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CUTTING EDGE

Cutting Edge: Induced Indoleamine 2,3 Dioxygenase Expression in Dendritic Cell Subsets Suppresses T Cell Clonal Expansion[1]

Andrew L. Mellor,[2] Babak Baban,* Phillip Chandler,* Brendan Marshall,* Kanchan Jhaver,[‡] Anna Hansen,* Pandelakis A. Koni,* Makio Iwashima,* and David H. Munn[†]*

***In mice, immunoregulatory APCs express the dendritic cell (DC) marker CD11c, and one or more distinctive markers (CD8α, B220, DX5). In this study, we show that expression of the tryptophan-degrading enzyme indoleamine 2,3 dioxygenase (IDO) is selectively induced in specific splenic DC subsets when mice were exposed to the synthetic immunomodulatory reagent CTLA4-Ig. CTLA4-Ig did not induce IDO expression in macrophages or lymphoid cells. Induction of IDO completely blocked clonal expansion of T cells from TCR transgenic mice following adoptive transfer, whereas CTLA4-Ig treatment did not block T cell clonal expansion in IDO-deficient recipients. Thus, IDO expression is an inducible feature of specific subsets of DCs, and provides a potential mechanistic explanation for their T cell regulatory properties. The Journal of Immunology,* 2003, 171: 1652–1655.**

Minor subsets of murine CD11c$^+$ dendritic cells (DCs)[3] coexpressing other markers (CD8α, B220, DX5, DEC205) suppress T cell responses and promote tolerance, rather than immunity, to specific Ags (1–6). Expression of the tryptophan-catabolizing enzyme indoleamine 2,3 dioxygenase (IDO) in human and murine cells inhibits Ag-specific T cell proliferation in vitro and suppresses T cell responses to fetal alloantigens during murine pregnancy (7–10). Correlations between IDO expression and immunoregulatory outcomes have been confirmed in several experimental systems (11–15). Recently, Grohmann et al. (16) reported that the synthetic immunomodulatory reagent CTLA4-Ig induces IDO expression in CD11c$^+$ DCs from murine spleen. Using this method to induce IDO expression, we show that CTLA4-Ig induced IDO expression selectively in specific DC subsets. In a model of T cell adoptive transfer, we show that CTLA4-Ig completely blocked CD8$^+$ T cell clonal expansion and that this property was IDO-dependent.

Materials and Methods

Mice

C57BL6 (B6), CBA/Ca, and F_1(CBA × B6) mice were bred in a specific pathogen-free facility. BM3 TCR transgenic mice have been described previously (7, 17, 18). IDO-deficient mice were generated using a DNA construct to target the IDO gene in murine embryonic stem cells (129/SvJ). A complete copy of the murine IDO gene was isolated from a genomic library (129/Sv) and a selectable marker (neomycin) was inserted to replace exons 3–5 (which encode critical portions of the enzyme catalytic site). In addition, a translational "stop" codon (TAG) was introduced into exon 2 sequences. Embryonic stem cells were electroporated, and clones carrying targeted IDO alleles were injected into blastocysts. Male chimeric mice were mated with B6 or CBA females, and offspring were mated to produce IDO$^{-/-}$ mice with either B6 or CBA backgrounds. The IDO-deficient mice bred normally, and exhibited no spontaneous autoimmune disorders or alteration in immune system development, with the exception of the features described herein.

Abs and immunohistochemistry

All commercial Abs were obtained from BD PharMingen (San Diego, CA) with the exception of anti-CD8 (DAKO, Carpinteria, CA). Anti-clonotypic (Ti98) Ab was used to detect BM3 T cells, as described previously (18). Polyclonal rabbit anti-murine IDO Abs were raised against synthetic peptides (CSAVERQDLKALEKALHD and KPTDGDKSEEPSNVESRGC) encoding amino acid sequences from murine IDO, conjugated to OVA. Serum from immunized rabbits was purified by peptide affinity chromatography. Histological sections (5 μm) were prepared from formalin-fixed paraffin-embedded tissues. Cytocentrifuge preparations of sorted cells were air-dried and fixed in formalin for 10 min. Endogenous peroxidase activity was blocked using hydrogen peroxide (0.3%, 10 min), then tissue sections were treated with proteinase K (10 min; DAKO) followed by Universal Blocking Reagent (8 min; BioGenex, San Ramon, CA), then incubated with anti-IDO Ab (1–2 h, 1/100 dilution in PBS). Primary Ab was detected with biotinylated goat anti-rabbit secondary (20 min; BioGenex) and peroxidase-conjugated streptavidin, (20 min; BioGenex) with 3-amino-9-ethylcarbazole chromogen, essentially as described for anti-human IDO Ab (10). Controls for specificity included use of preimmune rabbit serum as primary Ab, preincubation of primary Ab with neutralizing peptide

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[3] Abbreviations used in this paper: DC, dendritic cell; IDO, indoleamine 2,3 dioxygenase; 1mT, 1-methyl-tryptophan.

