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Cutting Edge: Human Eosinophils Regulate T Cell Subset Selection through Indoleamine 2,3-Dioxygenase

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Allergy involves eosinophilia and Th2 polarization. Indoleamine 2,3-dioxygenase (IDO)-catalyzed conversion of tryptophan to kynurenines (KYN) regulates T cell function. We show that human eosinophils constitutively express IDO. Eosinophils treated with IFN-γ showed an 8-fold increase in IDO mRNA within 4 h; IL-3, IL-5, and GM-CSF had no effect on baseline IDO expression. IL-3 pretreatment of eosinophils reduced IFN-γ-induced IDO mRNA expression below baseline. Conversely, GM-CSF, but not IL-5, resulted in a 2-fold increase in IFN-γ-induced IDO. Treatment with IL-3, IL-5, GM-CSF, or IFN-γ alone expressed IDO enzymatic activity (the presence of KYN in supernatants 48 h postculture). CD28 cross-linking resulted in measurable KYN in culture supernatants, inhibitable by a neutralizing anti-IFN-γ. Coculture of eosinophils with an IFN-γ-producing T cell line, but not IL-4-producing T cell clone, led to apoptosis and inhibition of CD3 or CD3/CD28-induced proliferation. Eosinophils infiltrating asthmatic lung and associated lymphoid tissue exhibited intracellular IDO immunoreactivity. Eosinophils may, therefore, maintain Th2 bias through IDO. The Journal of Immunology, 2004, 173: 5909–5913.

Indoleamine 2,3-dioxygenase (IDO), an IFN-γ-inducible enzyme, catalyzes the rate-limiting step in the oxidative catabolism of tryptophan. Kynurenines (KYN), the main products of this enzyme, exert immunosuppressive effects through induction of apoptosis and inhibition of proliferation in T cells (1). Induction of IDO in dendritic cells (DCs) occurs through autocrine effects of IFN-γ following ligation of CD80/CD86 on DCs by CTLA-4 on T cells and was recently shown to be a major mechanism of DC-induced T cell apoptosis (2). Th1 may be more sensitive to the effects of KYN than Th2 cells (3, 4).

Allergic asthma is a disease associated with the preponderance of Th2 cells and associated cytokines. The basis of such Th2 imbalance is not yet fully understood. However, exposure to nonmicrobial Ags during allergen sensitization and challenge may lead to the development of DC subsets that can promote Th2 cell differentiation in situ (5). Indeed, Akdis et al. (6) recently demonstrated that the Th2 predominance seen in atopic disease is due to preferential apoptosis of activated memory/effect Th1 cells. Thus, it is conceivable that an increase in IDO-expressing cells may be a basis for the Th2 polarization associated with allergic asthma.

Eosinophils (Eos) are consistently increased in number during allergic asthma (7). Eos are generally considered putative effector cells in asthma through the release of their granule-stored cationic proteins (8). However, Eos, like DCs, can interact with T cells and promote Th2 polarization (9, 10). We have investigated whether IDO induction in Eos is a potential mechanism in the regulation of Th2 polarization. Our results support such notion and ascribe a capacity for Eos to maintain Th1-Th2 imbalance seen in allergic asthma through expressing functionally active IDO in lymphoid tissues, leading to depletion of tryptophan from the lymphoid microenvironment and induction of apoptosis in lymphocytes by Eos-derived KYN.

Materials and Methods

Reagents and Abs

Rabbit anti-human IDO Ab was generated in-house (11), rIL-2, IL-3, IL-5, and GM-CSF were obtained from BD Biosciences (BD Pharmingen, Mississauga, Ontario, Canada). IFN-γ and IFN-γ-neutralizing Ab were obtained from R&D Systems (Minneapolis, MN). PHA, 1-methyl-DL-tryptophan and KYN were purchased from Sigma-Aldrich (St. Louis, MO). Anti-IL-4-PE, anti-IFN-γ-PE, anti-CD4-FITC, and the appropriate isotype controls, including functional grade anti-CD3, anti-CD28, and functional grade neutralizing Abs against IL-4 and IL-12, were obtained from eBioscience (San Diego, CA).

Cell isolation and culture

Blood samples (100 ml) were collected from atopic donors according to the Helsinki Convention. Eos purification was achieved using an AutoMACS system with immunomagnetic beads (Miltenyi Biotec, Gladbach, Germany), as described previously (12). The purity and viability of Eos obtained usually reached >99%. Eos were suspended in RPMI 1640 (supplemented to contain 100 μg/ml tryptophan). Cells (4 × 10⁶/ml) were plated in six-well culture plates, treated with 200 U IFN-γ/ml, and incubated at 37°C for 24–48 h. IL-3, IL-5, or GM-CSF (10 ng/ml) was added to promote survival. In some
Experiments, IL-3, IL-5, or GM-CSF were combined with IFN-γ to investigate the potential effects of these survival factors on IFN-γ-induced IDO expression or activity. A positive control consisting of the monocytic cell line THP-1 cells, treated with 200 U of IFN-γ, was also set up. Negative controls consisted of lymphocytes treated with same dose of IFN-γ. In some experiments, Eos were also cultured with IFN-γ in the presence of 1-methyl-tryptophan (800 μM), a specific IDO inhibitor, or anti-human IFN-γ Ab (10 μg/ml). A colorimetric method was used to determine the concentration of KYN in culture supernatant as previously described (13).

