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Tryptophan Deprivation Induces Inhibitory Receptors ILT3 and ILT4 on Dendritic Cells Favoring the Induction of Human CD4⁺CD25⁺ Foxp3⁺ T Regulatory Cells

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Tryptophan catabolism through IDO activity can cause nonresponsiveness and tolerance acting on T cells. Given the crucial importance of dendritic cells (DCs) in the initiation of a T cell response, surprisingly little is known about the impact of IDO activity and tryptophan deprivation on DCs themselves. In the present study, we show that human DCs differentiated under low-tryptophan conditions acquire strong tolerogenic capacity. This effect is associated with a markedly decreased Ag uptake as well as the down-regulation of costimulatory molecules (CD40, CD80). In contrast, the inhibitory receptors ILT3 and ILT4 are significantly increased. Functionally, tryptophan-deprived DCs show a reduced capacity to stimulate T cells, which can be restored by blockade of ILT3. Moreover, ILT3ⁿ⁻⁴⁻ILT4⁻⁻ DCs lead to the induction of CD4⁺CD25⁺ Foxp³⁺ T regulatory cells with suppressive activity from CD4⁺CD25⁻ T cells. The generation of ILT3ⁿ⁻⁴⁻ILT4⁻⁻ DCs with tolerogenic properties by tryptophan deprivation is linked to a stress response pathway mediated by the GCN2 kinase. These results demonstrate that tryptophan deprivation establishes a regulatory microenvironment for DCs, enabling these cells to induce T regulatory cells. The impact of IDO thus extends beyond local immune suppression to a systemic control of the immune response. The Journal of Immunology, 2009, 183: 145–154.

Both stimulatory and inhibitory mechanisms are required to balance the activity of the adaptive immune system. Accumulating evidence suggests that the capacities to stimulate and to inhibit are vested in one cell type, the dendritic cell (DC). The crucial role of DCs encompasses the presentation of peptides via MHC-peptide complexes and the delivery of costimulatory signals to naïve T cells. Costimulation is complex and involves the integration of activating and inhibitory signals. The sum of these signals determines the outcome of the T cell response, ranging from a productive response to T cell unresponsiveness and immunological tolerance (1–4).

Tolerogenic DCs have been described and generally show reduced expression of costimulatory molecules such as CD80 and CD86 and induce unresponsiveness in CD4⁺ Th cells (5, 6). Under certain circumstances, specific Ig-like transcripts (ILTs), membrane-bound or soluble Ig superfamily receptors, can be induced on the cell surface of DCs (7–11). ILTs have either activating or inhibiting activities; ILT2 through ILT5 and leukocyte Ig-like receptor 8 belong to the group of membrane-anchored inhibitory receptors with cytoplasmic ITIMs (12). The increased expression of ILT3 and ILT4 on APCs has been repeatedly reported to be associated with tolerogenic properties of these cells (10–14). In addition, an increased expression of ILT3 on DCs is required for the induction of CD4⁺Foxp³⁺ T regulatory cells (Tregs) (8, 10, 13).

IDO catalyzes the degradation of the essential amino acid tryptophan (Trp), and thereby depletes the cellular microenvironment of Trp. IDO expression is induced mainly by IFNs and LPS in cells from various organs and within tissue-invasive cells during inflammation (4, 15, 16). IDO activity of APCs, epithelial cells, fibroblasts, or tumor cells has been demonstrated to block T cell responses toward allogeneic fetuses, grafts, and tumors, and may play a role in the prevention of allergic disease (17–24).

Downstream metabolites of Trp, which are produced by IDO activity in murine DCs, are able to convert otherwise immunogenic and IDO-incompetent murine DCs into tolerizing DCs (25). This alteration requires the nearby production of Trp metabolites, which are taken up and metabolized by IDO-incompetent DCs to the end product quinolinolate in an IFN-γ-rich environment. However, Trp deprivation itself, and not downstream catabolites of Trp degradation, could have a direct effect on DCs. The critical reduction of the availability of this essential amino acid in the microenvironment of DC could alter their differentiation and biology by the induction of an integrated stress response. Trp deficiency specifically activates the general control nondepressing 2 (GCN2) kinase in murine T cells, which initiates downstream signaling for compensatory adaptation (26). Whereas the tolerogenic outcome of a low-Trp level

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may be bad for the host in infections, it is believed that in autoimmunity, Trp deprivation may help to balance peripheral tolerance (4). In vivo, lower Trp levels were measured systemically in circumstances connected to chronic inflammation such as in viral diseases including HIV infection, but also in chronic autoimmune disorders such as lupus erythematosus (27-30). Our group recently established that in lichen planus, an autoimmune skin disease of unknown etiology, myceloid DCs do express high IDO activity. It is conceivable that monocytes, differentiating into DCs and migrating into the skin, sense low-Trp conditions and turn into phenotypical and functional tolerogenic DCs favoring the induction of Tregs that eventually try to dampen the autoimmune reaction (31). In NOD mice, it was found that an impaired Trp catabolism is causative to the defective tolerance (32). In systemic lupus erythematosus and in viral infections, there is a lot of local and systemic IDO activity (29, 33). In these chronic infections, the induction of tolerogenic DCs by low Trp may add to the observed immunosuppression seen in these patients. Direct evidence for a low-Trp environment in vivo comes from a study by Fujigaki et al. (34). This group found dramatic reductions in the concentration of Trp in homogenized mouse lung tissue (~2 μM Trp) during an acute pulmonary infection with *Toxoplasma gondii*.