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(1.2 μg of Ab/10 μg of peptide), and tissues from IDO-deficient mice stained with anti-IDO Ab.

CTLA4-Ig

CTLA4-Ig (cytolytic isoform with an IgG2a Fc domain) was obtained from Chimerigen (catalog no. 70201; Allston, MA,) or Sigma-Aldrich (catalog no. C4483; St. Louis MO).

Flow cytometry

Flow cytometric analyses were performed on cells stained with fluorochrome-conjugated mAbs (BD PharMingen or DAKO) using on FACSCalibur machine (BD Biosciences, Mountain View, CA). Preparative cell sorting was performed using a Mo-Flo four-way flow cytometer (Cytomation; DAKO) equipped with 488 nm of argon (for FITC, PE, PE-CY5) and 647 nm of krypton (for allophycocyanin) lasers. Cells were gated based on forward and side scatter properties and on marker combinations to select cells of interest.

T cell adoptive transfer

Recipient male mice (6- to 8-wk-old) were injected i.v. with pooled splenocytes (between $0.5–1.0 \times 10^7$) from BM3 male donors (6- to 8-wk-old). After 96 h, single cell suspensions of splenocytes were stained with Ab and subjected to flow cytometric analyses, as described (7, 18). Mice treated with CTLA4-Ig received four i.p. injections as follows: 30, 6 h before (100 μg/injection) and 24, 48 h after (50 μg/injection) BM3 cells were injected ($t = 0$). Some mice were treated with 1 methyl D,L tryptophan (Sigma-Aldrich) or placebo (Innovative Research, Sarasota, FL) by implanting slow-release polymer pellets impregnated with, or without, IDO inhibitor under the dorsal skin of anesthetized mice 6 h before injecting BM3 cells as described (7). Doses were calculated to release ~20 mg/day of 1 methyl D,L tryptophan.

Results

CTLA4-Ig selectively induces IDO expression in specific DC subsets

To examine the effects of CTLA4-Ig, we prepared tissue sections from spleen of F_1(CBA × B6) mice treated with CTLA4-Ig and stained them with a rabbit polyclonal anti-IDO peptide Ab (Fig. 1). Very few cells expressed IDO in spleen from untreated mice (Fig. 1*a*). Many more IDO^+ cells were detected in spleen 24 h after CTLA4-Ig injection (Fig. 1, *b* and *c*). Clusters of IDO^+ cells, mostly small round mononuclear cells (Fig. 1*c*), were dispersed throughout splenic red pulp areas, while very few were located in lymphoid follicles. Treatment with isotype-matched IgG2a Abs had no effect on IDO expression (data not shown). Comparable outcomes were obtained with CTLA4-Ig obtained from two suppliers (see *Materials and Methods*).

To phenotype IDO^+ splenocytes following CTLA4-Ig treatment, we sorted Ab-stained splenocytes using preparative (Mo-Flo) flow cytometry, prepared cytospins, and stained sorted cells with anti-IDO Ab (Table I). Intensely stained (IDO^+) cells expressed murine myeloid cell markers (CD11c and/or CD11b) while nonmyeloid cells ($CD11c^-CD11b^-$) contained no stained cells (sort 1). Among $CD11c^+$ subsets, DCs with three partially overlapping phenotypes contained cells uniformly and intensely stained by anti-IDO Ab (Table I, bold text). Two IDO^+ populations corresponded to nonplasmacytoid ($CD11c^+CD8\alpha^+B220^-$) and plasmacytoid ($CD11c^+B220^+CD19^-$) DC subsets, described previously (3, 19). Fig. 1*f* shows an example of intense homogenous IDO Ab staining, in this case on sorted plasmacytoid DCs ($CD11c^+B220^+CD19^-$). By comparison, $CD11c^-B220^-CD19^-$ cells (mostly T cells) sorted in parallel were not stained (Fig. 1*e*). Intensely stained cells were also present in sorted $CD11c^+DX5^+CD8\alpha^+$ (uniformly stained) and $CD11c^+DX5^+CD8\alpha^-$ (~60% stained) subsets, corresponding to bitypic NK DCs described previously (5). Collectively, IDO^+ cells in these three DC subsets accounted for <50% of total $CD11c^+$ splenocytes in each sort and IDO Ab did not stain

