Interferon-gamma triggered indoleamine 2,3-dioxygenase competence in human monocyte-derived dendritic cells induces regulatory activity in allogeneic T cells

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Abstract

The role of the tryptophan-metabolizing enzyme indoleamine 2,3-dioxygenase (IDO) in down-regulating human allo-responses has recently been controversially debated. We here demonstrate that human monocyte-derived dendritic cells (mDCs) can be endowed with sustained IDO competence in vitro by 48-hour activation with lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). IFN-γ also amplified pro-inflammatory cytokine secretion during activation. Yet, upon re-culture after activation cytokine production ceased, while IDO enzymatic activity continued. Manipulation of tryptophan metabolism did not affect pro-inflammatory cytokine release, suggesting that IFN-γ triggers IDO activity and pro-inflammatory cytokine release as distinct cellular programs. IDO competent DCs down-regulated allogeneic T-cell responses, but this IDO-mediated effect was overcome by slightly modifying cell culture conditions. Nevertheless, the CD4+CD25+ T-cell fraction stimulated by IDO competent DCs displayed substantial suppressor activity. This suppressive activity (i) required allogeneic stimulation for its induction, (ii) affected third party T cells and (iii) was reduced by the IDO inhibitor methyl-thiohydantoin-tryptophan. It became also manifest when DC/T-cell co-cultures were initiated with naïve (CD4+CD25–CD45RA+) T cells, indicating the differentiation of adaptive regulatory T cells. Together, these findings suggest that IFN-γ triggered IDO competence in human mDCs constitutes a critical factor for endowing allogeneic T cells with regulatory activity.
Introduction

Indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme in tryptophan catabolism, has recently attracted attention for its proposed role in tolerance induction. IDO, when expressed in antigen presenting cells (APC), such as dendritic cells (DCs), establishes a microenvironment which at the DC/T-cell interface is depleted of tryptophan and enriched with tryptophan metabolites. Because of this alteration of the intercellular microenvironment, IDO activity has been suggested to impair T-cell responses. The mechanisms of IDO-mediated inhibition include that T cells stimulated under tryptophan-depleted conditions are impaired to undergo full cell cycle progression and are susceptible to apoptotic cell death. Furthermore, it has been reported that IDO expressing DCs can expand naturally occurring regulatory T cells. In a murine model Fallarino et al. recently showed that IDO activity supported the generation of adaptive regulatory T cells. Likewise, human IDO expressing tumor cells have been reported to induce CD4+CD25+ regulatory T cells. Most recently, the previously recognized immunoregulatory activity of human plasmacytoid DCs (pDCs) has been related to IDO activity. Thus, IDO-mediated down-regulation of T-cell responses has been suggested to be involved in a multitude of immunoregulatory processes, e.g. pregnancy, tumor growth and the induction of tolerance in transplantation (reviewed in).

IDO expression is not a constitutive feature of human DCs in homeostatic immunologic conditions but requires induction. Among the multiple mediators of IDO induction (reviewed in), interferon-γ (IFN-γ) plays a prominent role. IFN-γ has generally been considered a prototypic pro-inflammatory cytokine (reviewed in); compelling evidence, however, supports the ability of IFN-γ to promote anti-inflammatory responses. The ability of IFN-γ to induce IDO has recently been suggested as a critical factor linked to this anti-inflammatory activity.

In our previous studies addressing possible mechanisms of the immunodeficient state after hematopoietic stem cell transplantation (HSCT), we found that post-HSCT monocytes were
particularly sensitive to respond to an exposure to IFN-γ with an accelerated release of tryptophan metabolite kynurenine, and, thus, to turn into suppressor cells. This finding suggested the possibility that an augmented IDO activity in HSCT recipients might be involved in tolerance induction. On this background we began to test the hypothesis that the targeted induction of IDO enzymatic activity in human APC populations represents a means to achieve T-cell tolerance.

Dendritic cells, in order to prime immune responses, need to be activated. Recent studies have shown that a timely limited exposure of DCs to lipopolysaccharide (LPS) induces the release of pro-inflammatory cytokines and a Th1-polarized response24,25. In contrast, prolonged activation results in exhaustion of the DCs to secrete pro-inflammatory cytokines and supports a Th2-polarized and humoral response. In this context the role of IFN-γ is puzzling. On the one hand, IFN-γ has been reported to boost pro-inflammatory cytokine release by DCs thus acting as a pro-inflammatory compound26,27. On the other hand, IFN-γ by its capacity to induce IDO might dampen inflammation.

In the present study we demonstrate that abundant and sustained IDO protein expression and enzymatic activity, hereafter termed IDO competence, in LPS activated human monocyte-derived DCs is in fact dependent on the presence of IFN-γ during activation. The enzymatic activity of IDO, however, persists even when the IFN-γ triggered capability of DCs to secrete pro-inflammatory cytokines has ceased. Such IDO competent and cytokine exhausted DCs, set to stimulate allogeneic T cells in vitro, support the de novo differentiation of T cells with explicit regulatory function.
Material and Methods

Samples

All experiments were performed using human blood obtained from healthy volunteers or from blood donors at the blood bank of the General Hospital of Vienna upon giving informed consent, in accordance with the Declaration of Helsinki. Approval was obtained from the Children's Cancer Research Institute Institutional review board for these studies. Venipunctures were performed by experienced medical staff under standard sterile conditions.