Clarification of how low-Trp levels may contribute to DC tolerance and may affect differentiating DCs that migrate to the respective inflamed tissues with high IDO activity is relevant to a better understanding of the immunopathology of chronic diseases, and might provide the basis for new immunotherapeutic strategies targeting DCs. In the present study, we provide evidence that human monocyte-derived DCs generated under low-Trp conditions (5 μM Trp) are impaired in their stimulatory capacity toward CD4⁺ T cells. Low-conditioned DCs showed high expression of the inhibitory receptors ILT3 and ILT4 and favor, in an ILT3-dependent manner, the induction of CD4⁺CD25⁺Foxp3⁺ T cells with suppressive function.

**Materials and Methods**

**Reagents**
The following mAbs were used for flow cytometric analysis: RD1-labeled T6R61 against CD1a (IgG1; Beckman Coulter); PE-labeled mAb against CD14 (IgG2a; BD Biosciences); PC5-labeled mAb to ILT3 (IgG1; Beckman Coulter); FITC-labeled mAb to CD4 and PE-labeled mAb against CD25 (IgG1; all from BD Biosciences); Alexa Fluor 647-labeled mAb against Foxp3 (IgG1; BD Biosciences); and unlabeled mAbs to CD40, CD10, CD11a, CD11b, CD11c, CD85 (Foxp3), CD45 (all IgG1), and mAbs to ILT2 and CD32 (FcγRII; all IgG2b; BD Biosciences). Anti-IDO mAb was used, as described previously (35). Purified mAbs HBA1a detecting CD83a was from Sigma-Aldrich. mAbs to ILT3 and ILT4 (IgG4a) were from R&D Systems. FITC-labeled F(ab)'2 of goat anti-mouse Ab and mouse serum were from Dianova. Mouse mAb Lib43 against anti-MHC class II, and anti-MHC class I mouse mAb W6/32 were provided by N. Koch and J. Neumann (Division of Immunobiology, University Institute of Genetics, Bonn, Germany). The 7-aminoactinomycin D (7-AAD) and 2-ME were from J. Neumann (Division of Immunobiology, University Institute of Genetics, Bonn, Germany). The 7-aminoactinomycin D (7-AAD) and 2-ME were from J. Neumann (Division of Immunobiology, University Institute of Genetics, Bonn, Germany). The 7-aminoactinomycin D (7-AAD) and 2-ME were from J. Neumann (Division of Immunobiology, University Institute of Genetics, Bonn, Germany). The 7-aminoactinomycin D (7-AAD) and 2-ME were from J. Neumann (Division of Immunobiology, University Institute of Genetics, Bonn, Germany). The 7-aminoactinomycin D (7-AAD) and 2-ME were from J. Neumann (Division of Immunobiology, University Institute of Genetics, Bonn, Germany). The 7-aminoactinomycin D (7-AAD) and 2-ME were from J. Neumann (Division of Immunobiology, University Institute of Genetics, Bonn, Germany).

**Isolation of monocytes and T cells**
Human monocytes and T cells were obtained from healthy donors. Written informed consent was obtained from all patients, and the protocol was approved by the local ethic committee. Monocytes were isolated from peripheral blood with a density gradient centrifugation with Lymphoprep (Axis-Shield) for 30 min at 1000 × g. Autologous T cells were isolated from PBMCs using a nylon-wool column. For T cell proliferation assays with subsequent functional assessment of induced CD4⁺CD25⁺ T cells, PBMCs were isolated from buffy coat blood by density gradient centrifugation with Lymphoprep. CD4⁺CD25⁺ T cells and CD14⁺ monocytes were separated by negative selection via magnetic beads using CD4-CD25- and CD14-isolation kits (Miltenyi Biotec), respectively, and an autoMACS Separator (Miltenyi Biotec).

**Generation of DCs**
Monocytes (1 × 10⁶/ml) were cultured in the presence of 500 U/ml GM-CSF and 500 U/ml IL-4 in RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, and 100 μg/ml antibiotics/antimycotics in 24-well plastic plates. To generate DCs under Trp-deprived (DCs-lowTrp) and normal conditions (DCs-highTrp), low-Trp medium, containing 5 μM Trp, and standard medium (30 μM Trp), were used, respectively. On day 2 of culture, 250 U/ml IL-4 and 250 U/ml GM-CSF were added to the medium; on day 4, half of the medium was replaced with fresh medium containing 500 U/ml GM-CSF and 500 U/ml IL-4. Immature DCs (iDCs) were used on day 6, and maturation was achieved by the addition of polyriboinosinic-polyribocytidilic acid (poly(I:C), 25 μg/ml; Sigma-Aldrich) for 24 h.

