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Ligation of B7-1/B7-2 by Human CD4$^+$ T Cells Triggers Indoleamine 2,3-Dioxygenase Activity in Dendritic Cells[1]

David H. Munn,[2]*† Madhav D. Sharma,* and Andrew L. Mellor*‡

Human monocyte-derived dendritic cells (DCs) are capable of expressing the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO), which allows them to suppress Ag-driven proliferation of T cells in vitro. In DCs that express IDO, the activity of the enzyme is tightly regulated, with the protein being constitutively expressed, but functional activity requiring an additional set of triggering signals supplied during Ag presentation. We now show that triggering of functional IDO obligately requires ligation of B7-1/B7-2 molecules on the DCs by CTLA4/CD28 expressed on T cells. When this interaction was disrupted, IDO remained in the inactive state, and the DCs were unable to inhibit T cell proliferation. Inhibition could be fully restored by direct Ab-mediated cross-linking of B7-1/B7-2. Although both CD4$^+$ and CD8$^+$ T cells were susceptible to inhibition once IDO was induced, the ability to trigger functionally active IDO was strictly confined to the CD4$^+$ subset. Thus, the ability of CD4$^+$ T cells to induce IDO activity in DCs allowed the CD4$^+$ population to dominantly inhibit proliferation of the CD8$^+$ population via the bridge of a conditioned DC. We hypothesize that IDO activation via engagement of B7-1/B7-2 molecules on DCs, specifically, engagement by CTLA4 expressed on regulatory CD4$^+$ T cells, may function as a physiologic regulator of T cell responses in vivo. ***The Journal of Immunology*****, 2004, 172: 4100–4110.**

Maintaining tolerance in the peripheral T cell compartment is an active, on-going process (1, 2). One critical factor governing whether peripheral T cells respond or become tolerant to Ag is the nature of the APC (3, 4). Although APCs are important regulators of peripheral tolerance, the specific molecular mechanisms by which they control T cell responses are still being elucidated.

One mechanism by which APCs may regulate T cell responses is through the expression of indoleamine 2,3-dioxygenase (IDO),[3] an enzyme that degrades the essential amino acid tryptophan (5). We and others have shown that the expression of IDO by human monocyte-derived macrophages (6) and DCs (7, 8) allows these APCs to inhibit T cell proliferation in vitro. Transfection of recombinant IDO into non-APC cell lines confers the ability to inhibit Ag-specific T cell responses (9). In vivo, using murine models, a role for IDO has been demonstrated in maternal tolerance toward the allogeneic fetus (10, 11), suppression of T cell responses to MHC-mismatched allografts (10, 12), control of T cells in autoimmune disorders (13–15), and suppression of immune responses to tumors (16, 17).

A mechanistic link has also been demonstrated between the tolerance-inducing activity of recombinant CTLA4-Ig Fc fusion protein (CTLA4-Ig), a synthetic fusion protein that binds with high affinity to the costimulatory molecules B7-1 and B7-2, and the fact that CTLA4-Ig also induces IDO expression in DCs (18). Engagement of B7-1/B7-2 molecules on murine DCs by CTLA4-Ig was found to activate a signaling pathway leading to induction of IDO in vitro, and pharmacologic inhibition of IDO in vivo completely abrogated the tolerogenic effect of CTLA4-Ig in a mouse islet cell transplant model (18). Recently, the physiologic CTLA4 receptor expressed by murine regulatory T cells (Treg) has been shown to mediate an analogous IDO-inducing effect in vitro (19). We have reported that the up-regulation of IDO by CTLA4-Ig in vivo is highly selective, being restricted to the CD8α^+ subset of DCs and to B220$^+$ (plasmacytoid) DCs, but despite the limited number of such cells, IDO expression could completely suppress clonal expansion of large cohorts of adoptively transferred alloreactive T cells (20). Thus, in a variety of murine models, IDO expression by DCs functions as an inducible immunosuppressive mechanism, which may be triggered at least in part by the Treg system.

In the human system, IDO expression has been observed by immunohistochemistry in placenta, tumor-draining lymph nodes, and primary tumors (17, 21), but the functional immunoregulatory role of IDO in humans is less well defined. To model IDO-mediated immune regulation by human DCs we have developed an in vitro culture system in which monocyte-derived DCs can be enriched for a population expressing high levels of IDO (7). Using this system we have demonstrated that maturation does not in and of itself abrogate IDO-mediated suppression, allowing some DCs to manifest a phenotype that is both mature and actively suppressive (7).

In mature IDO$^+$ DCs the functional activity of IDO is tightly regulated, with the IDO protein being constitutively expressed, but enzymatic activity manifest only in response to specific triggering signals (7). The most potent trigger for functional activity was found to be exposure to activated T cells during Ag presentation. We initially hypothesized that this might be due to T cell-derived IFN-γ, because IFN-γ is produced early during T cell activation (22, 23), and IFN-γ from T cells induces IDO in human monocyte-derived macrophages (6). However, we found that simply treating isolated DCs with rIFN-γ did not recapitulate the IDO-inducing

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[3] Abbreviations used in this paper: IDO, indoleamine 2,3-dioxygenase; CD28-Ig, CD28-Ig Fc fusion protein; CTLA4-Ig, CTLA4-Ig Fc fusion protein; DC, dendritic cell; 1MT, 1-methyl-D-tryptophan; Treg, regulatory T cell.

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effect of activated T cells and, in fact, produced the diametrically opposite effect, i.e., marked down-regulation of IDO protein, mRNA, and enzymatic activity (7). Thus, activated T cells supplied an additional signal, over and above IFN-γ, which was obligately required to trigger functional IDO. Based on observations from the murine system, we hypothesized that this IDO-inducing signal might be ligation of B7 molecules on the DCs by their counter-receptors (CTLA4 and CD28) on T cells.

Materials and Methods

DC culture system and T cell isolation

Human monocytes (>90% purity) and lymphocytes (>80% T cells, balance of B and NK cells, <1% monocytes) were isolated by leukocytophoresis and counterflow elutriation (6) under a protocol approved by our institutional review board. To generate DCs, monocytes were cultured for 7 days in serum-free X-vivo15 medium (BioWhittaker, Walkersville, MD) supplemented with GM-CSF (50 ng/ml; R&D Systems, Minneapolis, MN) and IL-4 (50 ng/ml; R&D Systems) on days 0, 2, and 4 as previously described (7). Unless otherwise specified, all DCs were matured by adding the following mixture (24) during the final 48 h of culture: TNF-α (1100 U/ml; BD PharMingen, San Diego, CA), IL-1β (1870 U/ml; BD PharMingen), IL-6 (1000 U/ml; BD PharMingen), and PGE_2 (1 μg/ml; Sigma-Aldrich, St. Louis, MO). Under these conditions, IDO^+ cells constituted up to 60–70% of all DCs (in contrast to the DCs generated in bovine serum-based medium, which typically contained only 20–30% IDO^+ DCs). The nonadherent (IDO^+) fraction was harvested by gentle aspiration (without vigorous pipetting) and used in all assays. Optimal fractionation of IDO^+ cells was obtained at intermediate (rather than high) levels of GMCSF, and good results were also obtained with a single initial feeding of GM-CSF without further supplementation.

The adherent cells from these cultures were $CD14^+$ and appeared to represent a transitional or macrophage-like fraction, as previously described (7). They did not show significant IDO-mediated suppression and were not used in this study. In our system, the distinction between adherent and nonadherent fractions did not represent a unique, fixed lineage commitment, because the relative proportions of the two populations could be influenced by the culture conditions (and, for example, in bovine serum-based cultures, both IDO^+ and the IDO^{neg} cells were nonadherent (7)). However, the conditions used in this study generated a reliably enriched population of IDO-expressing DCs, which could be readily recovered in the nonadherent fraction.

For isolated T cell subsets, elutriator-purified lymphocytes were incubated with either anti-CD4 (clone T-310; DAKO, Carpinteria, CA) or anti-CD8 Ab (clone DK25, DAKO) for 30 min on ice, washed, and isolated by immunomagnetic bead sorting (Miltenyi Biotec, Auburn CA). The purity of each fraction was 90–95%. Normally, to avoid artifacts introduced by staining and sorting, elutriator-purified lymphocytes were used without fractionation, unless CD4 and CD8 subsets were specifically required. However, direct head-to-head comparison of unfractionated lymphocytes vs sorted/remixed (CD4 and CD8) T cells from the same apheresis showed similar patterns of response (as shown in *Results*), so the sorting process did not appear to adversely affect the T cells.