Cell culture medium

DC differentiation and activation was performed in AIM V cell culture medium (Gibco, Invitrogen, CA, USA) containing 2% human plasma (Octaplas, Octapharm, Vienna, Austria), and 1% v/v L-glutamine (PAA Laboratories, Pasching, Austria), hereafter termed complete medium. T-cell stimulation and suppressor assays (see below) were performed in complete medium supplemented with 25mM Hepes (PAA Laboratories). All cell cultures were maintained in humidified air containing 5%CO₂ at 37°C.

Cell isolation

Peripheral blood mononuclear cells (PBMC) were enriched from whole blood by density centrifugation (Lymphoprep, Nycomed, Oslo, Norway). Monocytes were separated from PBMC by selective adherence to polystyrene surfaces. Alternatively, the monocyte enrichment was done via counter-flow centrifugal elutriation (Elutra Cell Separation System, Gambro BCT, Lakewood, USA)²⁸. Both techniques typically resulted in a >85% CD14+ population. These monocyte enriched cell populations were plated in culture flasks (Iwaki, Sterilin, Aberbargoed, UK) at a density of 0.3-0.5x10⁶ cells/cm². Immature DCs (iDCs) were generated by culture in complete medium supplemented with 1000U/ml GM-CSF (CellGenix,
Freiburg, Germany) and 400U/ml IL-4 (CellGenix) for 5 days. For DC activation, iDCs were exposed to 50ng/ml LPS (Calbiochem, La Jolla, CA, USA; E. coli O111:B4) with or without 1000U/ml IFN-γ (Boehringer Ingelheim, Ingelheim, Germany) for 4 or 48 hours. The quality of DC generation was monitored by visual and flow cytometric evaluation of a typical DC morphology and expression of cell surface markers, respectively.

Highly enriched T-cell populations were prepared as described. In brief, total CD4+ T cells as well as naïve CD4+CD45RA+ T cells were isolated from total PBMC by magnetic cell sorting (Miltenyi-Biotec, Bergisch Gladbach, Germany) according to manufacturer’s instructions, typically resulting in a >95% enrichment of the targeted cell population. Purity and viability were monitored by flow cytometry.

**Flow cytometry**

The following monoclonal antibodies (mAb) were used: Isotype control, IgG1-FITC, IgG2a-PE, IgG1-PE, IgG2a-perCP, IgG1-APC; anti-CD45-perCP (clone 2D1), anti-CD3-APC (clone SK7), anti-CD4-perCP (clone SK3), anti-CD25-PE (clone 2A3), anti-CD86-APC (clone 2331), anti-CD14-perCP-Cy5.5 (clone M5E2), anti-CD45RA-PE (clone HI100), anti-CTLA4-PE-Cy5 (clone BNI3) (all from BD Biosciences, San Jose, CA, USA); anti-HLA class II-FITC (clone CR3/43), anti-HLA class I-PE (clone W6/32) (Dako, Vienna, Austria); anti-CD80-PE (clone MAB104) (Immunotech, Beckman Coulter, Fullerton, CA, USA). For detection of FOXP3 in T cells we used clone PCH101 (eBiosciences, San Diego, CA, USA) or clone 259D/C7 (BD Biosciences). Both antibodies showed similar results. Flow cytometric analyses to identify or to sort different cell populations were performed using a FACSCalibur or the FACSArray flow cytometer (BD BioSciences). Analyses of list mode data were done using the CellQuest or DivaCell software (BD BioSciences).

**T-cell stimulation and mixed lymphocyte reaction (MLR)**
Highly enriched total CD4+ T cells (5x10^5) or CD4+CD45RA+ T cells (5x10^5) were co-cultured with allogeneic DCs (5x10^5, unless otherwise indicated) or autologous DCs, (which were pulsed with tetanus toxoid, Calbiochem) for 7 days in 24-well plates (Iwaki). Where indicated, DC/T-cell co-cultures were supplemented with fresh complete medium on day 4. Proliferative responses were quantified by [3H]thymidine incorporation, as described^23. Alternatively, T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and proliferation was calculated as the per cent CFSE negative cells^30. The competitive IDO inhibitors 1-methyl-DL- tryptophan (1-MT, 300µM) (Sigma Aldrich, St Louis, MO, USA) or methyl-thiohydantoin-tryptophan (MTHT, 50µM, Sigma)^31 were used to test for IDO dependent effects. Of note, the capacity of MTHT to block tryptophan degradation was comparable to that of the more commonly used IDO inhibitor 1-MT (Supplementary Table 1), and MTHT had no pro-proliferative effect on PBMC (not shown).

Suppressor Assay

At the termination of the DC/T-cell co-cultures, the non-adherent cells were recovered by gentle pipetting. The CD4+CD25– and CD4+CD25+ populations were separated by flow cytometry (resulting routinely in ≥99% purity). Subsequently, the CD4+CD25– or CD4+CD25+ T cells (0.5x10^5) were co-cultured with 0.5x10^5 fresh autologous or third party CD4+ T cells, as indicated, in 96-well plates (Nunc, Thermo Fischer, Langenselbold, Germany) and in a final volume of 200µL medium/well. The fresh T cells were stained with CFSE and were stimulated with immobilized anti-CD3mAb (250ng/ml, Clone UCHT-1, Sigma). Suppression of proliferation was calculated by comparing the per cent CFSE negative fresh T cells in co-cultures with CD4+CD25– T cells to co-cultures with CD4+CD25+ T cells according to the following formula: % inhibition = [1-(% CFSE– T cells co-cultured with CD4+CD25+ T cells/% CFSE– T cells co-cultured with CD4+CD25– T cells)] x100.
IDO expression and activity