**Detection of apoptosis**
Apoptotic and nonviable CD1a⁺ T cells were determined using FITC-labeled annexin V (BD Biosciences) and 7-AAD. DCs were harvested after culture and incubated at 4°C with mAb against CD1a or an Ab with isotype-matched control for 20 min at a final concentration of 2.5 μg/ml and then further subjected to the manufacturer’s protocol. The percentage of annexin V/7-AAD-positive and -negative DCs was analyzed by flow cytometry.

**Assessment of pinocytic activity**
The pinocytic activity of iDCs was assessed using FITC-labeled BSA (FITC-BSA; Sigma-Aldrich), and was performed according to the manufacturer’s protocol. Briefly, 1 × 10⁶ iDCs were incubated with FITC-BSA at a final concentration of 50 μg/ml for 30 min at 37°C, or at 4°C as negative control. Then, cells were washed twice with ice-cold stopping buffer and analyzed by flow cytometry.

**Immunostaining and flow cytomteric analysis**
Cell analysis was performed, as previously described (36). Immunostaining and intracellular Foxp3 staining with 0.1% saponin were performed, as reported previously (36). At least 1 × 10⁶ cells were analyzed with a FACS Canto (BD Biosciences). In brief, fluorescence intensities of various Abs were determined as the relative fluorescence index (rFI); the median fluorescence intensity (MFI) for each Ag of the vital CD14⁺, CD1a⁺, or CD4⁺ cell population was determined. The rFI was assessed as follows: rFI = (MFI(AG) - MFI(isotype control))/MFI(isotype control).

**RT-PCR and real-time PCR**
Total RNA was extracted from DCs using TRIzol (Invitrogen), according to the manufacturer’s instructions. Reverse transcription was done with 1 μg of total RNA, followed by PCR. The specific primer sequences for ILT3 were forward, 5'-ACGTATGCGAACGTTGAACACG and reverse, 5'-CTTGGAGATTAGGAGGTCTGC (493 bp) (37); for C/EBP-homologous protein (CHOP), forward, 5'-GAAACGCGAACAGATGTTGCA TTCCCC and reverse, 3'-GTTGGATGAGGTGTCATCATTGGCA (309 bp) (38); and for β-actin, forward, 5'-GACGGGGAATCGTCTGCTGACATT and reverse, 3'-GATGAGGTGAAGTGATGTCGTC (240 bp). The PCR cycle numbers for the detection of ILT3 and CHOP were 36, and 26 for β-actin.

Real-time PCR for Foxp3 was performed in a 20-μl reaction volume containing 2 μl of 10x reaction buffer; 4 mM Mg²⁺, 200 nM of each primer; for β-actin: 3 mM Mg²⁺, 300 nM of each primer, 0.2 μl of SYBR Green (diluted 1/1000 in DMSO), 0.5 μM of Hotstar Taq (Qiagen), and 2 μl of cDNA, using the Rotorgene 6000 (Corbett Research), as already described (39). Reaction conditions were 15 min at 95°C, followed by 45 cycles of 10 s at 94°C, 20 s at 58°C, and 20 s at 72°C. Copy numbers were determined using a plasmid standard and normalized to expression of β-actin. Primers were as follows: Foxp3 forward, 5'-GAGGCTATGGAAA CAGCACAT and Foxp3 reverse, 5'-CCTTGGTACCAGTGTAATG; β-actin forward, 5'-GATGAGGTGAAGTGATGTCGTC; and β-actin reverse, 5'-CAGCAGGTTGCTGACCTC. All assays were performed according to the manufacturer’s instructions. All analyses were conducted in triplicates.
T cell proliferation assays

T cell proliferation assays were performed in 96-well round-bottom plates for 7 days in normal T cell medium (RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 100 μg/ml antibiotics/antimycotics, and 4 mM 2-ME) at 37°C. Autologous iDCs were treated with 50 μlimes flocculation/ml tetanus toxoid (TT; Chiron Behring). Four hours later, poly(I:C) (25 μg/ml) was added as a maturation stimulus for 24 h. Autologous T cells (1 × 10⁵ cells/well) were cultured in 200 μl of T cell medium with the respectively prepared mature DCs (mDCs; ratio DCs:T cells, 1:100). To determine the role of ILT3, anti-ILT3 mAb (10 μg/ml) was added to 1 × 10⁵ DCs/well 30 min before T cells (1 × 10⁵ cells/well) were added. Twenty-four hours before harvesting, cells were labeled with 0.5 μCi/well

FIGURE 1. Morphology, viability, and CD1a expression of DCs under Trp-deprived conditions. A. Morphology of DCs on day 6 generated under Trp deprivation (DCs<sub>low-Trp</sub>) and in normal culture medium (DCs<sub>Trp</sub>). Original magnification, ×100 and ×400. Results are representative for all donors tested (n ≥ 22). B. Viability of DCs<sub>low-Trp</sub> and DCs<sub>Trp</sub> on day 6 of differentiation. Upper panel. Staining with 7-AAD and FACS analysis reveals that the viability was not affected by DCs<sub>low-Trp</sub>. Representative data are shown (n ≥ 22). Lower panel, Annexin V/7-AAD staining shows that Trp depletion does not induce a higher rate of apoptosis in DCs<sub>low-Trp</sub> compared with DCs<sub>Trp</sub>. Representative data are shown (n = 3). C. Percentage of values of vital CD1a<sup>+</sup> DCs (left panel) and the rFl of CD1a expression (right panel) along the differentiation pathway from monocytes to DCs<sub>low-Trp</sub> (○) and DCs<sub>Trp</sub> (■). The induction kinetics of the surface marker CD1a was similar in both culture conditions. Results are expressed as mean ± SEM (n = 6).