V-bottom MLR system

For MLRs, nonadherent DCs were mixed with 5×10^5 allogeneic lymphocytes (unfractionated) or with the number of sorted CD4 or CD8 cells that would have been present in the original 5×10^5 cells. Unless otherwise specified, an APC:T cell ratio of 1:20 is shown in all figures. (In the figures, data are shown at a single APC:T cell ratio for simplicity, but comparable results were obtained at various APC:T cell ratios.)

MLRs were performed for 5 days in 250 μl of complete medium (10% FCS in RPMI 1640). Alternatively, where indicated, MLRs were performed in a chemically defined medium comprising tryptophan-free RPMI 1640 supplemented with BSA, insulin, transferrin, and cholesterol, as previously described (6). At the beginning of MLRs, replicate groups of five wells received no additive, 200 μM 1-methyl-D-tryptophan (1MT; Sigma-Aldrich), or 250 μM L-tryptophan (Sigma-Aldrich). (To prepare 1MT, a 100× stock solution (20 mM) was dissolved in 0.1 N NaOH, then adjusted to pH 7.4.) After 5 days of MLR, proliferation was measured by 4-h thymidine incorporation assay. In all MLRs, V-bottom culture wells (Nalge-Nunc, Rochester, NY) were used, as previously described (7).

For mitogen-activated T cell proliferation, purified lymphocytes were activated with immobilized anti-CD3 Ab plus soluble anti-CD28 (clone CD28.2, as used above), as previously described (6).

Blocking Abs

Neutralizing mAb against human IFN-γ (clone B-B1) was purchased from BioSource International (Camarillo, CA) and used at a supersaturating concentration of 100 μg/ml. Blocking anti-CTLA4 mAb (clone BNI3; BD PharMingen) was used at a concentration of 1 μg/ml. This clone has been used to block CTLA4 function on human T cells in vitro (25). Results were confirmed with a second clone against a different epitope (clone 8H5; Ancell, Bayport, MN). Anti-CD28 mAb (clone CD28.2; BD PharMingen; 1 μg/ml) was selected because it both blocks the CD28-B7 interaction (as shown below) and provides costimulation to T cells via CD28 cross-linking. Most intact dimeric anti-CD28 Abs provide costimulation (26), and this was empirically confirmed for this clone using purified T cells activated with immobilized anti-CD3 Ab (6). Results with this mAb were confirmed with a second clone (5D10; Ancell). Abs were added at the start of MLRs. The strategy of blocking CTLA4/CD28 (rather than blocking B7-1/B7-2) was chosen because Abs against B7-1/B7-2 might cross-link their targets, thus inadvertently generating the signal we wanted to block (27). In preliminary validation experiments, none of the blocking Abs used had any effect when T cell proliferation was stimulated by fresh monocytes plus anti-CD3 mAb (a system in which there is no IDO-mediated suppression of T cells).

To confirm that the anti-CD28 and anti-CTLA4 Abs were blocking Abs, we measured their ability to inhibit binding of soluble recombinant CD28-Ig Fc fusion protein (CD28-Ig) and CTLA4-Ig fusion proteins to DCs. DCs were incubated for 30 min on ice with either biotinylated CD28-Ig (Ancell) or CTLA4-Ig (Chimerigen, Allston, MA), both at 10 μg/ml. All incubations and washes contained 0.02% sodium azide to reduce capping. Duplicate tubes received either the blocking Ab being tested or an equivalent amount of isotype-matched mouse IgG. Cells were washed and fixed, and the bound ligand was quantitated by FACS using PE-conjugated anti-human IgG detection reagent (for CTLA4-Ig) or streptavidin-PE (for CD28-Ig).

Anti-CD80 mAb (clone L307.4) and anti-CD86 mAb (clone IT2.2; both from BD PharMingen; used at 1 μg/ml) have been shown to generate intracellular signals in B cells when cross-linked (28, 29). In preliminary validation experiments we found that isolated DCs did not require any additional cross-linking of these Abs to show a biologic effect (because DCs themselves are potent cross-linkers of Abs, as demonstrated by their ability to cross-link anti-CD3 Abs and activate T cells). However, in MLRs, where DCs were present at much lower densities, we found that a cross-linking reagent was required for the full effect. This cross-linking could be provided either by $F(ab')_2$ of goat anti-mouse Ab (1 μg/ml; Jackson ImmunoResearch, West Grove, PA) or by biotin-avidin cross-linking (60 ng/ml streptavidin with biotinylated primary Ab); both strategies produced similar results. The CD80/CD86 Abs were optimally effective when added on day 2 of MLR, just before the onset of T cell proliferation.

HPLC measurement of kynurenine

Culture supernatants (150 μl) were extracted with 1.3 ml of methanol, the precipitate was removed by centrifugation, and the supernatant was dried under vacuum. Samples were resuspended in an initial mobile phase (deionized water), injected onto a C_{18} column (Luna C-18; 250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA), and eluted with a linear gradient of acetonitrile in water (0–80% over 20 min). Absorbance was measured at 225 nm and compared against standard curves for tryptophan, kynurenine, and 1MT. The limit of detection for this assay was 1.2 μM.

Flow cytometry

Phenotyping Abs and reagents were obtained from BD PharMingen unless specified. Nonadherent DCs and adherent APCs were stained with anti-CD123-biotin (clone 7G3), followed by streptavidin-PerCP, and anti-CD11c-allophycocyanin (clone S-HCL-3). After fixation and permeabilization (Cytofix/Cytoperm), cells were stained with affinity-purified rabbit Ab against human IDO (generated as previously described (7)), followed by R-PE-labeled anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories; cross-adsorbed against mouse, human, and bovine IgG). For all experiments, the negative control for IDO staining was the anti-IDO Ab preadsorbed with a 50-fold molar excess of the immunizing peptide (DLIESGQLRERVEKLNMLC). Dendritic cells were gated on forward and side scatter. For FACS on MLRs, the cocultures were harvested after 48 h, treated for 5 min with EDTA to disrupt clusters, then stained as described. For analysis of T cells, B cells were excluded from the lymphocyte gate by CD20 labeling.

Immunohistochemistry

Cytocentrifuge preparations were fixed for 10 min in 10% formalin, then primary Abs were applied: either rabbit anti-human IDO (0.05 μg/ml) or anti-CTLA4 (clone BNI3; 1 μg/ml). IDO was detected with rabbit-specific secondary Ab conjugated to Alexa-488 (Molecular Probes, Eugene, OR) cross-adsorbed against mouse IgG; CTLA4 was detected with anti-mouse Ab conjugated to Alexa-568, cross-adsorbed against rabbit IgG (Molecular Probes). Cells were harvested from MLRs with minimal manipulation to preserve T cell-DC conjugates for confocal analysis (30).

Statistical analysis

Multiple treatment groups were compared within individual experiments by ANOVA. All experiments were repeated at least three times unless more replicates are specified.

Results

Optimization of conditions to detect IDO-mediated inhibition of T cell proliferation

The culture system for monocyte-derived DCs was optimized specifically to generate suppressive DCs and differed somewhat from other systems. Relevant variables included the use of a highly purified starting population of monocytes, the use of X-vivo15 medium without serum supplementation, maturation with a TNF-based cytokine mixture, and harvesting only the nonadherent cell fraction, all as described in *Materials and Methods*. Fig. 1*a* shows that the nonadherent cells from these cultures were preferentially enriched for DCs expressing IDO compared with the adherent cell fraction from the same cultures. The nonadherent cells (hereafter IDO$^+$ DCs) had the phenotype previously described (7), including coexpression of CD11c and CCR6 and low, but consistent, levels of CD123 (IL3-Rα; Fig. 1*a*). These markers were present before maturation and were unchanged after maturation. After maturation, the IDO$^+$ DCs were >80% CD83$^+$ and expressed high levels of HLA-DR, CD80, and CD86, consistent with a mature phenotype (7).

Mature IDO$^+$ were used as stimulators in allogeneic MLRs. The sensitivity of the MLR assays for detecting IDO-mediated suppression could be significantly enhanced by using a V-bottom well geometry, rather than round or flat-bottom wells (Fig. 1*b*). V-bottom wells enforced close, sustained contact between APCs and T cells, and we have previously shown that IDO-mediated suppression requires cell-cell contact (6). The degree of IDO-mediated suppression (shown by the arrows in Fig. 1*b*) was quantitated by comparing each MLR with duplicate assays performed in the presence of the IDO inhibitor 1MT (31). The V-bottom well geometry did not affect the overall depletion of tryptophan in the cultures (a proxy for IDO activity), nor did it have any intrinsic effect on T cell proliferation (as shown by the fact that proliferation was identical in flat and V-bottom wells when IDO was blocked by 1MT). However, the V-bottom system allowed much more sensitive detection of IDO-mediated suppression. (In this regard, round-bottom wells were intermediate, being superior to flat-bottom wells but less effective than V-bottom wells.)

