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Functional expression of indoleamine 2,3-dioxygenase by murine CD8α$^+$ dendritic cells

Francesca Fallarino, Carmine Vacca, Ciriana Orabona, Maria L. Belladonna, Roberta Bianchi, Brendan Marshall[1], Derin B. Keskin[1], Andrew L. Mellor[1], Maria C. Fioretti, Ursula Grohmann and **Paolo Puccetti**

Department of Experimental Medicine, University of Perugia, Via del Giochetto, Perugia 06122, Italy
[1]Institute of Molecular Medicine and Genetics, Medical College of Georgia, 1120 15th Street, Augusta, GA 30912, USA

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Abstract

Immunoregulatory antigen-presenting cells (APC) play an important role in maintaining T cell homeostasis and self-tolerance. In particular, recent evidence demonstrates a role for inhibition of T cell proliferation by macrophage tryptophan catabolism involving the activity of the enzyme indoleamine 2,3-dioxygenase (IDO). Dendritic cells (DC) have also been shown to exert immunoregulatory effects mediated by tryptophan catabolism and to cause T cell apoptosis. In the present study, we have comparatively analyzed the expression of IDO activity by murine macrophages and splenic DC. By means of PCR, Western blotting and measurements of enzyme functional activity, we obtained evidence that, different from macrophages, DC constitutively express IDO. Following activation by IFN-γ, the latter cells, in particular the CD8α$^+$ subset, exhibit high functional activity and, unlike macrophages, mediate apoptosis of T_h cells *in vitro*. Therefore, in the mouse, CD8α$^+$ DC may be unique APC capable of fully expressing the IDO mechanism functionally.

Inhibition of T cell proliferation by tryptophan catabolism protects the fetus from maternal immune responses (1,2). The expression, however, of indoleamine 2,3-dioxygenase (IDO) by different cell types could have broader immunological significance in tolerance and immunoregulation. Although inhibition of T cell proliferation by macrophage tryptophan catabolism may be pivotal in suppressing unwanted T cell responses under physiopathologic conditions (3), we have recently obtained evidence that splenic dendritic cells (DC) in the CD8α$^+$ subset can mediate IDO-dependent apoptosis of T cells, suggesting that tryptophan metabolism may also act to regulate T cell homeostasis through the selective deletion of T cells (4–6). Hence, it is important to assess the relative contributions of distinct antigen-presenting cells (APC), IDO-dependent processes and the associated mechanisms that inhibit T cell responses. In the case of macrophages, it has been demonstrated that these cells may veto T cell proliferation by removing tryptophan, the rarest essential amino acid, from local tissue microenvironments (3,7). Based on the observation that IDO-expressing DC can mediate apoptosis of T cell clones and that the effect is reversed by the addition of an IDO inhibitor (4–6), we have proposed that the induction of apoptosis may be an additional mechanism whereby tryptophan catabolism effects immunoregulatory phenomena. In the present study, we have comparatively analyzed macrophages and DC for expression and induction of IDO activity under similar experimental conditions. We have obtained evidence that the patterns of constitutive expression, activation by IFN-γ and T cell regulation may be significantly different in the two types of APC.

Macrophages have several effector mechanisms at their disposal to mediate immunoregulatory effects, including production of cytotoxic or cytostatic agents, nitric oxide and cytokines. Although IDO-mediated tryptophan catabolism has recently been added to this list (7,8), the overall immunosuppressive effects of IDO are not entirely known and may

Correspondence to: P. Puccetti

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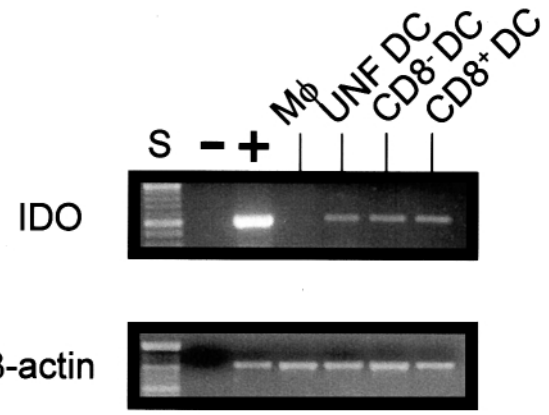