IDO protein expression in DCs was investigated by immunoblot analysis using a mouse monoclonal anti-human IDO antibody, kindly provided by O. Takikawa. IDO enzymatic activity was determined by measuring the levels of tryptophan and kynurenine in the cell culture supernatants by high pressure liquid chromatography as previously described. In brief, tryptophan was detected by its natural fluorescence at 286nm excitation and 366nm emission wavelengths. 3-nitro-L-tyrosine, used as an internal standard, and kynurenine were determined by ultraviolet absorption at 360nm. An albumin-based external standard mix was prepared and included 50µM tryptophan (Serva, Heidelberg, Germany), 10µM kynurenine (Sigma) and a frozen serum pool. Upon addition of 25µL 2M trichloroacetic acid (Merck, Darmstadt, Germany) the reaction vials were immediately vortexed and centrifuged at 12000g (13 000rpm) for 6 minutes at room temperature to precipitate protein. The concentration of the components was calculated according to peak heights and was compared with 3-nitro-L-tyrosine as a reference standard.

Cytokine release

For the examination of cytokine secretion, cell culture supernatants were analyzed by the cytokine bead array (FlowCytomix, Bender Medsystems, Burlingame, CA, USA) according to manufacturer’s instructions. In some experiments, to detect a potential effect of tryptophan catabolism on DC cytokine secretion, cultures were supplemented with excess tryptophan (100µM, Sigma) or a combination of tryptophan catabolites (L-kynurenine, 3-hydroxy-kynurenine, 3-hydroxy-anthranilic acid, quinolinic acid, 25µM each, all from Sigma).

Statistical analysis

All statistical analyses were performed using the student’s t-test (paired, two-tailed). A p-value below 0.05 was considered to indicate statistical significance.
**Results**

**IDO induction in human monocyte-derived DCs.**

To enlighten the above described ambivalent effects of IFN-\(\gamma\) in directing opposing types of T-cell responses, we activated human monocyte-derived DCs with LPS in the presence or absence of IFN-\(\gamma\) for different time periods (4 or 48 hours) and examined their capacity to acquire IDO competence, to secrete cytokines and to stimulate allogeneic T cells. First, we found that either DC activation strategy induced a similar level of expression of cell surface markers commonly associated with DC maturation, with the notion that, in line with the aforementioned studies\(^{27}\), the levels of expression of co-stimulatory molecules CD80 and CD86 increased with time of activation (Fig 1A).

Remarkably, IDO competence was restricted to long-term LPS/IFN-\(\gamma\) activated DCs. In fact, only DCs exposed to LPS/IFN-\(\gamma\) for 48 hours expressed abundant amounts of IDO protein (Fig 1B, upper panel) and depleted the cell culture medium of tryptophan and accumulated high concentrations of kynurenine (Fig 1B, lower panel). In contrast, DCs activated with LPS only, even upon a long-term exposure (48 hours), displayed only minor IDO expression and tryptophan metabolizing capacity.

To assess continuing IDO competence after activation, the differently activated DCs were extensively washed and re-cultured in fresh complete medium. In these cultures, only DCs that had acquired IDO competence by long-term activation with LPS and IFN-\(\gamma\), rapidly and effectively metabolized tryptophan even after removal of the activation stimuli. Indeed, these re-cultured DCs induced a tryptophan deplete/kynurenine enriched cell culture milieu within 24 hours (Fig 1C, upper panel). Importantly, however, we noticed that DCs having been activated with LPS/IFN-\(\gamma\) short-term (4 hours) gradually acquired IDO competence. In fact, 48 hours after the onset of re-culture, the cell culture medium containing short-term LPS/IFN-\(\gamma\) activated DCs was similarly depleted of tryptophan and enriched for kynurenine as the cell
culture medium containing long-term (48-hour) LPS/IFN-γ activated DCs (Fig 1C, lower panel; p-value n.s.), hereafter termed delayed IDO competence. In contrast, DC activation with LPS only, irrespective of whether or not they developed IDO competence during activation, failed to display significant IDO competence upon re-culture (Fig. 1C). Of note, the presence of allogeneic T cells did not additionally affect levels of tryptophan depletion or kynurenine accumulation (data not shown), thus excluding that T cells or their cytokine production were required for the maintenance of these DCs’ IDO competence.

**IFN-γ triggering of cytokine secretion and IDO activity are discrete processes.**

Since it has been reported that the ability to secrete cytokines, similarly to the above described induction of IDO competence, is related to the time of activation\(^24\) we addressed the question of whether cytokine production and IDO activity are linked processes. In a subsequent series of experiments we monitored IDO activity and the release of pro-inflammatory cytokines, IL-12, IL-6 and TNF-α, in DCs activated by LPS ± IFN-γ in parallel. Several important observations were made: (1) The addition of IFN-γ to LPS stimulated both, production of pro-inflammatory cytokines\(^26,27\) and IDO activity in a time dependent manner and lead to accumulation of abundant amounts of cytokines along with abundant IDO enzymatic activity within a 48-hour activation period (Fig 2A). (2) However, when the activating agents were removed and DCs were re-cultured in fresh medium, their capacity to produce pro-inflammatory cytokines was markedly reduced but their capacity to effectively degrade tryptophan and accumulate kynurenine was maintained (Fig 2B). This finding suggests that IFN-γ stimulates DCs’ pro-inflammatory cytokine production and IDO competence in parallel but the time period of IDO enzymatic activity exceeds that of cytokine secretion.