Using the preceding culture system and MLR assay, we compared the activities of immature vs mature IDO$^+$ DCs (Fig. 1*c*). In the absence of 1MT, neither immature nor mature DCs were good stimulators of T cell proliferation. However, when IDO activity was blocked with 1MT, the mature DCs were revealed as excellent stimulators of T cell responses (Fig. 1*c*) despite their potent suppression when IDO was active. Identical results were obtained when the tryptophan-depleting effect of IDO was bypassed by adding excess tryptophan to the medium. Neither 1MT nor 10× tryptophan had any effect on T cell proliferation when the T cells were activated using immobilized anti-CD3/CD28 mAbs (data not shown), confirming that these agents did not have any intrinsic, nonspecific stimulatory activity for T cells.

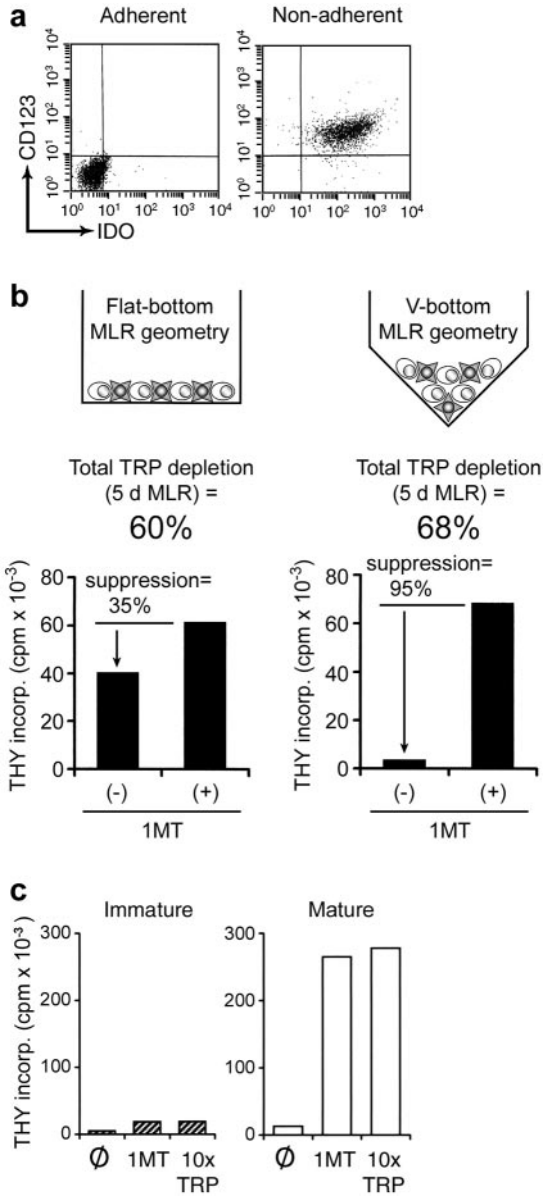

FIGURE 1. Culture and assay system for IDO-expressing DCs. Monocytes were cultured for 7 days in X-vivo15 serum-free medium supplemented with GM-CSF and IL-4, as described in *Materials and Methods*. *a*, Adherent and nonadherent cell populations from the same culture were harvested and stained for expression of CD123 vs IDO. *b*, Monocyte-derived DCs were matured (TNF-α, IL-β, IL-6, and PGE_2) during the final 48 h of culture, and enriched IDO$^+$ (nonadherent) DCs were harvested and used as stimulators in MLRs (APC:T cell ratio, 1:20; 5×10^5 T cells/well). Identical MLRs were performed in either flat- or V-bottom culture wells. Replicate groups received either no addition or 200 μM 1MT to inhibit IDO. The tryptophan concentration in the MLR supernatants was determined by HPLC at the end of the 5-day MLR and compared with that in cultures with medium alone. *c*, DCs, with or without maturation during the final 48 h, were used as stimulators in MLRs (APC:T cell ratio, 1:40). Replicate treatment groups received no addition (∅), 200 μM 1MT, or 250 μM L-tryptophan (10× TRP).

Ligation of B7-1/B7-2 prevents down-regulation of IDO in isolated DCs

Mature IDO$^+$ DCs activated in isolation (using recombinant IFN-γ, but without T cells) markedly down-regulated IDO (Fig.

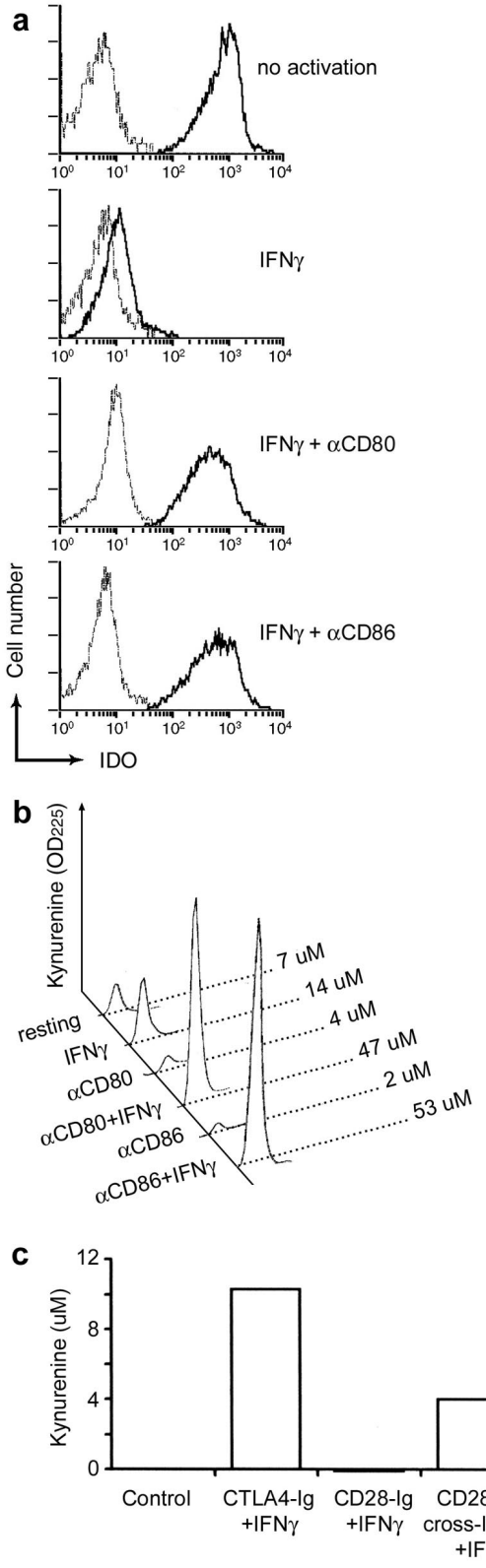

FIGURE 2. Effect of B7-1/B7-2 ligation on IFN-γ-induced down-regulation of IDO. *a*, Cytokine-matured IDO$^+$ DCs were activated in the absence of T cells using recombinant IFN-γ (100 U/ml, 24 h). IDO protein expression was measured by FACS. Compared with control cultures receiving buffer alone (no activation), the IFN-γ-treated cells showed marked down-regulation of IDO. This was prevented if the DCs were simultaneously treated with activating Abs against B7-1 (αCD80) or B7-2 (αCD86). *b*, Functional IDO enzymatic activity, measured as kynurenine production in culture supernatants, from IDO$^+$ DCs treated with Abs against CD80, CD86, or isotype-matched control Abs (resting). Each group is shown with and without IFN-γ. Traces show the kynurenine peak by HPLC analysis, with the calculated concentration of kynurenine (based on comparison against a standard curve) shown at the *right*. *c*, DCs were treated with IFN-γ (100 U/ml) plus CTLA4-Ig or CD28-Ig (both at 1 μg/ml) for 24 h. Control cultures received no additions. In the last group, the biotinylated CD28-Ig was cross-linked with streptavidin. Kynurenine was measured as described in *b*, and the basal production in resting DCs was subtracted from all groups.