FIGURE 2. Up-regulation of CHOP in DCs differentiated under Trp-deprived conditions (DCs<sub>low-Trp</sub>). Transcript expression of CHOP, a well-established marker for GCN2 activation, was analyzed by RT-PCR on day 6 in DCs. RT-PCR analysis of CHOP transcript expression reveals a marked up-regulation in DCs<sub>low-Trp</sub> compared with DCs<sub>Trp</sub>. One representative experiment is shown of three performed.

FIGURE 3. Low pinocytic activity of iDCs<sub>low-Trp</sub>. The uptake of FITC-BSA (50 μg/ml) by iDCs<sub>low-Trp</sub> was markedly decreased compared with iDCs<sub>Trp</sub>. Control cultures were established at 4°C to measure background staining. Representative data are shown (n = 5).
[1H]thymidine. All experiments were performed in quintuplicate or triplicate, cell yield permitting.

**Suppression assays**

Autologous T cell proliferation assays were set up with autologous TT-presenting DCs low-Trp and DCs +Trp, respectively. After 7 days, induced CD4+CD25+ T cells (Tregslow-Trp and Tregs +Trp) were isolated using the CD4+CD25+ Treg isolation kit (Miltenyi Biotec). To investigate the ability of Tregs to suppress polyclonally activated T cells, CFSE-labeled (0.5 μCi/well; Invitrogen) CD4+ T effector cells (1 × 10⁵ cells/well) were added. Cocultures were performed in a 96-well flat-bottom plate. Proliferation was measured over time (40, 68, and 92 h) and analyzed with a FACS Canto (BD Biosciences). To investigate the ability of Tregs to suppress TT-activated T cells, Tregs (1 × 10⁵ cells/well) were added to cocultures of CD4+ T effector cells (1 × 10⁵ cells/well) and TT-presenting DCs (1 × 10⁵ cells/well) for 7 days. During the last 24 h, cells were labeled with 0.5 μCi/well [3H]thymidine. Results are expressed as the mean of triplicate cultures.

**Statistical analysis**

Analyses were performed with SPSS 12.0 statistical software. Statistical analysis was done by using parametric Student’s t test and nonparametric Wilcoxon test. Values of p lower than 0.05 were considered as statistically significant. Results are expressed as mean ± SEM.
Results

Trp deprivation does not affect the generation of human myeloid DCs

We first studied the cellular response of human monocyte-derived DCs toward Trp deprivation. Upon addition of IL-4 and GM-CSF, monocytes isolated from peripheral blood rapidly became nonadherent in the presence of both normal (30 μM) and low concentrations (5 μM) of Trp (DCs+Trp and DCs-low-Trp, respectively), and cells of both cultures displayed the typical dendritic morphology (Fig. 1A). On day 6, cluster sizes and single-cell sizes of DCs-low-Trp were slightly smaller than in the case of DCs+Trp. The viability and the rate of apoptosis were not different under both culture conditions (Fig. 1B). The yield of CD1a+ cells on day 6 was similar in DCs+Trp and DCs-low-Trp (35 ± 5% vs 25 ± 7%; n = 22). The induction kinetics of the CD1a expression was similar in both culture conditions, in terms of both population size and expression levels (Fig. 1C). These data suggest that the generation of human myeloid DCs is not affected by low levels of Trp.

Trp deprivation of differentiating myeloid DCs leads to the up-regulation of CHOP indicative of the GCN2 stress response

Because low-Trp conditions unexpectedly allowed the generation of myeloid DCs, we investigated whether this phenomenon may be due to an active stress response mediated by activation of the GCN2 kinase that could serve metabolically to adapt the DCs to Trp deprivation (26). To examine whether the GCN2 pathway is activated in DCs following Trp depletion, we measured the level of C/EBP-homologous protein (CHOP), a well-established marker for GCN2 activation (26, 40). RT-PCR results showed a marked up-regulation of CHOP mRNA in DCs deprived of Trp compared with DCs+Trp at day 6 (Fig. 2).

DCs differentiated under Trp-deprived conditions show altered pinocytic activity

The capacity to take up Ag is a hallmark of functionally competent iDCs. To establish whether iDCs-low-Trp are altered in this ability, the pinocytic uptake of FITC-labeled BSA was measured by flow cytometry (Fig. 3). Remarkably, iDCs-low-Trp displayed a markedly decreased pinocytic activity compared with iDCs+Trp. These data suggest that the uptake of Ags is impaired in iDCs-low-Trp.