Next, to examine, whether cytokine production is affected by IDO activity, we manipulated tryptophan degradation and kynurenine accumulation during the DC activation
period. DCs were activated with LPS/IFN-γ for 48 hours in the presence of excess tryptophan (100µM) or of exogenous tryptophan metabolites (25µM each) or blockade of IDO activity by MTHT and 1-MT. While these manipulations resulted in the expected changes of tryptophan and kynurenine concentrations in cell culture supernatants (Fig 2C, right panel) the levels of pro-inflammatory cytokines remained essentially unaffected (Fig 2C, left panels). In addition, a delayed blockade of IDO activity by MTHT did not alter the release of the above cytokines (data not shown). Taken together, these findings suggest that in monocyte-derived and LPS-activated DCs IFN-γ triggers pro-inflammatory cytokine secretion and IDO activity as distinct cellular programs.

The inhibition of allogeneic T-cell proliferation by IDO competent DCs is sensitive to the cell culture condition.

Using the above described differently activated DCs as stimulators of allogeneic T cells allowed us to study a possible effect of IDO competence in human DCs on modulating allogeneic T-cell responses.

First, and consistent with previous observations, we found that IDO competent DCs had a low stimulatory capacity of allogeneic T cells. In fact, when we compared DCs activated with LPS/IFN-γ for 48 hours (IDO competent) to DCs activated with LPS/IFN-γ for 4 hours (delayed IDO competence) as stimulators of an MLR, the allogeneic T-cell response stimulated by IDO competent DCs was impaired. The extent of reduction of the proliferative response correlated to the number of IDO competent DCs added to the allogeneic T cells (Fig 3A), suggesting a suppressive effect on a per cell basis. The inhibition of the allo-responses by IDO competent DCs was reversible by MTHT-mediated blockade of IDO activity (Fig 3B), thus supporting an IDO dependent effect.

Somewhat unexpectedly, however, when we adapted cell culture conditions (see Material and Methods) to prevent exhaustion of the cell culture medium and to optimize proliferation
of allo-stimulated T cells, the impairment of the allo-responses by IDO competent DCs was no longer detectable, even when the DCs were added to allogeneic T cells in equal amounts. Either of the differently activated DC populations induced proliferation of allogeneic T cells to similar extents (Fig 3C). This finding is in agreement with former observations that the capacity of IDO competent DCs to suppress allogeneic T cell responses critically depends on cell culture conditions\textsuperscript{35,36}.

**IDO competent DCs, even when not suppressing proliferation, endow allogeneic CD4+ T cells with regulatory activity.**

When we examined the phenotype of allo-reactive T cells (CD4+CD25+CFSE negative) stimulated in the above described cell culture conditions, we noticed that the CD4+ T cells stimulated by either of the above differently activated DC populations showed similar levels of CD25, FOXP3 and CTLA-4 expression (Fig 4A), compatible with a regulatory phenotype. Yet, true regulatory activity of a CD4+CD25+ T-cell population may not be recognizable by phenotype\textsuperscript{37}, even when cells express the master regulatory marker FOXP3\textsuperscript{38}. Thus, to explicitly address a regulatory activity of CD4+CD25+ T cells, we tested the CD4+CD25+ T-cell fractions after stimulation by either of the differently activated DC populations for their suppressive capacity in a conventional suppressor assay. Briefly, after 7 days of MLR, T cells were recovered and were separated by flow cytometry into a CD4+CD25– and CD4+CD25+ T-cell faction and were subsequently added to fresh autologous T cells, which were stained with CFSE and stimulated with immobilized anti-CD3mAb.

First, as expected, we found that the CD4+CD25– T cells recovered from MLRs stimulated by the differently activated DCs had no suppressive capacity. The per cent CFSE negative fresh T cells after co-culture with the CD4+CD25– fraction recovered from MLRs stimulated by either of the differently activated DC populations were not different from controls (70% median, Fig 4B,C). However, when we examined the CD4+CD25+ T-cell
fractions retrieved from the same MLRs, the differences in their suppressor activity were striking. As depicted from a single experiment (Fig 4B), the CD4+CD25+ T cells recovered from an MLR stimulated by LPS/IFN-γ 4-hours activated DCs or LPS only 48-hours activated DCs (displaying delayed or minor IDO competence, respectively) only marginally suppressed the proliferation of fresh CD4+ T cells (Fig 4B, upper and middle panels). In clear contrast, the CD4+CD25+ T-cell fraction retrieved from an MLR which was stimulated by IDO competent DCs, potently suppressed the anti-CD3 mediated proliferation of fresh CD4+ T cells (Fig 4B, lower panel). This suppressive capacity of CD4+CD25+ T cells stimulated by allogeneic IDO competent DCs was consistently observed in 15 consecutively tested different stimulator/responder pairs (68% suppression, median, Fig 4C). Suppression was evident when the CD4+CD25+ T cells were added to the fresh T cells in equivalent amounts but only rarely at lower ratios (data not shown). However, the capacity of CD4+CD25+ T cells recovered from MLRs stimulated by IDO competent DCs to suppress was significantly higher (p<0.01) than the only minor suppressor activity of CD4+CD25+ T cells recovered from MLRs stimulated by DCs with delayed or minor IDO competence (25% suppression, median, Fig 4C).