2*a*). However, this down-regulation could be prevented if a second signal was supplied through ligation of B7 molecules (CD80 or CD86). Fig. 2*a* (*panels 3* and *4*) show that Ab-mediated cross-linking of CD80 or CD86 fully rescued the isolated DCs from IFN-γ-induced down-regulation of IDO. The FACS data were confirmed by analysis of IDO enzymatic activity (Fig. 2*b*), measured as accumulation of kynurenine in the culture supernatants. (Kynurenine is the first stable downstream metabolite produced by IDO. Although it is not a quantitative measure of enzyme activity, because its accumulation is influenced by the degree of conversion to subsequent downstream metabolites (32), it is a highly specific qualitative marker for IDO activity.) By itself, IFN-γ produced a small and transient increase in functional activity (the figure reflects the amount of kynurenine accumulating over the first 24 h), but after 24 h, the expression of IDO protein had been lost (see Fig. 2*a*), and IDO enzymatic activity returned to background levels. Likewise, CD80 or CD86 engagement alone produced a variable, but always minor, amount of IDO activity. However, IFN-γ and B7 engagement together acted synergistically, allowing IDO protein expression to be maintained (Fig. 2*a*) and achieving full enzymatic activity (Fig. 2*b*).

Both CTLA4 and CD28 are potential ligands for B7-1/B7-2. We compared the abilities of the respective recombinant fusion proteins, comprising the extracellular (B7-binding) domains of CTLA4 or CD28 linked to IgG Fc, to synergize with IFN-γ in the induction of IDO. Fig. 2*c* shows that CD28-Ig was markedly less effective on an equimolar basis than CTLA4-Ig at inducing IDO. Even when CD28-Ig was extensively multimerized by avidin-biotin cross-linking, it was still less effective than CTLA4-Ig at inducing IDO.

B7-1/B7-2 and their corresponding counterreceptors are expressed during MLR

The preceding experiments used only isolated DCs. We therefore asked which of the preceding molecules were actually present during MLRs. Fig. 3*a* shows that the DCs expressed both CD80 and CD86, and that the majority of activated T cells (identified by CD25 expression) expressed CD28 (Fig. 3*b*). Consistent with previous reports (33), we were unable to detect CTLA4 on the surface of most T cells by FACS analysis (data not shown), but we readily detected intense, localized accumulations of intracellular CTLA4 by fluorescence microscopy in a small subset of activated T cells (Fig. 3, *c* and *d*). Thus, B7-1, B7-2, CTLA4, and CD28 were all expressed in the MLR system.

Blocking CTLA4 and CD28 prevents induction of IDO during MLR

To test the hypothesis that engagement of B7 molecules triggered IDO expression during Ag presentation, we used blocking Abs against the extracellular domains of CTLA4 and CD28. These Abs inhibited binding of soluble CTLA4-Ig and CD28-Ig fusion proteins to IDO$^+$ DCs (Fig. 4*a*), confirming that the Abs blocked the physiologic interaction of their respective target molecules with

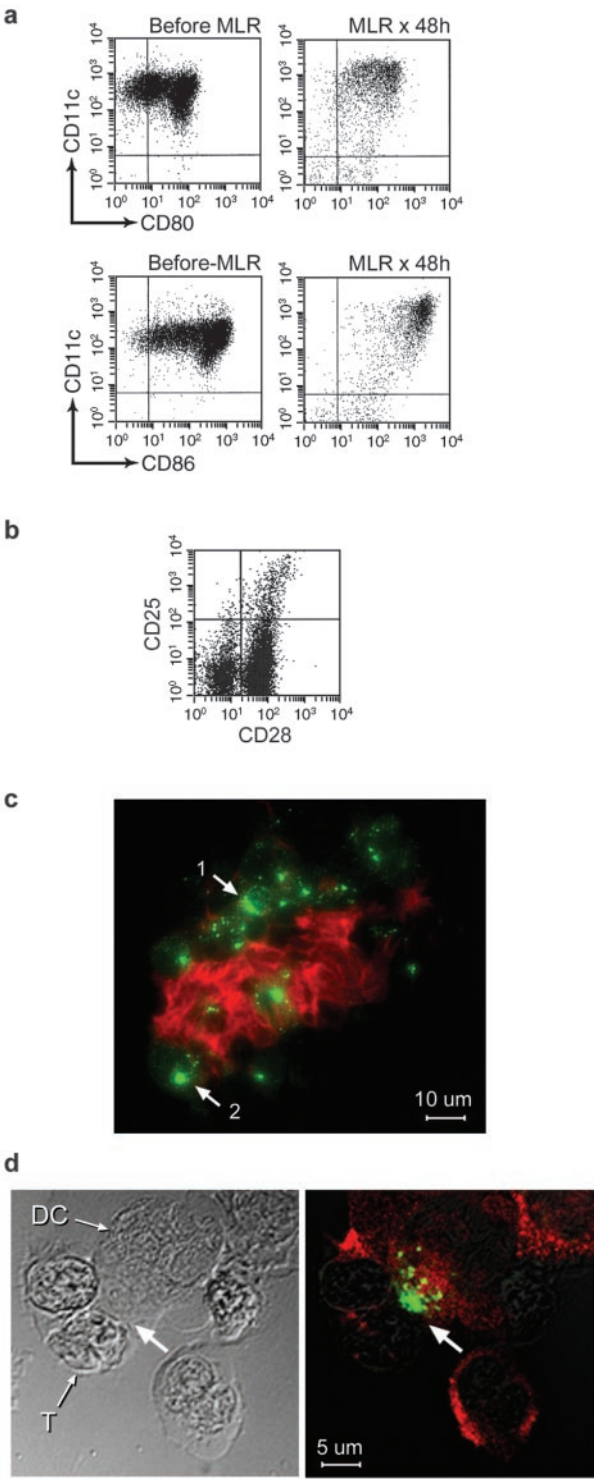

FIGURE 3. Expression of B7-1/B7-2 and CTLA4/CD28 in MLRs. Mature IDO$^+$ DCs were used as stimulators in allo-MLRs, then cocultures were harvested and stained for flow cytometry and immunofluorescence microscopy. *a*, Expression of CD80 and CD86 Ags on DCs (identified by light scatter gates): *left*, purified DCs before MLR; *right*, DCs from MLRs after 48 h. (Fewer cells are seen in the MLR dot plots because the T cells have been gated out.) *b*, Expression of CD28 on CD25$^+$ T cells taken from MLRs after 72 h. *c*, Cytocentrifuge preparation of MLR cocultures (72 h) showing IDO expression in DCs (red) and CTLA4 expression in T cells (green). Intracellular CTLA4 was observed in focal cytoplasmic collections (arrows). *d*, Analysis of a representative conjugate in which a zone of contact between a T cell and a DC was well preserved (white arrow). The *left panel* shows a Nomarski image of the field; the *right panel* shows a confocal image, with CTLA4 in green and IDO in red.

B7. To ensure that T cells were not deprived of costimulation, an activating clone of anti-CD28 Ab was used (6).

We found that neither Ab when used alone prevented IDO-mediated inhibition of T cells (Fig. 4*b*). However, when both Abs were added together (thus blocking all ligands for B7), the result was complete abrogation of IDO-mediated inhibition. Blocking CTLA4/CD28 restored T cell proliferation to the level seen when IDO-mediated inhibition was abrogated by 1MT or 10× tryptophan. Significantly, when CTLA4/CD28 were blocked, 1MT and 10× tryptophan lost all enhancing effect on T cell proliferation, thus confirming that the effect of blocking CTLA4/CD28 was specifically to abrogate the IDO-mediated component of suppression.

The preceding results were confirmed at the level of IDO enzymatic activity (kynurenine accumulation in the MLR supernatants), as shown in Fig. 4*c*. We found that blocking either CTLA4 or CD28 alone caused a significant decrease in overall kynurenine production (a finding not evident from the less sensitive T cell proliferation readout in Fig. 4*b*), thus indicating that the roles of CTLA4 and CD28 were not simply redundant. When the two Abs were used together, they abolished all detectable IDO activity, corresponding to the loss of suppressor activity seen in Fig. 4*b*. (Accumulation of kynurenine in the supernatant is a useful qualitative marker of IDO activity, but the kynurenine itself does not cause T cell inhibition, and the quantitative level of kynurenine does not predict the degree of T cell suppression.)