Fig. 1. Expression of IDO-specific transcripts in macrophages and DC either unfractionated or fractionated according to CD8 expression using a positive selection column in combination with CD8α microbeads. Cells were analyzed for constitutive expression of IDO-specific mRNA by RT-PCR. RNA was isolated from macrophages, unfractionated DC, $CD8^-$ DC (>99% pure) or $CD8^+$ DC (>90% pure) and the resulting cDNA was used in the PCR normalized to β-actin. The sequences of 5′ sense and 3′ antisense primers of murine IDO were as follows: 5′ [108–127]-GAA GGA TCC TTG AAG ACC AC and 3′ [587–606]-GAA GCT GCG ATT TCC ACC AA. After 30 cycles of amplification, 10 μl of the reaction mix was removed, analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The positive control (+) consisted of IDO-expressing MC_{24} cell transfectants. The minus symbol indicates no cDNA added to the amplification mix during RT-PCR. S, marker track.

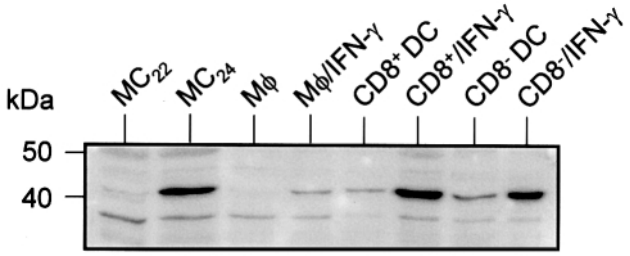

Fig. 2. IDO expression in macrophages and DC by Western blot analysis. IDO expression was investigated using a specific antibody in macrophages and $CD8^+$ or $CD8^-$ DC, either untreated or exposed to IFN-γ. Following incubation with medium or IFN-γ (200 U/ml for 18 h), cells were recovered and lysed in buffer containing 1% Nonidet P-40. After SDS–PAGE resolution, immunoblotting was performed with rabbit polyclonal IDO-specific antibody. Membranes were blocked in Tris-buffered saline containing 0.05% Tween 20, 5% non-fat dried milk and 1% BSA, and incubated sequentially with the antibody (1:3000) and horseradish peroxidase-conjugated anti-rabbit IgG (1:5000). Controls consisted of IDO-expressing MC_{24} transfectants and mock-transfected MC_{22} cells. No 42–45 kDa bands were observed in the MC_{24} transfectants and the other cell types using non-immune rabbit serum (not shown).

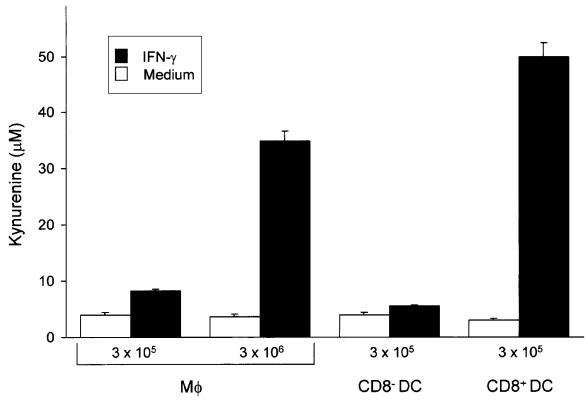

Fig. 3. Functional activity produced by macrophages and DC in terms of ability to metabolize tryptophan to kynurenine. Cells were assayed either as such or after activation with IFN-γ. Kynurenine levels were measured by HPLC. Results are the mean ± SD of replicate samples in one of several experiments with similar results.

depend on the type of IDO-expressing cell. Macrophages may suppress T cell proliferation by depleting tryptophan (3) and DC could mediate death of T cells through the production of pro-apoptotic tryptophan catabolites along the so-called kynurenine metabolic pathway (4–6). We have examined IDO expression in the two cell types to ascertain any difference in enzyme activity possibly leading to different *in vivo* roles.

Peritoneal macrophages and splenic DC were analyzed for constitutive expression of IDO-specific transcripts using primers designed in our laboratory on the basis of the known sequence of the murine IDO gene. Freshly harvested peritoneal macrophages (>99% $Mac\text{-}1^+$) (9) from naive DBA/2J mice were analyzed comparatively with splenic unfractionated DC expressing CD11c or DC fractionated according to CD8α expression ($CD8^+$ and $CD8^-$ DC) (4–6). Consistent with previous results using human macrophages in the absence of IFN-γ activation (3), murine macrophages failed to express IDO-specific messages. In contrast, considerable expression was observed in the unfractionated DC and in the $CD8^-$ and $CD8^+$ fractions in addition to IDO-transfected cells (MC_{24}) (10) used as a positive control (Fig. 1).