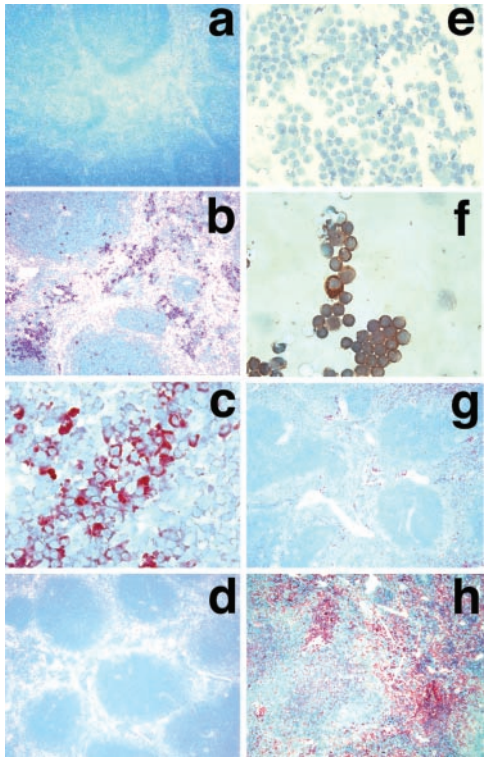

FIGURE 1. CTLA4-Ig selectively induces IDO expression in splenocytes. F_1(CBA × B6) mice were treated with PBS (*a*), or 100 μg of CTLA4-Ig (*b–f*). After 24 h, mice were sacrificed and spleen sections were stained with anti-IDO Ab in the presence (*d*) or absence (*a–c*) of excess peptide used to prepare anti-IDO Ab. Alternatively, splenocytes from CTLA4-Ig-treated F_1(CBA × B6) mice were stained with a mixture of Abs (B220, CD19, CD11c), sorted using a flow cytometer, spun down, and stained with IDO Ab (*e* and *f*). Data shown are sorted and stained, (*e*) non-B, non-DC splenocytes ($B220^-CD11c^-CD19^-$), and (*f*) plasmacytoid DCs ($B220^+CD11c^+CD19^-$). F_1(CBA × B6) IDO-sufficient (*g*), or IDO-deficient (*h*) mice were exposed to CTLA4-Ig (300 μg, see *Materials and Methods*), injected (i.v.) with BM3 splenocytes, and 96 h later, spleen tissues were stained with CD8 mAb. Original magnifications: *a*, *b*, and *d* (×200); *c*, *e*, and *f* (×1000); *g* and *h* (×100).

$CD8\alpha^-$ DCs ($CD11c^+B220^-CD8\alpha^-$). Other sorted populations stained heterogeneously and/or with lower intensities. Together with outcomes from immunohistochemical staining analyses, these data show that IDO expression was selectively and specifically up-regulated in some DC subsets following exposure to CTLA4-Ig. Alternatively, CTLA4-Ig treatment may have induced influx of IDO^+ cells into spleen.

IDO up-regulation in regulatory DC subsets blocks alloantigen-specific T cell responses in vivo

To examine the effect of induced IDO on APC functions, we injected H-$2K^b$-specific $CD8^+$ T cells from TCR transgenic mice (BM3) into recipient mice expressing H-$2K^b$ (F_1(CBA × B6)). We selected this system because BM3 T cells clonally expand, differentiate into CTLs and cause extensive immunopathology in recipient spleen following adoptive transfer (7, 17, 18, 20). In addition, BM3 T cells do not require CD28-mediated signals to activate (9, 21), rendering them relatively resistant to costimulatory blockade by CTLA4-Ig. Despite this, clonal expansion of donor BM3 $CD8^+$ T cells expressing clonotypic Ab ($Ti98^+$) following adoptive transfer into F_1(CBA × B6) mice was blocked completely (99% inhibition) when recipients were treated with CTLA4-Ig (Fig. 2). Consistent with