Finally, we asked whether IDO$^+$ DCs that failed to receive a signal via B7 during MLR would behave like the isolated DCs shown in Fig. 2*a*, i.e., would down-regulate IDO instead of up-regulating it. Fig. 4*d* shows that the DCs normally up-regulated IDO protein during MLR, typically by 2- to 3-fold (measured by FACS, gating selectively on the CD11c$^+$CD123$^+$ population to identify the DCs). In contrast, when CTLA4 and CD28 were blocked, IDO was significantly down-regulated during MLR, thus recapitulating the behavior of DCs activated in the absence of T cells.

cross-linking B7-1/B7-2 up-regulates IDO activity in MLR

We next asked whether artificially cross-linking B7-1/B7-2 would restore IDO activity in MLRs when CTLA4 and CD28 were blocked. For these experiments, anti-CD80/CD86 mAbs were used as described in Fig. 2. Fig. 5*a* shows that Abs against CD80 or CD86 added individually had little effect on IDO. However, simultaneously cross-linking both molecules fully restored inhibition, to a level comparable to that seen when CTLA4 and CD28 were active. This inhibition was reversed by 1MT, indicating that the inhibitory effect of B7-1/B7-2 ligation was due to induction of IDO. Cross-linking CD80/CD86 induced kynurenine production in the same pattern observed for T cell inhibition (Fig. 5*b*). In additional experiments (not shown), cross-linking B7-1/B7-2 also prevented down-regulation of IDO in MLRs when CTLA4 and CD28 were blocked (i.e., as in Fig. 4*d*). Thus, by three independent readouts (T cell suppression, kynurenine production, and maintaining IDO expression), ligation of CD80/CD86 restored functional IDO activity in MLRs.

In the experiments shown in Fig. 5, *a* and *b*, each treatment group received goat anti-mouse F(ab′)$_2$ to cross-link the anti-CD80/CD86 Abs. Fig. 5c demonstrates that this cross-linking step was important for full induction of IDO, consistent with a model in which clustering of B7 molecules was required to generate signaling (27, 34).

IFN-γ is required for full induction of IDO during MLR

In Fig. 2*b* we showed that both B7 engagement and IFN-γ were required to achieve full induction of IDO in isolated DCs. We

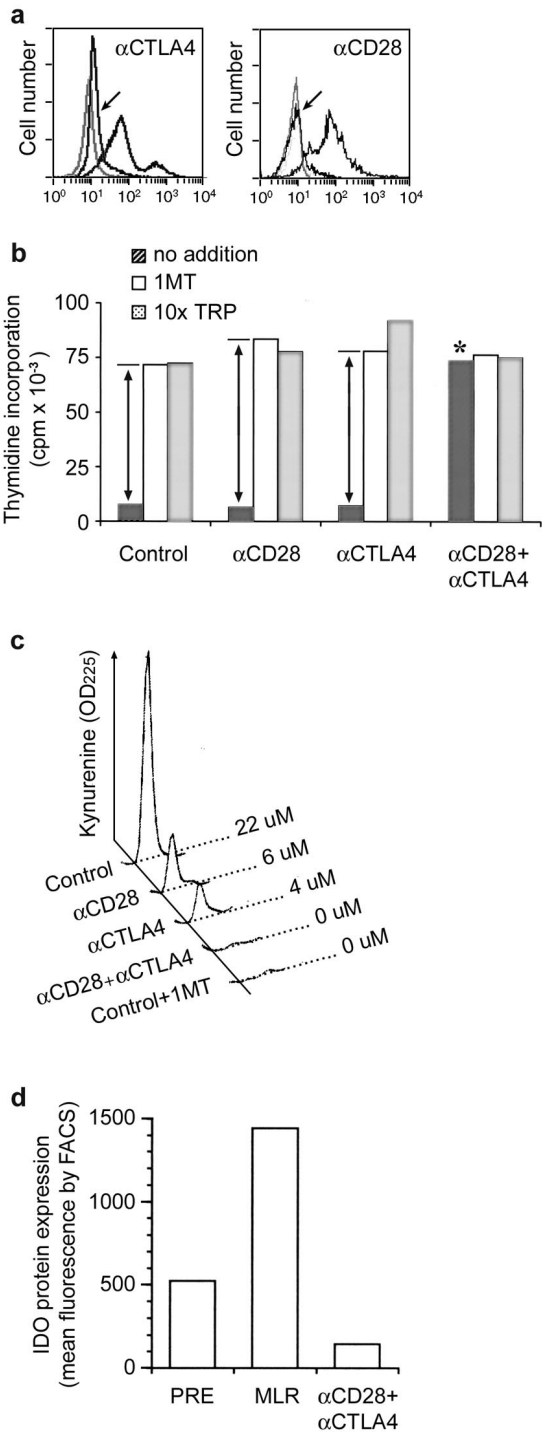

FIGURE 4. Blocking Abs against CTLA4 and CD28 prevent induction of IDO in MLRs. *a*, Validation of blocking Abs. Binding of soluble recombinant CTLA4-Ig (*left*) and CD28-Ig (*right*) to mature IDO$^+$ DCs was measured by flow cytometry (see *Materials and Methods*). The addition of anti-CTLA4 or anti-CD28 blocking Ab (arrows) inhibited binding of the respective fusion protein to near the background level (secondary fluorescent detection reagent alone, shown in gray). *b*, Blocking Abs prevent IDO-mediated suppression in MLRs. Mature IDO$^+$ DCs were used as stimulators in MLRs. Replicate groups received either no addition (control) or blocking Abs against CD28, CTLA4, or both. (Additional controls receiving the various isotype-matched irrelevant Abs showed proliferation identical with the control shown.) IDO-mediated inhibition (shown as arrows) was defined based on MLRs with and without 1MT. (Results with 1MT were confirmed by parallel groups receiving 10× TRP, as shown, which gave identical results.) ∗, Complete abrogation of IDO-mediated suppression by αCTLA4 and αCD28 Abs (p = NS compared with all 1MT groups by ANOVA, but $p < 0.01$ vs each of the other no addition groups). This was a reproducible result in >20 experiments with different donor-responder pairs. Experiments were confirmed with different sets of blocking Abs (see *Materials and Methods*), with identical results. *c*, Kynurenine concentration in supernatants at the end of 5-day MLRs, taken from an experiment similar to that in the previous panel. *d*, Expression of IDO protein in DCs by FACS analysis, shown before their additional to MLRs (PRE), after 48 h of coincubation with allogeneic T cells (MLR), or after 48 h of MLR in the presence of blocking Abs against CTLA4 and CD28, as in *b*. Bars show the mean fluorescence of IDO staining in the DC population (gated on the CD123$^+$ CD11c$^+$ cells).

therefore asked whether endogenously produced IFN-γ was required for induction of IDO during MLR. Fig. 6*a* shows that neutralizing Abs against IFN-γ significantly reduced the expression of IDO enzymatic activity during MLR. As measured by kynurenine production, the effect of neutralizing endogenous IFN-γ was almost as great as that of blocking CTLA4/CD28. The effect of IFN-γ neutralization on T cell suppression was not as complete (Fig. 6*b*), but was still significant. (As shown in Fig. 4*c*, it is easier to reduce overall kynurenine production than it is to abrogate suppression, suggesting that maximal levels of IDO are not necessary to accomplish suppression.)

Only CD4$^+$ T cells can trigger immunosuppressive IDO activity

Many helper/regulatory activities are mediated preferentially by the CD4$^+$ subset of T cells. We asked whether the ability to induce IDO via B7 ligation was associated only with CD4$^+$ T cells or was found in both CD4$^+$ and CD8$^+$ subsets. T cells were fractionated based on CD4 or CD8 expression, as described in *Materials and Methods*, and each fraction (plus the original unfractionated preparation) was used as responder cells in MLRs (Fig. 7*a*). All MLRs were stimulated by the same DCs, so any differences in IDO induction were attributable solely to the responder T cells. Fig. 7*a* shows that isolated CD4$^+$ T cells triggered potent IDO-mediated inhibition (>90% at all time points), which was comparable to the total lymphocyte population without fractionation. In contrast, isolated CD8$^+$ showed excellent proliferation with virtually no 1MT-reversible inhibition. Not only was this pattern different from the isolated CD4$^+$ cells, but it was also markedly different from the behavior of the same CD8$^+$ cells in the unfractionated mixture (where they were fully suppressed). Thus, isolated CD8$^+$ T cells either became refractory to IDO or failed to induce IDO.