IDO expression was also investigated by Western blot using a previously described rabbit polyclonal IDO-specific antibody (10) (Fig. 2). Considerable expression of the enzyme protein, with a mol. wt of ~42–45 kDa (11), was detected in macrophages and DC (either $CD8^+$ or $CD8^-$) following overnight activation with IFN-γ (200 U/ml). However, constitutive expression was not observed in macrophages, as opposed to DC, whose basal expression was quantitatively similar to that of IFN-γ-treated macrophages. In addition, considerable IDO expression was also observed in control, IDO expressing MC_{24} cell transfectants, but not in mock-transfected MC_{22} cells (10).

The functional activity of IDO produced by different APC was measured in terms of ability to metabolize tryptophan to kynurenine (5,6,12). Although not entirely quantitative, this assay provides a reliable means of measuring functional IDO activity, reflecting a multifactored combination of IDO expression, tryptophan transport into the cells and the intracellular conditions that post-translationally affect enzyme activity (13). Peritoneal macrophages and $CD8^-$ or $CD8^+$ DC, either as such or treated overnight with IFN-γ, were incubated with 100 μM tryptophan at 37°C for 4 h. Supernatants were harvested and kynurenine was measured by HPLC. Figure 3 shows that using 3×10^5 cells, limited or no activity was observed with macrophages irrespective of IFN-γ treatment, although significant activity was observed with 3×10^6 cells/well after activation with

IFN-γ. Rather surprisingly, we found that the $CD8^-$ fraction of DC did not exhibit significant enzyme activity despite an expression comparable to the $CD8^+$ subset on Western blot analysis. In contrast, high activity was induced by IFN-γ in the $CD8^+$ subset. In experiments not reported here, we also found that increasing the number of $CD8^-$ DC (treated or not with IFN-γ) up to 3×10^6/well would not increase the levels of enzyme functional activity. Therefore, it appears that murine $CD8^-$ DC may be subjected to significant post-translational regulation of IDO, as has recently been observed in activated human macrophages (11). Although our current data do not offer an explanation as to why IDO activity manifests only in the $CD8^+$ DC subset following IFN-γ treatment, a number of putative factors may affect IDO expression in the different cell types, including superoxide anion radicals (which act as activators) and nitric oxide (which inhibits enzyme activity) (11,14–16). Preliminary observations in our laboratory indicate that $CD8^-$ DC treated with IFN-γ produce significantly higher levels of nitric oxide than the $CD8^+$ counterpart.

We have already demonstrated that the expression of IDO makes $CD8^+$ DC treated with IFN-γ capable of effecting apoptosis of a T_h1 cell clone (5,6). Using a P815AB-specific $CD4^+$ T cell clone for measurement of apoptosis upon co-culture of the latter cells with $CD8^+$ DC exposed to IFN-γ, we have previously detected a percentage of apoptosis as high as 40% (17). We therefore became interested in comparatively analyzing the effects of macrophages and DC in the same model system. T_h1 cells were incubated for 3 days with the different APC in the presence of the cognate antigen peptide (P815AB), prior to assessment of apoptosis by Annexin V staining. In line with previous results, Fig. 4 shows that the addition of IFN-γ -treated $CD8^+$ DC to the T_h1 cell clone caused a significant proportion of these cells to undergo apoptosis. This effect was inhibited by the addition of the competitive inhibitor of IDO, 1-methyl-DL-tryptophan, to the co-culture of $CD8^+$ DC and $CD4^+$ T cells. In contrast, no significant apoptosis could be observed upon incubation of the T cells with macrophages or $CD8^-$ DC, either as such or after treatment with IFN-γ. In additional experiments, we found that increasing up to 10-fold the concentrations of macrophages would not result in significant T cell apoptosis (data not shown). Of interest, no apoptosis of T_h1 cells was mediated by $CD8^+$ DC in the absence of cognate peptide (see legend to Fig. 4). Therefore, $CD8^+$ DC appear to mediate TCR-dependent T cell apoptosis via IDO activation under the adopted experimental conditions.