Subsequently, we ascertained that the induction of suppressor T cells was in fact linked to IDO competence in stimulating DCs. In these experiments allogeneic T cells were stimulated by IDO competent DCs in the presence or absence of the IDO inhibitor MTHT. The presence of MTHT in the MLR reduced the suppressive capability of the CD4+CD25+ T cells as evidenced by a significantly increased proliferative response of the fresh T cells to stimulation with anti-CD3 (1.8 fold, median, p<0.05) (Fig 4D,E). Notably, the addition of MTHT to MLRs stimulated by short-term LPS/IFN-γ activated DCs had no effect on the capacity of CD4+CD25+ T cells to alter proliferation of fresh T cells (Fig 4E). These findings strongly support that IDO activity essentially contributes to the induction of regulatory activity in allogeneic T cells.
Next, to explore, whether allogeneic stimulation is critical for the induction of regulatory activity in T cells upon co-culture with IDO competent DCs, we compared the regulatory activity of T cells stimulated by allogenic (as above) and autologous IDO competent DCs. In the autologous setting IDO competent DCs presented a nominal antigen (tetanus toxoid). Unexpectedly, the experiments showed (Fig 5) that the CD4+CD25+ T cells upon stimulation by autologous IDO competent DCs, were unable to act as regulatory cells in subsequent suppressor assays, in contrast to T cells stimulated by allogeneic IDO competent DCs. This finding suggests an essential role of allogeneic stimulation39 for the generation of T-cell regulatory activity by IDO competent DCs.

To then determine, whether the regulatory activity induced by IDO competent DCs is restricted to suppressing autologous T cells, we compared the suppressive capacity of CD4+CD25+ T cells retrieved from MLRs stimulated by IDO competent DCs in suppressor assays containing fresh autologous or third party T cells. The results (Fig 6) showed a similar suppressor activity of CD4+CD25+ T cells upon co-culture with autologous or third party T cells, suggesting that the T-cell regulatory activity induced by IDO competent DCs is not restricted to the antigen through which it was activated40 but is more generally applicable.

Together, these results identify IDO competence in stimulating DCs as a critical factor contributing to the induction of a non-specific suppressor function in allogeneic T cells. The finding that DCs upon short-term activation with LPS/IFN-γ, which develop delayed IDO competence, induced only minor suppressor activity in allo-reactive T cells indicates that IDO competence must be present at the initiation of the MLR in order to induce suppressor function in responder T cells.

**IDO competent DCs induce regulatory CD4+CD25+ T cells from naïve CD4+CD25− T cells.**
Finally, we addressed the question of whether IDO competent DCs possess the capability to generate CD4+CD25+ suppressor T cells from naïve CD4+CD45RA+ T cells. The latter have been shown to be devoid of memory cells and devoid of the naturally occurring CD25+ regulatory T cells (data not shown). CD4+CD45RA+ or total CD4+ T cells were co-cultured with IDO competent DCs and after termination of the MLR, were assessed for the induction of suppressor activity as described above. Stimulation of CD4+CD45RA+ or CD4+ T cells with allogeneic IDO competent DCs induced similar proportions of CD4+CD25+ T cells (data not shown). Noticeably, the CD4+CD25+ T cells retrieved from an MLR, in which CD4+CD45RA+ T cells were responders, had equivalent suppressive capacity as CD4+CD25+ T cells retrieved from an MLR in which responders were total CD4+ T cells (Fig 7; p-value n.s.), indicating that IDO competent DCs can induce adaptive regulatory T cells.
Discussion

The essential conclusion of the present study is that IDO competence in human monocyte-derived DCs, even when not causing a quantitative down-regulation of proliferative responses, nevertheless possesses the capacity to support T-cell regulatory activity.

Recently, numerous observations have questioned the relevance of IDO competence in human DCs because of the findings that (i) IDO activity had no down-regulating effect on T-cell proliferation and (ii) the commonly used IDO inhibitor 1-methyl-tryptophan (1-MT) seemingly had an effect on cell proliferation apart from inhibiting IDO. To this end, our data show that upon a slight modification of the cell culture conditions, the inhibition of proliferative responses of allogeneic T cells by IDO competent DCs was no longer detectable. Despite different grades of IDO competence either of the examined DC populations induced quantitatively equivalent T cell responses. Thus, our data support the notion, that the T-cell dampening effect of IDO activity in human cells in vitro may critically depend on details of cell culture conditions. Moreover, they provide sound evidence that allo-reactive T cells stimulated by IDO competent DCs may, even when these T cells retain their ability to proliferate, acquire regulatory activity.

The role of phenotypically mature DCs as powerful stimulators of immune effector responses has lately been revisited and their ability to induce or activate regulatory T cells and to maintain tolerance has been appreciated. In a recent report, tolerogenic pDCs when activated through TLR9 ligation have been shown to expand regulatory T cells via the IDO pathway. Our data evidence that, likewise, human monocyte-derived DCs, upon activation by TLR4 ligation (LPS) and IFN-γ, acquire sustained IDO competence and can induce T-cell regulatory activity. In our ongoing experiments we found that also a PGE2 based DC activation strategy induced IDO competence and stimulated a regulatory T-cell response (BJ, unpublished observation, January 2009). Collectively, these findings support the view that IDO competence in DCs, regardless of the DC subtype and of the IDO inducing strategy,
constitutes a common and critical factor for enabling DCs to stimulate T-cell regulatory activity. Intriguingly, IDO competent DCs endowed even naïve allogeneic (CD4+CD25–CD45RA+) T cells with the ability to suppress suggesting that IDO competent DCs possess the capacity to induce adaptive regulatory T cells.