Fig. 7*b* shows that isolated CD8$^+$ T cells failed to trigger functional IDO enzymatic activity in MLRs, as assessed by kynurenine accumulation in the medium. In contrast, the CD4$^+$ T cells triggered IDO activity comparable to unfractionated cells. Thus, the lack of IDO-mediated inhibition in MLRs using isolated CD8$^+$ cells appeared entirely due to the failure of these cells to trigger IDO expression. The isolated CD8$^+$ cells remained sensitive to IDO-mediated suppression, as shown by the fact that recombining the sorted CD8$^+$ cells with sorted CD4$^+$ cells fully restored IDO-mediated inhibition (Fig. 7*c*). Thus, the IDO-inducing activity segregated strictly with the CD4$^+$ subset, but both CD4$^+$ and CD8$^+$ subsets were susceptible to inhibition once IDO was triggered by the CD4$^+$ cells.

Tryptophan depletion is required for inhibition of T cell proliferation by IDO

Mechanistically, IDO depletes tryptophan, but it also generates kynurenine and other downstream metabolites. At high concentrations, some of these metabolites can affect T cell proliferation and viability (35–37). To determine whether the effect of IDO in our

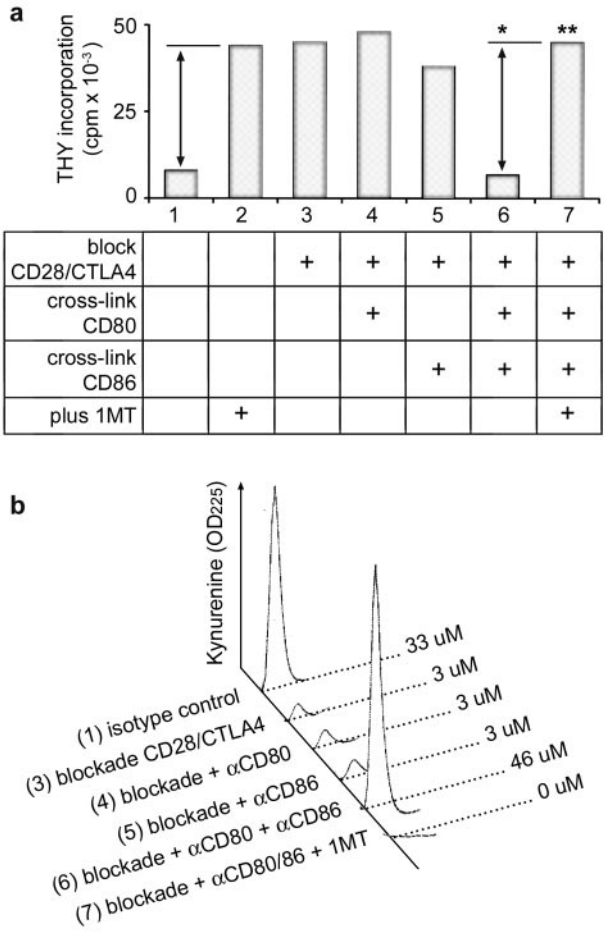

FIGURE 5. Cross-linking B7-1/B7-2 induces IDO in allo-MLR. *a*, Mature IDO$^+$ DCs were used as stimulators in allo-MLRs (*bar 1*). IDO-mediated inhibition (arrow) was defined relative to replicate MLRs treated with 1MT (*bar 2*). The addition of blocking Abs against CTLA4 and CD28, as described in Fig. 4*b*, abrogated IDO-mediated inhibition (*bar 3*). The further addition to this system of cross-linking Abs against CD80 or CD86 showed little effect when the Abs were added individually (*bars 4* and *5*), but the combination of both Abs triggered full IDO-mediated suppression (*bar 6*). Suppression was reversed with 1MT (*bar 7*), confirming that it was due to IDO. *, $p < 0.05$, groups 6 vs 3; **, p = NS, groups 7 vs 3 (by ANOVA). All groups received F(ab′)$_2$ goat anti-mouse IgG secondary Ab to cross-link the primary Abs. *b*, HPLC analysis of kynurenine production in MLR supernatants from an experiment similar to that in the previous panel. The group numbers in parentheses correspond to the groups in *a*. *c*, Comparison of anti-B7 Ab treatment with and without cross-linking. MLRs were conducted as in preceding experiments, except that replicate groups received either no cross-linking Ab (□), or F(ab′)$_2$ goat anti-mouse IgG (GαM; ■). Groups 1 and 2 are controls showing IDO-mediated inhibition of T cell proliferation and its reversal by 1MT. Group 3 shows abrogation of inhibition by blockade of CTLA4 and CD28. Group 4 shows the effect of anti-CD80 and -CD86 mAbs; a modest IDO-inducing effect was seen in the absence of exogenous cross-linking (dotted arrow), but cross-linking was required to induce full inhibition of T cell proliferation (solid arrow).

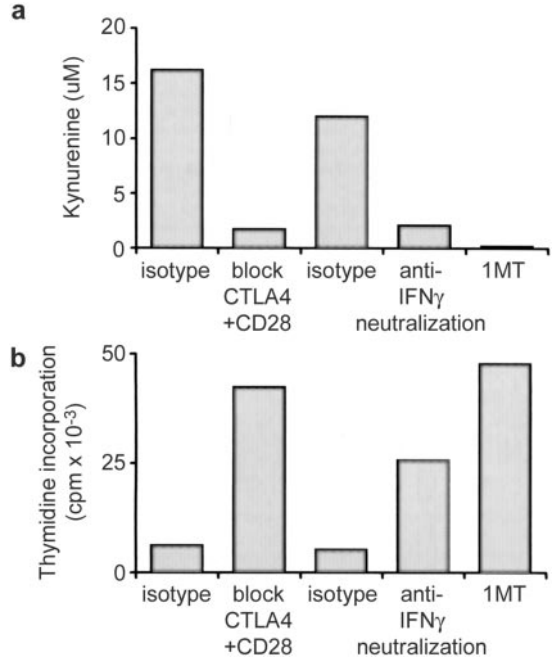

FIGURE 6. Production of IFN-γ during MLR is required for full induction of IDO. MLRs were performed as described in Fig. 5. Neutralizing Ab against human IFN-γ was added at the beginning of the MLR. *a*, IDO enzymatic activity (kynurenine production in the MLR supernatants) was significantly reduced when IFN-γ was neutralized, to a degree comparable to that seen with CTLA4 and CD28 blockade (performed as in Fig. 4*b*). Control MLRs received 1MT and showed no kynurenine production. *b*, The reduction in IDO activity seen when IFN-γ was neutralized was accompanied by a partial reversal of the DCs' ability to inhibit T cell proliferation.

system was due to depletion of tryptophan or production of toxic metabolites, we added increasing amounts of tryptophan to MLRs, then simultaneously measured the concentration of kynurenine (the first major metabolite) in the medium, and the degree of T cell suppression. As shown in Fig. 8*a*, adding more tryptophan progressively increased the amount of kynurenine produced, but it also progressively enhanced T cell proliferation (i.e., reversed the IDO-mediated suppression). At the highest concentrations of tryptophan, T cell proliferation reached the same level seen when IDO activity was blocked by 1MT. Thus, suppression was lowest in the groups in which metabolite production was highest, and highest where metabolites were lowest, arguing strongly against a causative role for toxic metabolites in our system. (These findings were consistent with our previous report showing that excess tryptophan overcomes suppression by IDO-expressing macrophages (6) and also with reports by others using a variety of cell types (13, 38, 39).)

We next asked whether adding exogenous metabolites (using kynurenine as a source of the various other downstream metabolites (40)) would restore suppression to MLRs in which the enzymatic activity of IDO was blocked. As shown in Fig. 8B, adding exogenous kynurenine did not restore suppression when IDO was blocked by 1MT.