The main observation in this report is that macrophages and DC, which are both known to express IDO activity, differ in their patterns of enzyme expression and may have distinct *in vivo* functions. In line with the notion that inhibition of T cell proliferation by macrophage tryptophan catabolism is important in suppressing unwanted T cell responses (3,7), we found that significant tryptophan-metabolizing activity is induced by IFN-γ in macrophages. However, DC appear to differ from macrophages in that: (i) they manifest significant IDO transcript expression under basal conditions and also exhibit enzyme protein detectable by Western blot analysis, (ii) they will express far superior enzyme activity on a per cell basis after treatment of the $CD8\alpha^+$ subset with IFN-γ, and (iii) the latter cells can mediate apoptosis of T cells *in vitro*. Therefore, $CD8\alpha^+$ DC may represent the only murine APC subset capable of expressing high levels of functional IDO. It is possible that the murine lymphoid $CD8^+$ DC lineage corresponds in humans to the progeny of DC precursors with a characteristic surface phenotype and a plasmacytoid appearance (18). It has been shown that human DC expressing significant IDO activity can mediate inhibition of T cell proliferation (12). Our present and previous observations (4–6) provide the first experimental evidence for the involvement of tryptophan degradation in T cell apoptosis and regulation of anergy of mature T lymphocytes by $CD8^+$ DC in the mouse. This may add to the complex role of DC in the control of immunity, and may provide novel mechanistic insights into how DC tolerize T cells to self-antigens and minimize autoimmune reactions (19–21).

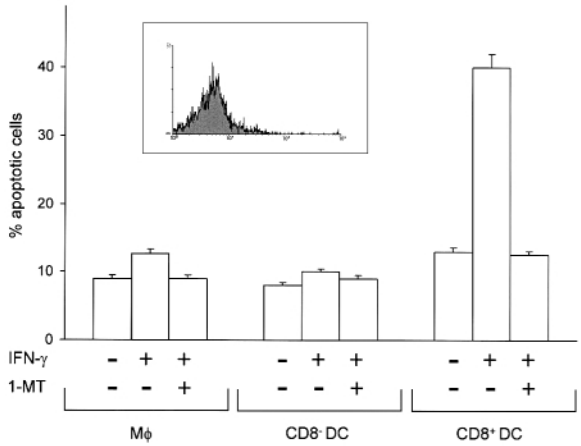

Fig. 4. IDO-dependent apoptosis of T cells as mediated by $CD8^+$ DC. Apoptosis was measured in $CD4^+$ T cells cultured in the presence of cognate peptide with macrophages, $CD8^-$ DC or $CD8^+$ DC either as such or after treatment with IFN-γ. The assay was performed as previously described (5,6). Cells (2.5×10^5) treated overnight with medium or IFN-γ (200 U/ml) were washed and cultured for 3 days with 5×10^5 F76 cells, a T_h1-type tumor-specific $CD4^+$ T cell clone, in the presence of 5 μM cognate peptide. At the end of the co-culture, cells were surface stained with anti-CD4–phycoerythrin and FITC-labeled Annexin V and propidium iodide (PI). For measurement of apoptosis, a gate was set on $CD4^+$ T cells and the percentage of cells in the very early stages of apoptosis was determined by Annexin V staining excluding PI^+ cells. Parallel co-cultures were established in the presence of 2 μM 1-methyl-DL-tryptophan (1-MT), an inhibitor of IDO activity. Controls (not shown) included co-cultures of $CD4^+$ T cells and IFN-γ-treated $CD8^+$ DC in the absence of cognate peptide (apoptosis of ~5%), as well as cultures of T cells and peptide in the absence of $CD8^+$ DC (apoptosis <5%). Each value is the mean ± SD of replicate determinations in one of four experiments. Inset: representative histogram of Annexin V staining of gated $CD4^+$ PI^- cells cultured with untreated $CD8^+$ DC in the presence of peptide.

In conclusion, although IDO is known to play a role in inflammation and maternal tolerance of fetal allografts (1,2,8,22), its exact modes of action are unclear. Our current findings, which demonstrate IDO-mediated apoptosis of T cells by $CD8\alpha^+$ DC, may add to the multiplicity of IDO effects *in vivo* as an immunoregulator.

Acknowledgements

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Abbreviations

APC	antigen-presenting cell
DC	dendritic cell
PI	propidium iodide
IDO	indoleamine 2,3-dioxygenase

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