DCs differentiated under low-Trp conditions display a CD40lowCD80low/ILT3highILT4high phenotype

We next analyzed the phenotype of myeloid DCs focusing on molecules important for their function (Fig. 4. A and B). The costimulatory molecules CD40 and CD80 as well as IgG FcγRII (CD32) showed a significantly lower expression on iDCs and mDCs-low-Trp compared with DCs+Trp. MHC class II and CLIP were significantly less expressed on the cell surface of iDCs-low-Trp than on DCs+Trp. MHC class I expression was lower on mDCs-low-Trp compared with DCs+Trp. In contrast, the inhibitory receptors ILT3 and ILT4 were significantly increased on iDCs-low-Trp compared with DCs+Trp. Kinetic experiments showed transient ILT3 expression under both culture conditions, but, remarkably, DCs-low-Trp showed a strong peak of ILT3 surface expression between days 2 and 3 (Fig. 4C), which was confirmed by RT-PCR analysis (Fig. 4D). Thereafter, ILT3 was continuously down-regulated, but expression remained significantly higher on DCs-low-Trp compared with DCs+Trp on days 6 and 7. Addition of Trp (30 μM Trp; corresponding to 1× concentration of standard culture medium) to DC cultures on day 3 failed to reverse the higher expression of ILT3 on DCs-low-Trp over DCs+Trp along their differentiation pathway (Fig. 4B). Taken together, DCs generated under low-Trp conditions show a CD40lowCD80low/ILT3highILT4high phenotype suggestive of tolerogenic functions.

DCs-low-Trp show significantly lower stimulatory capacity toward T cells than DCs+Trp

A lower expression of molecules that are positively involved in DC function as well as a higher expression of inhibitory receptors suggest that DCs-low-Trp may be altered in their T cell stimulatory activity. To test this, we compared the proliferation of TT-specific T cells activated by autologous DCs-low-Trp and DCs+Trp. T cell proliferation assays were performed in normal culture medium (30 μM Trp) for 7 days. CD4+ T cells (1 × 10⁵) were stimulated with TT in the presence of DCs-low-Trp (■) and DCs+Trp (▲), respectively (ratio DCs:T cells, 1:100). Proliferation was measured as [3H]thymidine incorporation on day 6. The addition of anti-ILT3 mAb (10 μg/ml) to these cocultures partially restored the inhibitory effect of DCs-low-Trp (■) on TT-sensitized T cells, whereas anti-ILT3 had no effect on T cells stimulated by DCs+Trp (▲). Results are expressed as mean ± SEM (n = 6). The relative stimulatory index (rSI) was assessed as follows: rSI = (mean cpm of triplicate wells (coculture)) – (mean cpm of triplicate wells (T cell control))/cpm of triplicate wells (T cell control).

ILT3 on DCs-low-Trp over DCs+Trp along their differentiation pathway (Fig. S1). Upon maturation, inhibitory receptors ILT2, ILT3, and ILT4 were significantly increased on mDCs-low-Trp over mDCs+Trp. The staining intensity of the death receptor CD95 (Fas) was significantly increased on mDCs-low-Trp over mDCs+Trp. The integrin CD11a was significantly increased on iDCs-low-Trp, and mDCs-low-Trp compared with DCs+Trp, whereas CD11b and CD11c were significantly decreased on iDCs-low-Trp compared with DCs+Trp, although all cells were positive for both markers. After maturation with poly(I:C), LPS, IFN-γ, or CD40L, the expression of CD83 was induced to the same extent on both mDCs-low-Trp and mDCs+Trp, implying that low-Trp levels do not inhibit the full maturation of DCs (Fig. 4B). Taken together, DCs generated under low-Trp conditions show a CD40lowCD80low/ILT3highILT4high phenotype suggestive of tolerogenic functions.

The online version of this article contains supplemental material.
strongly suggest that ILT3 is an important molecule in the tolerogenic capacity of DCs low-Trp.

Enhanced induction of CD4^+CD25^+Foxp3^+ T cells by DCs_{low-Trp}

Evidence suggests that Foxp3^+ Tregs are most efficiently induced if Ag is presented in subimmunogenic conditions, i.e., with low doses of Ag and with no or low costimulation (41). Therefore, we speculated that low-Trp-conditioned DCs with low pinocytic Ag uptake and low FcγRII, CD40, and CD80 favorably induce Tregs from CD4^+CD25^+Foxp3^+ T cells. To study this, we depleted enriched peripheral CD4^+ T cells of pre-existing Tregs by positively selecting the CD4^+CD25^+Foxp3^+ T cell population. The remaining