Regulatory T cells have in most studies been identified as CD4+CD25+ T cells expressing the transcription factor FOXP3. Substantial evidence now disagrees with the view that the human adaptive regulatory T cells can in fact be identified by the expression of CD25 or FOXP3 or CTLA-4 because these molecules have been reported to be induced to similar levels in recently activated effector T cells. In fact, also in the present study allogeneic CD4+ T cells stimulated by DCs with different degrees of IDO competence, showed a largely similar CD4+CD25+FOXP3+CTLA-4+ phenotype while their capacity to suppress was substantially different. Thus, the suppressive capacity could not be deduced from the marker expression profile of responding T cells.

In our studies, the addition of IFN-γ to LPS as a strategy to activate monocyte-derived DCs in vitro amplified both, the production of pro-inflammatory cytokines and IDO competence. The data further evidence that DCs after 48 hours of activation with LPS and IFN-γ lose the capacity to secrete pro-inflammatory cytokines but retain the capability to effectively metabolize tryptophan and accumulate kynurenine (Fig 2B). This divergent balance appears to shape the T-cell response. DCs activated in a short-term strategy (LPS/IFN-γ 4 hours) have been shown to be able to continue to secrete pro-inflammatory cytokines upon re-culture and, though gradually developing IDO competence, support a Th1-polarized T-cell response. These observations suggest an in vitro model system to enlighten the paradoxical roles of IFN-γ in directing opposing immune reactions. IFN-γ may trigger a signaling program in DCs to stimulate Th1-polarized T-cell responses, but this pro-inflammatory program is limited by time. IFN-γ concomitantly elicits IDO competence in DCs, which becomes prevalent after the production of pro-
inflammatory cytokines has ceased. Thus, IDO competence may represent a signature of an anti-inflammatory program, and may enable APCs to either suppress T-cell responses or induce T-cell regulatory activity.

Our data, by showing that the manipulation of tryptophan catabolism left cytokine production essentially unaffected, support the view that the IFN-γ triggered induction of pro-inflammatory cytokines and of IDO competence represent distinct cellular programs. However, to comprehensively address the question of a potentially linked regulation of IDO activity and cytokines, further in-depth studies including the molecular level are required. Thus far, this model is compatible with viewing IDO induction as an immunoregulatory feedback mechanism, representing a physiological limitation of potentially hazardous immune reactions14,48. The observation that T-cell regulatory activity was induced by allogeneic rather than by autologous IDO competent DCs is intriguing. One likely possibility to explain this observation may be the lower T-cell precursor frequency specific for cognate antigen (TT) as compared to that of T-cell precursors specific for allo-antigens. Studying in detail the mechanisms underlying this finding may have important implications for the understanding of IDO mediated tolerogenesis in vivo.

Irrespective of whether the findings presented herein, including the opposing activities of DCs activated by IFN-γ, translate in vivo, they may be significant for the design of DCs as therapeutic immunomodulators ex vivo49. Practically, to induce a regulatory T-cell response in vitro, one possible approach is to generate IDO competent DCs which would be incapable of secreting pro-inflammatory cytokines and would encounter T cells in a tryptophan deplete but kynurenine rich environment. Whether the DC activation strategy used in this study, which is to combine LPS with IFN-γ to generate IDO competence, is the most effective one, is currently extensively examined in our laboratory. Furthermore, the implication of our findings on the design of DC vaccination in vivo warrants further investigation.
In conclusion, we propose the following conceptual view for understanding the role of IFN-γ in activating human monocyte-derived DCs: IFN-γ stimulates DCs to release pro-inflammatory cytokines and to develop IDO competence in parallel, but with different time kinetics. As long as pro-inflammatory cytokine release prevails and IDO competence is not yet fully developed, the DCs may stimulate a pro-inflammatory T-cell response. However, when cytokine production ceases, the IFN-γ triggered IDO activity becomes prevalent and ongoing even after activation. The ongoing IDO activity may be particularly relevant when DCs encounter responder T cells. These IDO competent DCs dampen immune responses. The relevance of IDO competence in dampening immune responses may become evident as the DCs’ ability to suppress T-cell responses quantitatively. This effect, however, appears to be sensitive to details of cell culture conditions. Yet, even when the inhibitory effect is not obvious, IDO competence in DCs may nevertheless contribute to modify immune responses by endowing T cells with regulatory activity. Thus, future efforts to elaborate in full the potential of IDO competence and its usefulness in clinical application will have to take into account the various properties of IDO competence in DCs. In addition to the frequently observed suppression of T-cell responses, these properties include the recently reported capability of IDO-mediated apoptosis that particularly affects activated T cells, and the capability of DCs to endow T cells with regulatory activity. The appropriate use of the combined effects of IDO competence in APCs may be valuable in the generation of antigen-specific tolerance.
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Authorship

Contribution: B.J. designed and performed the experiments, analyzed data and wrote the paper. U.H. performed initial experiments and commented on the paper. D. F. performed HPLC studies measuring tryptophan and kynurenine concentrations and critically reviewed the manuscript. T.F. designed initial experiments and commented on the paper. A.H. designed and supervised the study, analyzed data and wrote the paper.

Conflict of interest disclosure: The authors declare no competing financial interests.

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Figure legends

Figure 1. Characterization of maturation status and IDO competence in human monocyte-derived DCs activated with LPS ± IFN-γ for different time periods.

