated with allogeneic iDC showed a strong suppressive activity in these cocultures, T cells from ICOS-deficient patients did not (Fig. 7, *left panel*). To investigate this phenomenon in more detail, naive $CD4^+$ Th cells were primed and differentiated with allogeneic iDC either in the absence or presence of blocking anti-ICOS-L mAb. In agreement with the results shown before, blockade of ICOS/ICOS-L interaction during the priming process of naive $CD4^+$ Th cells in the presence of tolerogenic iDC completely prevented the differentiation into suppressor T cells (Fig. 7, *right panel*). ICOS blockade resulted in alloreactive Th cells with normal proliferative capacity with no suppressive activity.

Our data indicate that in the absence of ICOS expression not only the induction of anergy but also the differentiation of resting $CD4^+$ T cells into T cells with a suppressive phenotype upon repetitive stimulation with iDC is abrogated. Thus, ICOS/ICOS-L interaction is essential for anergy induction and differentiation of $CD4^+$ T cells into suppressor T cells in the presence of tolerogenic iDC.

Discussion

We investigated the functional role of ICOS/ICOS-L interaction in the differentiation process of human $CD4^+$ T cells. First, we examined the effect of siRNA-mediated inhibition of ICOS expression by human $CD4^+$ T cells on their response to tolerogenic iDC. Additionally, we abrogated the ICOS/ICOS-L interaction using blocking anti-ICOS-L mAb. Moreover, we analyzed the differentiation of ICOS-deficient T cells derived from a subgroup of patients suffering from CVID. We found that in all cases, absence of ICOS/ICOS-L interaction completely abrogated the essential first step of Th cell differentiation into suppressive T cells: the induction of T cell anergy. In contrast to $CD4^+$ T cells from healthy controls, $CD4^+$ T cells from ICOS-deficient patients were completely resistant to anergy induction and differentiation into suppressor T cells, even after addition of IL-10 to the cultures. Furthermore, ICOS/ICOS-L blockade also prevented the differentiation of naive cord-blood-derived $CD4^+$ T cells into suppressor T cells after repetitive stimulation with tolerogenic DC. Taken together, these data indicate an essential role for ICOS in silencing $CD4^+$ T cell responses by tolerogenic iDC.

Although it has been published that $CD4^+$ T cells from ICOS-knockout mice show reduced proliferation (19), there are also contradictory studies in which normal proliferation of ICOS-deficient T cells after anti-CD3/CD28, Con A, or PMA/ionomycin stimulation was observed (22). In the present study, human ICOS-deficient T cells are hyperproliferative after stimulation with immature tolerogenic DC. This might be due to the stimulation with physiological APC like DC instead of polyclonal stimulation, which was used in other studies so far.

While it was recently shown that ICOS-L expression on mature plasmacytoid DC is involved in the induction of T cells with a suppressive phenotype in an inflammatory environment (17), our study demonstrates an essential role for immature myeloid DC in the induction of anergic T cells with regulatory capacities in a noninflammatory environment. We postulate that while mature plasmacytoid DC induce suppressor T cells during an infection in the periphery, immature myeloid DC play this crucial role in a steady-state situation.

Notably, the effect of ICOS in driving the development of suppressive T cells in this context is not simply mediated by IL-10: so far, ICOS expression on T cells had always been connected to IL-10 secretion by these cells as IL-10 itself may contribute to anergy induction and development of suppressive T cells. In this study, we report that loss of anergy induction in ICOS-deficient T cells is not due to missing IL-10 in this system. In our experiments, not only do ICOS-deficient T cells themselves produce significant amounts of IL-10 but also DC might be responsible for the presence of IL-10 in the cocultures. The increased total IL-10 concentration in the supernatants together with a still substantial IL-10 production of ICOS-deficient T cells indicates that the failure to observe anergy induction among these mutant cells cannot be due simply to an absence of IL-10 production. Additionally, a direct effect of ICOS-induced IL-10 in driving anergy induction in this system could be excluded since the substitution of IL-10 did not promote the development of anergic T cells in the ICOS-deficient system. These data seem to be in contrast with reports showing that resting human ICOS-deficient T cells produce less IL-10 after stimulation with anti-CD3/CD28 or anti-CD3/anti-ICOS (11), and murine ICOS^{high} T cells produce more IL-10 than T cells expressing intermediate or low levels of ICOS (8). However, in none of these studies was tolerogenic iDC used for stimulation. Moreover, analyzing freshly isolated T cells might reveal a different cytokine pattern than T cells differentiated in the presence of iDC. Since also other groups observe a basic IL-10 production after blocking the interaction of ICOS and its ligand, an exclusive role for ICOS in eliciting IL-10 secretion has to be questioned.