FIGURE 6. DCs_{low-Trp} favor the induction of CD4^+CD25^+Foxp3^+ T cells from CD4^+CD25^+Foxp3^+ T cells. A, CD4^+CD25^+ T cells are negative for Foxp3 after purification of peripheral CD4^+ T cells from CD4^+CD25^+ T cells (over 99% purity). B, Induction of Foxp3 in CD4^+CD25^+Foxp3^+ T cells after coculture with DCs_{low-Trp} and DCs_{Trp}, respectively. The density plot shows a clear shift toward a population of CD4^+ T cells positive for Foxp3 after culture of CD4^+CD25^+ T cells with DCs_{low-Trp}. Also, the histograms of Foxp3 in the gated CD4^+CD25^+ population show clearly Foxp3^+ T cells (~40%). Isotype controls are shown as gray area underneath the thin line; expression of Foxp3 is reflected by thick black lines. Two experiments are shown. Real-time PCR shows that levels of Foxp3 mRNA were increased in Tregs_{DCs_{low-Trp}} compared with Tregs_{DCs_{Trp}}. Data represent copies of Foxp3 mRNA/β-actin copies x 10^6 from three independent experiments (●); mean is expressed as bars. C, ILT3 on DCs_{low-Trp} is required for their enhanced capacity to induce CD4^+CD25^+Foxp3^+ T cells. Three-color analysis of CD4^+ T cells shows that anti-ILT3 mAb (10 μg/ml) prevents the induction of Foxp3 within the CD4^+ T cell population by DCs_{low-Trp}. The dot plots show Foxp3 in the gated CD4^+ population. This effect was not seen with cocultures of DCs_{Trp}. Representative data are shown (n = 2).
A T cell population was negative for Foxp3 (99% purity) and was used in these studies (Fig. 6A). The CD4^+CD25^+Foxp3^+T cells were stimulated for 7 days with TT, presented by autologous DCs low-Trp and DCs^+Trp, respectively. As shown in Fig. 6B, DCs^low-Trp induced clearly more CD4^+CD25^+Foxp3^+T cells (40%) than DCs^+Trp (6–16%) from CD4^+CD25^+Foxp3^+T cells. The higher up-regulation of Foxp3 in cocultures with DCs low-Trp was also confirmed by real-time PCR analysis (Fig. 6B).

To evaluate the role of ILT3 in the induction of Foxp3^+ Tregs by DCs^low-Trp, we blocked ILT3 in cocultures of purified CD4^+ effector cells with DCs^low-Trp or DCs^+Trp using anti-ILT3 mAb. As shown in Fig. 6C, the addition of an anti-ILT3 mAb prevented the induction of CD4^+CD25^+Foxp3^+T cells (50% Foxp3^+T cells without anti-ILT3 vs 11% with anti-ILT3), whereas there was only a marginal effect when DCs^+Trp were used (~9% Foxp3^+T cells without anti-ILT3 vs 6% with anti-ILT3). These data implicate the ILT3 expression induced on DCs by low-Trp conditions in the induction of CD4^+CD25^+Foxp3^+T cells.

The IDO pathway itself was recently described to be involved in the induction of human Tregs (42, 43). Therefore, we measured the expression of IDO in DCs^low-Trp vs DCs^+Trp after maturation with poly(I:C) (Fig. S2). IDO expression was nearly identical in DCs differentiated in both conditions, and thus, differences in IDO expression (i.e., increased IDO expression in DCs^low-Trp) are unlikely to contribute substantially to the increased generation of Tregs.

**FIGURE 7.** Suppressive function of CD4^+CD25^+Foxp3^+T cells induced from cocultures with DCs^low-Trp and DCs^+Trp, respectively, on polyclonally (A) and Ag-specific activated CD4^+T cells (B). A. Purified CD4^+T cells were CFSE labeled and stimulated with surface-bound anti-CD3 mAb. Induced CD4^+CD25^+T cells from cocultures with DCs^low-Trp (Tregs^DCslow-Trp^) or with DCs^+Trp (Tregs^DCs^+Trp^) were added in equal numbers (ratio Tregs:T cells, 1:1). After 40, 68, and 92 h, CFSE-labeled CD4^+T cells were analyzed by flow cytometry. Unstimulated CD4^+T cells served as control. Two experiments are shown. B. Purified CD4^+T cells (1 × 10^5) were stimulated with TT in the presence of autologous DCs (1 × 10^3). Induced CD4^+CD25^+Tregs from cocultures with DCs^low-Trp (Tregs^DCslow-Trp^) or DCs^+Trp (Tregs^DCs^+Trp^) were added (ratio Tregs:T cells, 1:1). Equal numbers of CD4^+T cells alone were used as control (■). Suppression assays were performed for 7 days, and proliferation was measured by incorporation of [3H]thymidine. Results are expressed as mean ± SEM (n = 5).
after coculture DCs\textsuperscript{low-Trp} with T cells. These results were confirmed by adding 10× Trp to the T cell cocultures, which failed to inhibit the induction of Tregs (Fig. S3).\textsuperscript{5}

\textbf{Induced CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} T cells by low-Trp-conditioned DCs display high suppressive activity}