(A) DCs after activation with LPS only or LPS in combination with IFN-γ for different time periods were examined for expression of DC cell surface molecules typically associated with maturation (shaded histograms, isotype control; open histograms, DCs after activation). One representative of 10 similar experiments is shown. (B) Upper panel: IDO protein expression in differently activated DCs examined by immunoblot analysis; Lower panel: The concentrations of tryptophan (grey bars; tryptophan, TRP, left scale) and kynurenine (black bars; kynurenine, KYN, right scale) in cell culture supernatants of the differently activated DCs were determined at the termination of the activation period. Results of one of 10 similar experiments are shown. (C) The concentrations of tryptophan and of kynurenine were examined as in Fig 1B in cell culture supernatants 24 hours (upper panel) and 48 hours (lower panel) after removal of the activating agents and re-culture in fresh medium. Results of one experiment, representative of 10 experiments, are shown. **, p<0.01; n.s., not significant.

Figure 2. The IFN-γ triggered pro-inflammatory cytokine production and IDO induction in human DCs represent distinct cellular programs.

(A) The amount of pro-inflammatory cytokine release at the end of the activation period was examined in cell culture supernatants of differently activated DCs (left panels). In the same culture supernatants tryptophan (grey bars) and kynurenine (black bars) concentrations were examined (right panel). The bars indicate the median of 4 experiments. The numbers given in brackets indicate the range. (B) DCs were activated with LPS/IFN-γ for 48 hours to induce IDO competence. Pro-inflammatory cytokine release was examined in cell culture supernatants after removal of the activating agents and re-culture of the activated DCs in fresh medium for a 48-hour period. The values of cytokine release upon re-culture are expressed as
per cent of the maximum release (= 100%) during the activation period (upper panel). In the same culture supernatants tryptophan and kynurenine concentrations were analysed (lower panel). Results of one experiment, representative of 2 experiments, are shown. (C) DCs were activated with LPS/IFN-γ for 48 hours in conditions of external manipulation of tryptophan metabolism, as indicated (TRP met, tryptophan metabolites). Pro-inflammatory cytokine release (left panels) and the concentrations of tryptophan and kynurenine (right panel) were examined. Results of one experiment, representative of 2 experiments, are shown.

**Figure 3. The reduced capacity of IDO competent DCs to stimulate allogeneic T cells can be overcome by a slight modification of cell culture conditions.**

(A) DCs were activated with LPS/IFN-γ for 4 hours (♦) or 48 hours (■) and co-cultured in graded amounts with allogeneic T cells (MLR) and their capacity to stimulate an allogeneic T-cell response was determined by [³H]thymidine incorporation. Results of one experiment, representative of 2 experiments, are shown. Error bars indicate the standard error of the mean (SEM). (B) DCs were activated with LPS/IFN-γ for 48 hours and were added in graded amounts to allogeneic T cells as in Fig 3A. MLRs were set up in the presence (grey squares) or absence (black squares) of the IDO inhibitor MTHT. The allogeneic T-cell responses were determined as the per cent CFSE negative T cells. Results of one experiment, representative of 2 experiments, are shown. (C) Differently activated DCs were co-cultured with the same amount of allogeneic T cells and fresh cell culture medium was added to the cell cultures at day 4. The allogeneic T-cell responses were determined as in Fig 3B.

**Figure 4. IDO competent DCs, even when not suppressing the allogeneic T-cell response, induce regulatory activity in allogeneic CD4+ T cells.**

(A) Differently activated DCs were used as stimulators of an MLR as in Fig 3C. At the end of the co-culture T cells were recovered and were examined for CD25, CTLA-4 and FOXP3
expression (shaded histograms, CD4+CD25− T cells; open histograms, CD4+CD25+ T cells). Results of one experiment, representative of 4 experiments are shown. (B) CD4+ T cells were recovered from MLRs, in which the differently activated DCs were used as stimulators as in Fig 4A, and were sorted by flow cytometry into a CD4+CD25− and a CD4+CD25+ population (left panel). These separate T-cell populations were subsequently co-cultured for 5 days with fresh autologous T cells (1:1) (suppressor assay). The latter were stained with CFSE. The proliferative responses of these T cells to stimulation with immobilized anti-CD3 mAb were assessed as the per cent CFSE negative cells. Inhibition of proliferation was calculated according to the formula noted in Material and Methods. One representative experiment is shown. (C) Cumulative results: Light grey bars: Median per cent CFSE negative T cells only without (left bar) or with anti-CD3 mediated stimulation (right bar); blank bars: Median per cent CFSE negative T cells co-cultured with CD4+CD25− T cells retrieved from MLRs stimulated by differently activated DCs, as indicated; dark grey bars: Median per cent CFSE negative fresh T cells co-cultured with CD4+CD25+ T cells retrieved from the same MLRs. Error bars indicate the range of experimental results of 15 consecutively tested donor/responder pairs. **, p<0.01. (D) DCs were activated with LPS/IFN-γ for 48 hours and used as stimulators of an MLR as in Fig 4A, in the absence (left panels) or presence (right panels) of MTHT. After the MLR, the CD4+ T cells were recovered and sorted and subjected to suppressor assays as in Fig 4B. Proliferative responses are shown by CFSE dilution of fresh T cells (open histograms, thick black line) upon co-culture with the sorted autologous CD4+CD25− (upper row) and CD4+CD25+ (lower row) T cells. Filled grey histograms: CFSE dilution of anti-CD3 stimulated fresh T cell only; open histograms, thin line: CFSE dilution of unstimulated fresh T cells only. The numbers indicate the per cent CFSE negative cells in suppressor assays. One representative experiment is shown. (E) Cumulative results: Light grey bars: Median per cent of CFSE negative fresh T cells only in the absence (left bar) or presence (right bar) of stimulation with anti-CD3 mAb (controls);
dark grey bars: Median per cent of CFSE negative fresh T cells after co-culture with CD4+CD25+ T cells retrieved from MLRs stimulated by DCs activated with LPS/IFN-γ for 4 hours or for 48 hours, as indicated, in the absence or presence of the IDO inhibitor MTHT. Error bars indicate the range of a total of 6 consecutive experiments. *, p<0.05.