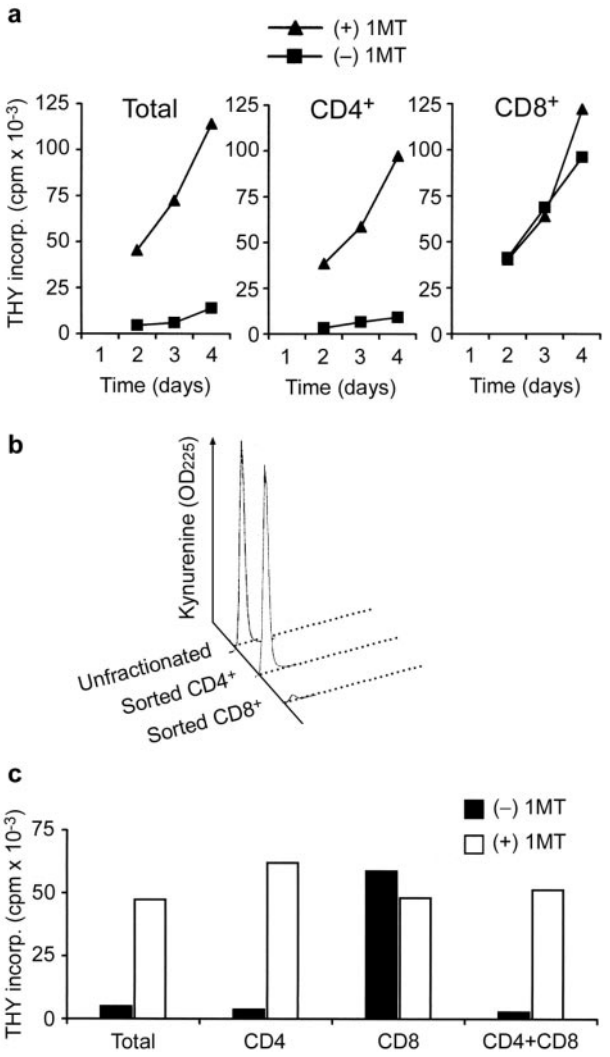

FIGURE 7. IDO activity is triggered by $CD4^+$ T cells, but not by $CD8^+$ T cells. *a*, Mature IDO^+ DCs were used as stimulators for sorted $CD4^+$ T cells, sorted $CD8^+$ T cells, or the original, unfractionated lymphocytes (Total). Thymidine incorporation was measured on days 2, 3, and 4 of MLR. Each assay was performed with and without 1MT, as shown, and all MLRs received identical DCs. (The small effect of 1MT in the $CD8^+$ group on day 4 was often absent, as shown in *c*.) *b*, Culture supernatants were harvested from MLR cultures on day 2 and analyzed by HPLC for kynurenine accumulation. The kynurenine peak is shown for each of the groups in *a*, without 1MT. (Kynurenine was measured at 48 h to minimize any effects of the proliferating T cells; similar results were also seen at later time points.) *c*, Mixing experiments (day 4 MLRs) showing that the readdition of sorted $CD4^+$ T cells to the $CD8^+$ fraction fully restored IDO-mediated suppression.

Finally, we asked whether IDO-mediated inhibition could occur when the concentration of tryptophan metabolites was negligible. MLRs were performed in chemically defined, serum-free medium (6) containing reduced levels of free tryptophan (10, 1 or 0 μM, as compared with the normal concentration in culture medium of 25–50 μM). As downstream metabolites are derived from tryptophan with a 1:1 stoichiometry, this imposed a de facto upper limit on the maximum concentration of metabolites that could be generated in the system. Fig. 8C shows that IDO+ DCs continued to show full IDO-mediated inhibition even at 1 μM tryptophan, demonstrating that suppression could occur even at low levels of metabolites. In these experiments, as the concentration of tryptophan in the medium was reduced, the ability of the T cells to proliferate when they were not suppressed (i.e., in groups receiving 1MT) progressively fell, consistent with our previous reports that T cells require tryptophan for proliferation (6, 41).

Taken together, the experiments in Fig. 8 indicate that the production of downstream metabolites was neither necessary nor sufficient for IDO-mediated inhibition in our system, but that the tryptophan-depleting effect of IDO was required.

Discussion

In the current study we demonstrate that engagement of B7-1/B7-2 molecules on human IDO^+ DCs delivers a signal that is obligately required for the triggering of IDO activity during Ag presentation. In the absence of this signal, the DCs failed to up-regulate functional IDO activity, and lost the ability to inhibit T cells. These findings emphasize that even in those DCs that have established an IDO^+ phenotype during differentiation, the decision to express the functional enzymatic activity remains subject to regulation at the time of Ag presentation.

We also provide mechanistic evidence that an IDO-expressing APC can serve as a regulatory bridge connecting two independent T cell populations. In our system the $CD4^+$ subset accounted for all the observed ability of activated T cells to induce IDO in DCs (Fig. 7). Pure $CD8^+$ T cells (with no $CD4^+$ cells present) did not induce IDO during MLRs and showed no evidence of IDO-mediated suppression or enhancement by 1MT. Thus, all the B7-dependent, IDO-inducing effects of activated T cells shown in Figs. 4 and 5 appeared solely mediated by the $CD4^+$ subset. However, although the $CD8^+$ T cells could not trigger IDO, both $CD8^+$ and $CD4^+$ T cells could be suppressed by IDO once it was triggered by $CD4^+$ cells. Thus, even though $CD8^+$ cells proliferated strongly in response to IDO^+ DCs when tested in isolation, the same $CD8^+$ cells were suppressed in the presence of $CD4^+$ T cells (Fig. 7*c*). We propose that IDO thus serves as an intermediate effector mechanism in this system, allowing the $CD4^+$ T cells to dominantly regulate the responses of $CD8^+$ T cells via the intermediary suppressive DCs (Fig. 9). Conceptually, this is analogous to the model in which $CD4^+$ Th cells can condition or "license" a DC so that it becomes competent to activate $CD8^+$ T cells (42, 43). In our system, however, the license is to suppress rather than to activate.

The possibility that inhibitory APCs might form a bridge between Treg and responder T cells has been proposed as a mechanism contributing to the phenomena of linked suppression and dominant tolerance (44). Consistent with this possibility, there is experimental evidence that anergic T cells (45, 46), $CD25^+CD4^+$ Treg (47), and $CD8^+$ Treg (48, 49) can all negatively affect their APCs. However, it has been unclear how this might serve to inhibit other T cells; the assumption has usually been that the APCs somehow become incompetent (e.g., via loss of costimulatory ability). We now show that linked suppression via a conditioned APC can also be an active, dominant process, mediated via the molecular mechanism of IDO.

In our system it appeared that either of the B7 counter-receptors, CTLA4 and CD28, were able to deliver a sufficient signal to trigger IDO (based on the fact that both receptors had to be blocked before inhibition was lost). However, bulk MLRs are not an ideal system in which to discriminate the biologically relevant differences between CTLA4 and CD28, because of the potential for cross-talk and bystander effects. Recombinant CD28-Ig fusion protein was significantly less efficient than CTLA4-Ig at triggering IDO (cf., Fig. 2*c*), which is consistent with the higher affinity of CTLA4 for B7 molecules (50). Functionally, CTLA4 is also known to play an important immunosuppressive and tolerogenic role in the immune system (51, 52), and it has been reported to be obligatory for induction of IDO in DCs by murine Tregs (19).