DCs generated in low-Trp conditions stimulated the appearance of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} T cells. The last set of experiments was designed to test whether these phenotypical Tregs were indeed regulatory cells, i.e., capable of suppressing T cell reactions. To study this, we tested the capacity of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} T cells to suppress the proliferation of polyclonally activated CD4\textsuperscript{+} T cells (Fig. 7A) and Ag-activated CD4\textsuperscript{+} T cells (Fig. 7B). Isolated CD4\textsuperscript{+}CD25\textsuperscript{+} T cells induced after culture of CD4\textsuperscript{+} T cells and DCs\textsuperscript{low-Trp} (Tregs\textsuperscript{DCslow-Trp}) displayed a high suppressive activity on polyclonally activated CD4\textsuperscript{+} T cells in that they profoundly inhibited cell division of CD4\textsuperscript{+} T cells during a 92-h incubation period (Fig. 7A). CD4\textsuperscript{+}CD25\textsuperscript{+} T cells after coculture with CD4\textsuperscript{+} T cells and DCs\textsuperscript{+Trp} (Tregs\textsuperscript{DCs+Trp}) showed much less suppression during 92 h, commensurate with their smaller share of Foxp3\textsuperscript{+} T cells. Similarly, Ag-stimulated CD4\textsuperscript{+} T cells were suppressed in their proliferation to ~50\% by addition of Tregs\textsuperscript{DCslow-Trp} after 7 days (Fig. 7B). If Tregs\textsuperscript{DCs+Trp} were added to a TT-specific coculture, only ~55\% inhibition of CD4\textsuperscript{+} T cell proliferation was seen after 7 days. In conclusion, low-Trp-conditioned DCs induce Tregs with early and profound suppressive activity on polyclonally activated and Ag-specific CD4\textsuperscript{+} T cell proliferation.

\textbf{Discussion}

This study identifies a novel mechanism by which low-Trp levels may add to immunosuppression. We show that an environment deprived of Trp generates human monocyte-derived DCs with a marked up-regulation of the inhibitory receptors ILT3 and ILT4 and an enhanced capacity to induce CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} Tregs in an ILT3-dependent manner. These results demonstrate that low-Trp levels establish a regulatory microenvironment not only for T cells, but also for differentiating DCs.

DCs that differentiate under low-Trp conditions do not undergo cell death at a significant rate and are capable of differentiation and maturation. This was evidenced by their dendritic morphology; expression of CD1a, CD11b, and CD11c; up-regulation of CD83; and up-regulation of CD40, CD80, MHC class I, and class II molecules during TLR-induced maturation.

The phenotypic and functional alterations of DCs generated in low external Trp are most likely linked to intracellular Trp deprivation, followed by GCN2 activation. Amino acid insufficiency can cause a rise in uncharged tRNA in the cytosol, which activates the GCN2 stress response kinase domain and initiates downstream signaling (40). Several observations suggest that GCN2 activation could be a general feature of how IDO achieves its tolerogenic effects (26, 44–47). The combination of Trp starvation and Trp catabolites has been shown to result in a GCN2 kinase-dependent down-regulation of the TCR ξ-chain and the secondary induction of a regulatory phenotype in naive CD4\textsuperscript{+} T cells (47). GCN2 kinase activity has recently been shown to be induced in murine CD19\textsuperscript{+} DCs by a reduced access to Trp caused by high IDO activity (15). Because this integrated stress response selectively up-regulates a program of downstream response genes and is possibly responsible for the metabolic adaptation of DCs in our culture system, we tested whether the GCN2 kinase was also induced in human DCs deprived of Trp. CHOP/gadd153 expression is indicative of GCN2 activation because GCN2-knockout T cells showed no CHOP expression under low-Trp conditions (26). In our study, CHOP was low expressed in normally generated DCs at day 6. In contrast, in DCs\textsuperscript{low-Trp} there clearly was a strong induction of CHOP at day 6 of differentiation.

DCsdifferentiating in low Trp appeared to become committed to a regulatory phenotype early on, because external addition of Trp on day 3 did not down-regulate ILT3 (Fig. S1).\textsuperscript{5} In several studies, it has been established that high expression of ILT3 and ILT4 is a general feature of tolerogenic or regulatory DCs (8, 14, 48, 49). Studies of human heart transplant recipients showed that CD8\textsuperscript{+}CD28 alloantigen-specific T suppressor cells induce the up-regulation of ILT3 and ILT4 on monocytes and DCs with low expression of costimulatory molecules and induction of Ag-specific unresponsiveness in CD4\textsuperscript{+} Th cells (13, 14). The myelomonocytic cell line KG1, transduced with ILT3 and ILT4, has been shown to induce Tregs with suppressive activity (37). In low-Trp-conditioned DCs (DCs\textsuperscript{low-Trp}), the decreased expression of the costimulatory molecules CD40 and CD80 is most likely due to a block in their transcription (50). Inhibitory receptors such as ILT2, ILT3, and ILT4 can inhibit NF-κB signaling, which in turn is required for the expression of CD40 and CD80 (14, 51). However, the lower surface expression of MHC class II molecules on DCs\textsuperscript{low-Trp} is not a consequence of a lower intracellular pool of these proteins following Trp deficiency. Western blot analysis and pulse-chase experiments demonstrated that protein concentrations and the biosynthesis of MHC class II and invariant chain molecules were not impaired in iDCs\textsuperscript{low-Trp} compared with DCs\textsuperscript{+Trp} (Fig. S4).\textsuperscript{5} Rather, MHC class II and invariant chain molecules were retained intracellularly on their way from the endoplasmic reticulum to the endocytic compartments and did not reach the cell surface. The increased expression of ILT3 and ILT4; the reduced surface expression of MHC class I, MHC class II, FcγRII, CD40, and CD80 molecules on DCs\textsuperscript{low-Trp}; as well as the low pinocytic activity can be expected to reduce the cell’s ability to present Ag and to stimulate T cells. In our experiments, blocking of ILT3 by a neutralizing mAb partially, but not completely, restored T cell proliferation in cocultures with DCs\textsuperscript{low-Trp} compared with DCs\textsuperscript{+Trp}. These data can be explained by the fact that the increased generation of Tregs\textsuperscript{DCslow-Trp} was prevented by anti-ILT3 mAb, but the observed reduced Ag uptake and reduced expression of costimulatory molecules on DCs\textsuperscript{low-Trp} would still not permit full CD4\textsuperscript{+} T cell stimulation. All factors may act jointly to impair the observed stimulatory capacity of DCs\textsuperscript{low-Trp} toward TT-sensitized, autologous T cells (range: from 20 to 70\% lower CD4\textsuperscript{+} T cell proliferation compared with DCs\textsuperscript{+Trp}).