In absence of ICOS expression, we were not able to detect any effect of exogenously added IL-10, whereas in ICOS-sufficient T cells, IL-10 is capable of inducing anergy. Thus, we favor the view that IL-10 and ICOS are working together in a sense that stimulation of T cells via ICOS might enable IL-10 to mediate its effects through the up-regulation and stabilization of the IL-10R expression on activated T cells. This effect can be explained in part by the high expression of ICOS-L and low amounts of CD80/86 on iDC. This results in a dominant signaling via ICOS, which then determines the fate of the corresponding T cell population through up-regulation of the IL-10R. On the other hand, high expression of CD80/86 on mDC leads to a dominant signaling through CD28 and the development of a T cell subset expressing low levels of IL-10R and thus rendering them insensitive to the tolerogenic effects of IL-10. Our results demonstrate that ICOS-deficient CD4$^+$ Th cells are insensitive to the tolerogenic effects of immature DC. This supports the hypothesis that ICOS plays an essential role in the induction of induced Tregs (iTregs) by tolerogenic DC.

Linking our findings of impaired induction of anergic and suppressive T cells in ICOS-deficient T cells back to the phenotype of ICOS-deficient patients, the question arises, are these patients are specifically prone to develop autoimmune diseases? Clinical studies performed in these patients revealed autoimmune neutropenia, splenomegaly, and inflammatory bowel disease (11, 21). The intestine is one site where the organism constantly has contact with foreign Ags, and especially here, adaptive-induced Tregs ("Tr1" cells) are thought to play an important role in balancing protective immune responses and tolerance to foreign Ags. Therefore, inflammatory bowel disease present in ICOS-deficient patients is a very interesting fact and might be a hint for the importance of this molecule in adaptive tolerance mechanisms. However, ICOS-deficient patients mainly suffer from infections due to their limited B cell numbers; autoimmune diseases in the absence of pathogens are not thought to be one of the most prominent features in these patients. This might be in part due to the fact that naturally occurring thymic-derived Tregs in these patients are not reduced or significantly altered. Additionally, different subsets of induced Tregs exist; the impaired development of a single subset might be balanced by higher numbers of other subsets. Furthermore, the dramatic reduction of PBMC ratios and specifically CD4$^+$ T cells in peripheral blood of ICOS-deficient patients could illustrate a potential mechanism counterbalancing potential autoreactive T cells by simply reducing the total number of T cells in the circulation.

Another interesting fact is described more recently in a article of Burmeister et al. (23), showing that in mice under homeostatic conditions, absence of ICOS results in reduced ratios of effector-memory T cells and Tregs. The results are in agreement with the observation of reduced T cell numbers in the blood of ICOS-deficient patients. This important finding would be interesting to test after ICOS blockade in T cells in more detail, because the limited data that we could obtain with the blood of ICOS-deficient patients did not reveal significant differences so far.

Several reports underline the importance of altering unbalanced immune responses by influencing peripheral tolerance to cure autoimmune diseases. For example, it has been shown that epicutaneous immunization with autoantigenic peptide induces suppressive T cells, which are capable of preventing EAE in the mouse model for multiple sclerosis (24). Understanding the mechanisms of tolerance induction in more detail might highlight potential targets for attractive future therapeutic strategies in autoimmune diseases.

DC have been studied extensively as modulators of alloreactive and autoreactive T cell responses. Tolerogenic DC presenting Ag without adequate costimulatory signals necessary for T cell activation prevent inflammatory immune responses by induction T cell anergy and/or differentiation into Tregs. These tolerogenic DC have a great therapeutic potential as tools to ameliorate or prevent graft rejection or graft-vs-host disease or to treat autoimmune diseases (24). For example, i.v. infusion of donor-derived immature myeloid DC 7 days before transplantation prolonged the survival of heart or pancreatic islet grafts in untreated recipient mice (25). Even though there are various subsets of DC in mice and men, the tolerogenicity is not restricted to one specific subset: We have previously shown that immature human myeloid DC induce IL-10-producing Tregs from naive T cells in a steady-state situation (3), whereas mature plasmacytoid DC induce Tregs in an inflammatory context (14).

In this article, we analyzed whether the loss of ICOS in ICOS-deficient individuals and thus an impaired ICOS/ICOS-L interaction will influence the development of anergy. Our data indicate that blocking of the ICOS/ICOS-L pathway will lead to the loss of anergy induction by iDC. This knowledge might give rise to new potential therapeutic strategies by providing ICOS and ICOS-L as possible molecular targets. Manipulation of the ICOS/ICOS-L pathway may therefore be a promising way to influence immunotherapy in humans. One might speculate that a positive signal through ICOS—by administration of stimulating agents in combination with tolerogenic DC—might enhance transplantation tolerance. On the other hand, blockade of ICOS/ICOS-L interaction might shift the balance from cancer-protecting Tregs, which have been found to be present in high numbers in many tumor tissues (25), to a less tolerant environment with more cancer-aggressing effector T cells. This may have significant implications for therapeutic strategies to break tumor-mediated immunosuppression.

Taken together, our data indicate an important and so far unnoted function for ICOS/ICOS-L interaction in the development of adaptive tolerance by myeloid iDC and therefore render ICOS an interesting target for an immunotherapeutic intervention.

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Disclosures

The authors have no financial conflict of interest.

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