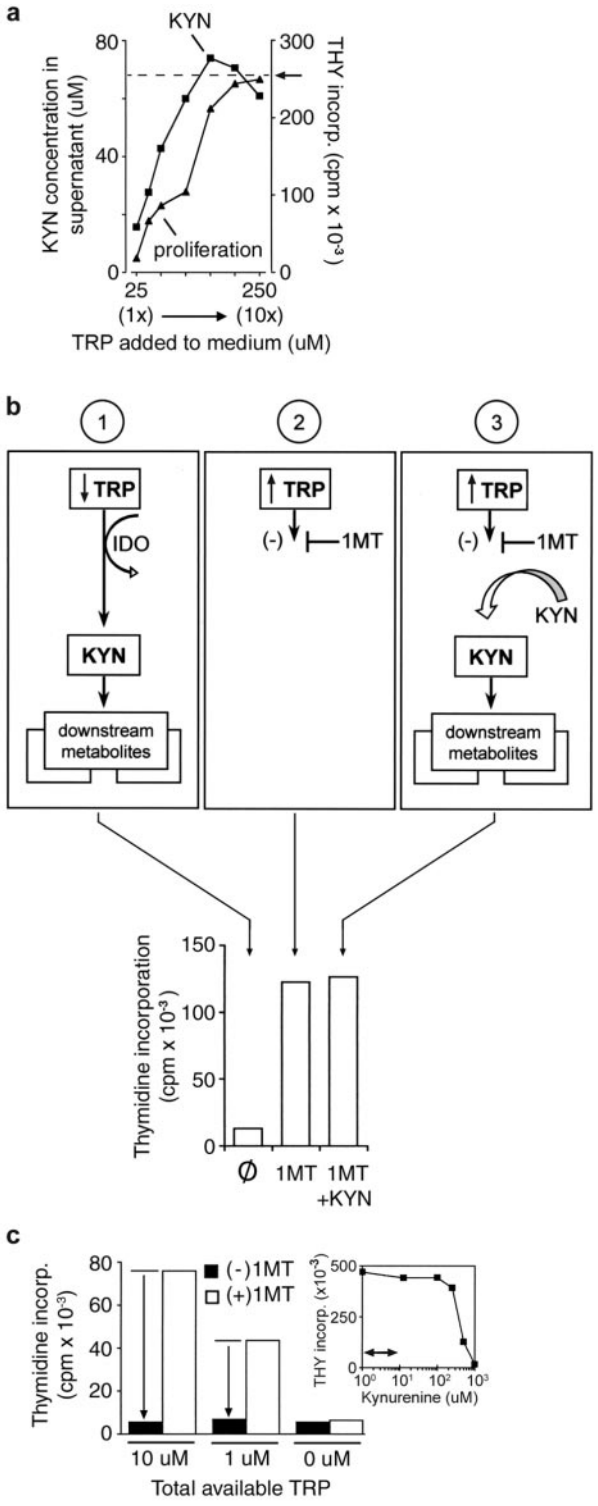

FIGURE 8. Inhibition of T cells requires depletion of tryptophan, but is not due to production of downstream metabolites. *a*, MLRs were performed using mature IDO$^+$ DCs as stimulators. Replicate treatment groups received graded doses of tryptophan (25–250 μM, corresponding to 1–10 times the normal medium concentration). Kynurenine production (KYN) was measured in the supernatant at the end of the 5-day MLR (squares, *left axis*). T cell proliferation was measured by thymidine incorporation (triangles, *right axis*). The arrow indicates the level of T cell proliferation seen in control MLRs in which IDO was blocked with 200 μM 1MT. *b*, Exogenous kynurenine does not restore inhibition in MLRs treated with 1MT. MLRs were performed as in the previous panel, with no additions (*diagram 1*), with 200 μM 1MT (*diagram 2*), or with 1MT plus 100 μM kynurenine (to bypass IDO and provide a source of downstream metabolites; *diagram 3*). Kynurenine was added at a concentration of 100 μM (twice the maximum that could occur if all the tryptophan in the medium were simultaneously converted to kynurenine). *c*, IDO$^+$ DCs remain inhibitory under conditions of low TRP. MLRs were performed in chemically defined medium (see *Materials and Methods*) containing the amounts of TRP shown. *Inset*, Dose-response curve for the inhibition of mitogen-activated T cell proliferation by kynurenine. Kynurenine was added at the concentration shown to purified T cells activated with immobilized anti-CD3/CD28, as previously described (6). The arrow indicates the maximum range of kynurenine concentrations that could occur in the MLRs shown in the bar graphs (1–10 μM), which are well below the inhibitory range.

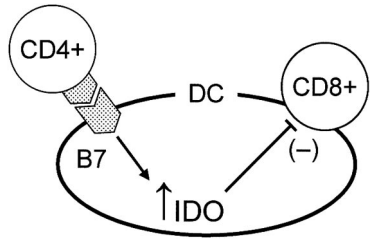

FIGURE 9. Proposed model in which CD4$^+$ T cells dominantly suppress CD8$^+$ T cell responses by activating IDO in an intermediary DC. In this model, CD4$^+$ T cells (or a subset thereof) recognize Ag on an IDO-competent DC, triggering functional IDO activity via ligation of B7 molecules. The IDO activity then suppresses CD8$^+$ T cells that recognize other Ags presented by the same DC, even though the CD8$^+$ cells themselves do not trigger IDO. In the figure, the DC is as depicted presenting Ag to both cells concurrently, but the interaction with the CD4$^+$ cell might precede that with the CD8$^+$ cell, as suggested previously (42).

Therefore, we hypothesize that CTLA4, rather than CD28, is likely to be the biologically relevant trigger for IDO in vivo.

We do not yet know which subset within the CD4$^+$ T cell population is responsible for triggering IDO in human DCs. It is tempting to speculate that it might be the CD25$^+$CD4$^+$ Tregs, as has been recently suggested in mice (19), particularly as certain Tregs constitutively express high levels of CTLA4 (53, 54). However, Treg subsets in humans are less clearly defined than those in mice, and even the CD25-negative CD4$^+$ T cells have been shown to acquire regulatory attributes after activation in vitro (55). In our hands, preliminary fractionation experiments have not revealed a straightforward segregation of IDO-inducing activity with the CD25$^+$CD4$^+$ subset in humans (unpublished observations), although improved fractionation may well reveal a specific subset of IDO-inducing cells within the larger CD4$^+$ population. Even if there prove to be species-related differences in Treg subsets, the key point is that a population of CD4$^+$ T cells is able to regulate other T cells, including all of the CD8$^+$ cells, via induction of IDO in APCs.

The specific type of APC serving as the bridge in this system is likely to be a critically important biologic variable. In our in vitro culture system, we chose conditions that biased the monocyte-derived DCs toward high expression of IDO, so that suppression would predominate. Under different conditions, however, different DCs might be generated that would not express immunosuppressive IDO regardless of B7 ligation by T cells. Thus, the properties of the responding APC (i.e., whether B7 ligation leads to IDO activation) is a critical variable. In an in vitro system such as ours, the distinction between types of DCs is rather artificial, as these attributes are dictated by the culture conditions, and cultured DCs do not recapitulate the complex subsets of human DCs that exist in vivo (56). Nevertheless, we hypothesize that the ability to induce high levels of IDO, or not, in response to B7 ligation constitutes a

real and biologically important distinction between different types of DCs in vivo. We have recently shown that there exist marked differences between different subsets of mouse DCs in their ability to up-regulate IDO when challenged by CTLA4-Ig in vivo (20). Whether this represents specific, committed, IDO-competent subsets of DCs, a stage of maturation at which IDO is expressed, or both remains to be determined. However, several reports suggest that IDO-expressing APCs possess potent immunosuppressive and tolerogenic properties in murine models (18–20, 57). Our in vitro system allows us to model at least one human counterpart of these B7-inducible, IDO-expressing DCs.

Our system for deriving such cells differs from other DC culture systems in that it has been optimized specifically to study suppression. One relevant modification includes the use of X-vivo15 medium without serum supplementation, which we and others (58) have observed favors adherence of a significant proportion of cells. This results in an enrichment for IDO-expressing cells in the nonadherent fraction (e.g., compared with monocytes cultured in bovine serum-based medium, where we find that the proportion of IDO^+ cells is small, and almost all cells are nonadherent regardless of IDO expression (7)). Although there is still a 100-fold range of IDO expression by FACS in our IDO-positive cells, the relative enrichment for IDO allows suppression to predominate over activation in MLRs. This is important, because we have previously shown that when MLRs are stimulated by a mixed population of IDO^+ and IDO^{neg} DCs, it can be impossible to detect the IDO-mediated suppression against the background of predominating T cell activation unless the IDO^+ cells are suitably enriched (7).

Even when an enriched population of IDO^+ cells is available, the ability to measure T cell suppression also requires an assay system sensitive to the property of interest, as demonstrated by the importance of the sustained cell-cell contact obtained in V-bottom wells (cf., Fig. 1*b*). We see an identical impact of well geometry in MLR assays using mouse cells, where we know that the DCs are potently immunosuppressive in vivo (20). Thus, the optimized MLR assay allows us to measure a real and biologically important property; without a suitable assay, this property will be missed. This is potentially important, because increasing evidence suggests that immunoregulatory APCs can be biologically significant in vivo even when they comprise only a minority of cells. Adoptive transfer experiments in mice suggest that even a limited number of tolerogenic DCs can promote systemic tolerance (59). In mixing experiments, a small population of tolerogenic DCs has been reported to create systemic unresponsiveness despite coadministration of a majority of nonsuppressive DCs (57). And we have recently reported that activation of IDO in even a restricted subset of DCs is sufficient to systemically suppress Ag-driven T cell responses in vivo (20). These observations underscore the importance of having a system in which to study IDO-mediated immunoregulation in humans.

Taken together, our data suggest that IDO-mediated suppression is tightly regulated. It is contingent on the correct subset of T cells (presumably a regulatory population of $CD4^+$ cells) sending the correct B7-mediated triggering signal to the correct (IDO-competent) subset of DCs. Only then does IDO-mediated suppression come into play. Given the potent biologic effects of a dominant immunosuppressive mechanism such as IDO, this multilayered regulation may be both appropriate and necessary.

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