Figure 5. IDO competent DCs stimulate T-cell regulatory activity upon allogeneic rather than autologous stimulation.

DCs were activated with LPS/IFN-γ for 48 hours and used to stimulate an allogeneic or an autologous, TT triggered, response (see Material and Methods). CD4+ T cells were recovered from the DC/T-cell co-cultures and sorted and subjected to subsequent suppressor assays as in Fig 4. Left panels: The CD4+ T cells were retrieved from DC/T-cell co-cultures stimulated by allogeneic DCs. Right panels: The CD4+ T cells were retrieved from DC/T-cell co-cultures stimulated by autologous, TT pulsed, DCs. Proliferative responses of fresh CFSE stained and anti-CD3 stimulated T cells are shown by CFSE dilution (open histograms, thick black line) upon co-culture with the sorted CD4+CD25− (upper row) and CD4+CD25+ T cells (lower row). Filled grey histograms: CFSE dilution of anti-CD3 stimulated fresh T cells only; open histograms, thin line: CFSE dilution of unstimulated fresh T cells only. The numbers indicate the per cent CFSE negative cells in suppressor assays. Results of one experiment, representative of 3 experiments, are shown.

Fig 6. CD4+CD25+ T cells stimulated by allogeneic IDO competent DCs suppress the proliferation of third party T cells.

DCs were activated with LPS/IFN-γ for 48 hours and used as stimulators of an MLR and the CD4+ T cells retrieved from the MLR were subjected to subsequent suppressor assays as in Fig 4. Left panels: CFSE stained fresh T cells and CD4+ T cells were autologous. Right panels: CFSE stained fresh T cells and CD4+ T cells were allogeneic (3rd party). Proliferative
responses of fresh T cells are shown by CFSE dilution (open histograms, thick black line) upon co-culture with sorted CD4+CD25− (upper row) and CD4+CD25+ T cells (lower row). Filled grey histograms: CFSE dilution of anti-CD3 stimulated fresh T cell only; open histograms, thin line: CFSE dilution of unstimulated fresh T cells only. The numbers indicate the per cent CFSE negative cells in suppressor assays. Results of one experiment, representative of 3 experiments, are shown.

Fig 7. IDO competent DCs induce the differentiation of adaptive regulatory T cells.

Total CD4+ T cells or naïve CD4+CD45RA+ T cells were co-cultured with IDO competent DCs (activated with LPS/IFN-γ, 48 hours) and, after termination of the MLR, were sorted by flow cytometry into a CD4+CD25− and a CD4+CD25+ population. These separate T-cell populations were subsequently co-cultured with fresh autologous T cells (1:1) which were stained with CFSE. The bars indicate the median per cent of CFSE negative cells after 5 days of culture. Light grey bars: per cent CFSE negative fresh T cells only without (left bar) or with anti-CD3 stimulation (right bar) (controls); blank bars: per cent CFSE negative fresh T cells co-cultured with CD4+CD25− T cells retrieved from the MLR; dark grey bars: per cent CFSE negative fresh T cells co-cultured with CD4+CD25+ T cells retrieved from the MLR; Error bars indicate the range of experimental results of 4 consecutively tested donor/responder pairs. p=not significant.
Figure 3

A

[\text{[^3]H] thymidine incorporation} \text{cpm} \times 10^3

\begin{align*}
10\% \text{ DC} & \quad 20\% \text{ DC} & \quad 50\% \text{ DC} \\
\end{align*}

B

\text{per cent CFSE negative T cells}

\begin{align*}
10\% \text{ DC} & \quad 20\% \text{ DC} & \quad 50\% \text{ DC} \\
\end{align*}

C

LPS/IFN-g 4h

\begin{align*}
50\%
\end{align*}

LPS 48h

\begin{align*}
49\%
\end{align*}

LPS/IFN-g 48h

\begin{align*}
55\%
\end{align*}
Figure 6

source of CFSE stained T cells

<table>
<thead>
<tr>
<th>CD25-</th>
<th>88%</th>
<th>81%</th>
</tr>
</thead>
<tbody>
<tr>
<td>autologous</td>
<td></td>
<td>3rd party</td>
</tr>
<tr>
<td>CD25+</td>
<td>31%</td>
<td>30%</td>
</tr>
</tbody>
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CFSE
Figure 7

Efficacy of DC activation strategies for primary MLR. The per cent of CFSE negative T cells in control, total CD4+ and naive CD4+ cells treated with LPS/IFN-γ for 48 h are depicted. Data are shown as mean ± SD. n.s. = not statistically significant.
Interferon-gamma triggered indoleamine 2,3-dioxygenase competence in human monocyte-derived dendritic cells induces regulatory activity in allogeneic T cells

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