The exact cellular and molecular mechanisms involved in the generation of peripheral Tregs are not fully understood (52). It has been suggested that a portion of Tregs is derived from rapidly dividing peripheral memory CD4\textsuperscript{+}CD45RO\textsuperscript{+} T cells or from activated CD4\textsuperscript{+} T cells by Ag stimulation (52, 53). Recently, Hill et al. (54) found that IDO enzymatic activity in LPS-stimulated DCs contributes to the expansion of Tregs. Furthermore, it is known that the IDO pathway itself is able to induce the differentiation of naive CD4\textsuperscript{+} T cells into Tregs with suppressive function (42, 43). In these studies, differentiation into Tregs was dependent on the expression of IDO in activated plasmacytoid DCs. In our system, we could show that it is the increased expression of the inhibitory receptors such as ILT3 on DCs\textsuperscript{low-Trp} that endows these cells with the ability to preferably induce Tregs. mDCs\textsuperscript{low-Trp} express IDO to the same extent as DCs\textsuperscript{+Trp} (Fig. S2),\textsuperscript{5} and thus, differences in Treg induction are unlikely to be the result of the IDO pathway. Moreover, the addition of 10× Trp to the T cell cocultures could not reverse the induction of Tregs in cocultures with DCs\textsuperscript{low-Trp} (Fig. S3).\textsuperscript{5} The cytokine profile of DCs\textsuperscript{low-Trp} and DCs\textsuperscript{+Trp} after stimulation with poly(I:C) or LPS did not differ significantly, and
thus, cytokines are unlikely to have influenced the function of the DCs in our system (Fig. S5, A and B). Only the addition of anti-ILT3 mAb prevented the induction of Tregs with DCsslow-Trp. A recent study shows further that subimmunogenic doses of Ag and low costimulatory activity favor the conversion of CD4+ CD25+ T cells into Foxp3-expressing CD4+CD25+ T cells, whereas Foxp3+ cell populations can be favorably expanded by immunogenic presentation of Ag (41). This suggests that in our system, in which DCsslow-Trp, which are poor at pinocytosis and express low levels of costimulatory molecules, stimulate T cells and also cause the conversion of CD4+ CD25+ into Foxp3+ Tregs. Induction of Foxp3 with concomitant Treg function can rapidly disappear or be only a sign of activated T effector cells (52). However, our data show that CD4+ CD25+ Foxp3+ T cells derived from cocultures of CD4+ CD25+ T cells with low-Trp-conditioned DCs can profoundly inhibit proliferation of polyclonally activated CD4+ T cells. Ag-specific CD4+ T cell proliferation was inhibited by TregsDCslow-Trp to ~80% after 7 days. This effect was much smaller when CD4+ CD25+ T cells were used from coculture with DCsTrp. The result that CD4+ CD25+ T cells from coculture with DCsTrp are also somewhat suppressive is not surprising because Tregs seem to be part of any normal immune activation (and indeed a smaller percentage of stimulated T cells was Foxp3+ in this population). Importantly, TregsDCslow-Trp were always much more potent in terms of T cell suppression than TregsDCslow-Trp*, very likely as the result of a significantly greater number of Foxp3+ T cells induced by DCsTrp compared with DCsTrp*. A further explanation could be a more stable regulatory function of TregsDCslow-Trp, perhaps through epige
cnetic mechanisms such as changes in methylation of the Foxp3 promoter (55). This difference could also indicate a higher level of Ag specificity in the population of TregsDCslow-Trp, which could lead to a more efficient suppression of the T-cell recall response.

Further studies are needed to investigate DC phenotype and functions in chronic inflamed conditions in vivo in which low-Trp levels might occur. Differentiating DCs are largely affected from low-Trp levels by turning their phenotype into a regulatory one. This result may have implications for DC-based immunotherapy, especially for therapy in transplantation, autoimmune diseases, and allergies.

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References