Table I. *Phenotypic analysis of IDO$^+$ cells from CTLA4-Ig-treated F_1 (CBA × B6) mice*

Phenotype	Cell Type	IDO$^+$ (%)[a]
Sort 1		
CD11c$^+$ and/or CD11b$^+$	**Myeloid**	**ht**[b]
CD11c$^-$CD11b$^-$	Nonmyeloid	0
Sort 2		
CD11c$^+$B220$^-$CD8α^+	**CD8+ DCs**	**100**
CD11c$^+$B220$^-$CD8α^-	CD8− DCs	0
Sort 3		
B220$^+$CD11c$^+$CD19$^-$	**pDCs**[c]	**100**
B220$^+$CD11c$^-$CD19$^+$	B cells	0
B220$^-$CD11c$^-$CD19$^-$		0
Sort 4		
DX5$^+$CD11c$^+$CD8α^+	**NK DCs**	**100**
DX5$^+$CD11c$^+$CD8α^-	NK DCs	~60
DX5$^+$CD11c$^-$	NK cells	0

[a] Percent of cells stained intensely with anti-IDO Ab.
[b] Heterogeneous expression on <50% of cells.
[c] Plasmacytoid DCs.

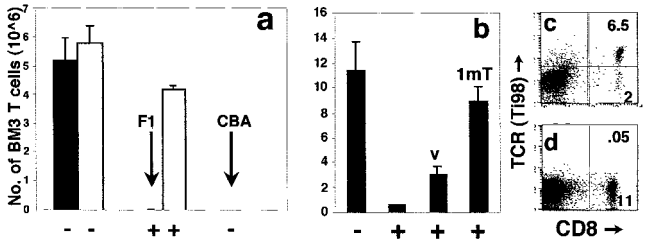

FIGURE 2. IDO-dependent suppression of BM3 T cell clonal expansion in vivo. BM3 splenocytes were injected (i.v.) into F_1(CBA × B6) or (CBA) recipient mice and 96 h later numbers of donor CD8$^+$ T cells in recipient spleen were assessed by flow cytometry (7). Recipient mice were IDO-sufficient (■) or IDO-deficient (□) mice. Treatments with CTLA4-Ig (+) or PBS (−) are indicated below each panel. Arrows indicate data for F_1 and CBA recipient mice that show no T cell clonal expansion (*a*). Treatments with IDO inhibitor (1mT) or vehicle pellets alone (v) are indicated above data bars (*b*). Histograms (two-color) show representative staining profiles of cells from CTLA4-Ig-treated IDO-deficient (*c*) and IDO-sufficient (*d*) mice, 96 h after BM3 transfer, which were used to calculate donor T cell numbers (CD8$^+$, Ti98$^+$) shown in *a*. Numbers indicate percentage of total events falling into *upper* (donor Ti98$^+$CD8$^+$ T cells) and *lower* (recipient Ti98$^-$CD8$^+$ T cells) *right quadrants*. Experiments were replicated three times. Error bars show 1 SD.

this, very few CD8$^+$ cells were detected in spleen tissues stained with anti-CD8 Ab (Fig. 1*g*) and spleens exhibited normal tissue architecture. In stark contrast, CTLA4-Ig treatment of IDO-deficient mice with otherwise identical genetic backgrounds (F_1(CBA × B6)) had only marginal inhibitory effects on BM3 T cell clonal expansion (25% inhibition) following adoptive transfer (Fig. 2, *a* and *c*), which produced large numbers of CD8$^+$ cells in spleen tissue sections (Fig. 1*h*), accompanied by massive disruption of normal spleen architecture. The low numbers of donor T cells in these CTLA4-Ig-treated IDO-sufficient recipients were comparable with low numbers present in control CBA (H-2K^b-negative) mice analyzed in parallel (Fig. 2*a*). The low level of CTLA4-Ig-mediated inhibition observed in IDO-deficient recipients may arise from costimulatory blockade of CD28-dependent, H-2^b-specific donor T cells with rearranged endogenous TCR genes (our unpublished data). Thus, the ability of CTLA4-Ig to suppress clonal expansion of H-2K^b-specific CD8$^+$ T cells in this experimental system was almost exclusively IDO-dependent.

We used an alternative approach to evaluate whether IDO activity was required for CTLA4-Ig mediated suppression of BM3 T cell expansion in vivo by surgically inserting slow-release polymer pellets impregnated with pharmacologic IDO inhibitor, 1-methyl-tryptophan (1mT) (2) into IDO-sufficient recipients, before injecting donor BM3 splenocytes (see *Materials and Methods*). Though tissue inflammation due to surgery and pellet insertion enhanced BM3 responses, pellets containing IDO inhibitor were significantly more effective in enhancing elicited BM3 clonal expansion (Fig. 2*b*). Thus, pharmacologic inhibition of IDO activity, like genetic inactivation of IDO, overcame the suppressive effects of CTLA4-Ig. Thus, up-regulated IDO expression had potent immunosuppressive effects on aggressive CD8$^+$ T cell responses following adoptive transfer of BM3 splenocytes.

Discussion

We show that IDO up-regulation in response to CTLA4-Ig occurred selectively and specifically in splenic DC subsets with particular phenotypes. Though this response was restricted to <50% of CD11c$^+$ splenocytes, IDO up-regulation had a profound inhibitory effect on the ability of alloantigen-specific CD8$^+$ T cells to undergo clonal expansion following adoptive transfer.

Phenotypic analyses revealed that DC subsets induced to express uniformly high amounts of IDO were related to DC subsets previously reported to exhibit immunoregulatory properties in some systems. DCs expressing CD8α, first described by Shortman and colleagues (4), can promote tolerance to peripheral self-Ags and tumor-associated Ags, though they can also promote immunity (4, 13, 22–25). Isolated splenic CD8α^+ DCs express IDO and suppress delayed-type hypersensitivity responses, except when treated with IDO inhibitor (26, 27). We found that 100% of nonplasmacytoid CD8α^+ DCs (CD11c$^+$B220$^-$CD8α^+) expressed high levels of IDO following CTLA4-Ig treatment in vivo, as did cells corresponding to plasmacytoid DCs. Plasmacytoid DCs have tolerogenic or immunostimulatory potential, depending on conditions and their state of activation or maturation (6, 28). Moreover, plasmacytoid DCs may overlap the CD8α^+ DC population when they mature (29). We also detected high levels of IDO expression in bitypic DCs coexpressing DC (CD11c) and NK (DX5) markers, also reported to have regulatory properties (5). Because IDO up-regulation is an obligate mechanism by which CTLA4-Ig exerts most of its immunosuppressive activity in our experimental system, classifying DCs based on IDO up-regulation in response to CTLA4-Ig exposure in vivo provides a potential mechanistic explanation for their regulatory effects.

The consequences of up-regulating IDO in minor CD11c$^+$ DC subsets were profound in the BM3 adoptive transfer system, which leads to massive, sublethal destruction of splenic tissues mediated by cytolytic donor CD8$^+$ T cells after 3–4 days (7, 17, 18, 20). Indeed, sublethal irradiation of recipient mice leads to lethal T cell-mediated graft-vs-host disease. Prior treatment with CTLA4-Ig completely abrogated BM3 T cell clonal expansion, but only if recipients had intact IDO genes. Pharmacologic inhibition of IDO activity only partially restored BM3 T cell clonal expansion, possibly due to low inhibitor bioavailability in vivo (our unpublished data). Thus, CTLA4-Ig

mediated costimulatory blockade did not block BM3 clonal expansion, while up-regulated IDO expression had potent inhibitory effects on elicited T cell responses. These outcomes provide mechanistic evidence extending recent findings that CTLA4-Ig-mediated inhibition of pancreatic islet allograft rejection was abrogated in mice treated with pharmacologic IDO inhibitor (16).

IDO-deficient mice exhibit otherwise normal immune system development and function and do not display spontaneous autoimmunity (our unpublished results). This suggests that IDO-mediated suppression is not essential for maintenance of central or peripheral tolerance to self-Ags under homeostatic conditions. The inability of IDO-deficient mice to suppress potentially lethal T cell responses when pretreated with CTLA4-Ig revealed a defect in acquired tolerance. Hence, induced IDO expression may contribute to acquisition of tolerance to neoantigens (30). This implies that therapies based on the use of autologous isolated IDO$^+$ DCs or selective up-regulation of IDO expression in some DC subsets may improve clinical outcomes for patients with autoimmune diseases or following tissue transplantation. Viewed from this perspective, the documented ability of tumors (10, 31) and pathogens, such as HIV (32–34) to recruit IDO$^+$ DCs and up-regulate IDO expression suggests that the IDO mechanism may contribute to both beneficial and pathologic immunosuppressive pathways (30).

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