**Generation of T cell lines and clones**

T cells isolated by negative selection using immunomagnetic beads (Miltenyi Biotec) were activated with immobilized anti-CD3 (10 μg/ml) or anti-CD3/anti-CD28 on a 24-well tissue culture plate. Cells were activated for 4 days in the presence of anti-IFN-γ, anti-IL-12, and rIL-4 to generate IL-4-producing lines. IFN-γ-producing lines were generated in the presence of anti-IL-4, rIL-12, and recombinant human IFN-γ. Following three rounds of activation and rest, cells were cloned in 96-well tissue culture plates at 0.6 cells/well and tested for IFN-γ or IL-4 expression by intracellular cytokine staining and flow cytometry, analyzed using Flowjo software (Treestar, San Carlos, CA), following activation over a period of 5-h incubation (37°C) with ionomycin (500 ng/ml) and PMA (5 ng/ml).

**Proliferation assays**

Activated cells were labeled for visualization using the Vybrant CFDA-SE (CFSE) cell tracker kit (Molecular Probes, Eugene, OR) and reactivated with CD3/anti-CD28 before coculture with Eos. Eos were pretreated with 200 U IFN-γ for 4 h, washed twice, and resuspended in RPMI 1640 (supplemented to include 100 μg/ml tryptophan and 20 U of IL-2) before coculture (4 days) with T cell lines and clones at different Eos:T cell ratios. Baseline fluorescence of labeled T cells was determined before coculture. Using the proliferation platform on the Flowjo software, a statistical model was used to track fluorescence peaks representing different generations of proliferating CFSE-labeled cells in culture, after gating out dead cells by forward scatter, side scatter, and propidium iodide fluorescence.

**RT-PCR and quantitative real-time RT-PCR**

Total nucleic acid was isolated from Eos using a Qiaquick RNasey Mini kit (Qiagen, Mississauga, Ontario, Canada). To analyze IDO transcript expression, RT-PCR was conducted using intron-spanning primers to amplify a 230-bp fragment of IDO cDNA: forward, 5'-AGAAGTGGGCTTTGCTCTGC-3' and reverse, 5'-AGAAGTGGGCTTTGCTCTGC-3'. Control primers were designed at 18S ribosomal RNA: forward, 5'-GTAACCCGTTGAAC-3' and reverse, 5'-AGAAGTGGGCTTTGCTCTGC-3'. Fluorescence was detected at 490 nm using the iCycler IQ real-time PCR detection system (Bio-Rad, Hercules, CA). PCR products from IDO and 18S ribosomal RNA fragments were gel purified and the concentration was determined colorimetrically to calculate the DNA copy number of the purified product. For quantitative PCR, amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Following normalization with 18S RNA, the DNA copy number was calculated for each treatment and expressed as the mean transcript number.

**Western blot analysis**

After incubation with medium or IFN-γ for 24–48 h, cells were harvested, pelleted by centrifugation, and resuspended in lysis buffer. Equal amounts of protein (30 μg) were loaded onto a 5% acrylamide stacking gel and separated by SDS-PAGE using a 14% separating gel. Following transfer of separated proteins; nitrocellulose membranes were blocked and probed overnight at 4°C with a rabbit anti-human IDO Ab. The membrane was probed for 1 h at room temperature with mouse anti-rabbit HRP-conjugated Ig (Amersham Biosciences, Montreal, Quebec, Canada) and immunoreactivity detected by chemiluminescence.

**CD28 cross-linking**

Cross-linking was performed as previously described (10). Briefly, each well of a six-well culture plate was coated with 50 μg/ml anti-mouse IgG F(ab')₂, for 2 h at 37°C. After washing three times with PBS, wells were coated with 25 μg/ml anti-mouse human CD28 Ab (or purified mouse IgG1 isotype control) and incubated for 2 h at 37°C. Following another wash step, 10⁸ Eos in RPMI 1640 were added to each well and incubated at 37°C for 3 days. A control well contained anti-IFN-γ-neutralizing Ab (10 μg/ml).

**Immunohistochemical detection of IDO in Eos**

Immunohistochemistry was conducted using an Ag-retrieval technique as previously described (14). Primary antisera consisted of a purified rabbit anti-human IDO polyclonal IgG with a polyclonal rabbit IgG as negative control. Colocalization of IDO and BMK-13 (a mouse monoclonal anti-human Eos major basic protein Ab) immunoreactivity was analyzed by digital deconvolution restoration microscopy using a DeltaVision microscopy system (Applied Precision, Issaquah, WA).

**Statistical analyses**

Results are presented as mean values ± SE using Student’s t test.

**Results and Discussion**

Peripheral blood Eos express IDO

We first investigated whether IDO is expressed in Eos obtained from atopic donors. Untreated Eos were found to express IDO-specific mRNA compared with monocytes, which only expressed IDO after IFN-γ treatment (Fig. 1A). IDO protein expression in Eos was confirmed using Western blotting to show the presence of a 42-kDa protein that specifically reacted with IDO Ab (Fig. 1C). However, we also detected immunoreactivity with a higher molecular mass Ag (45 kDa). The specificity of this higher molecular mass band was confirmed following a wash step, which incorporated 3 M urea to remove low-avidity nonspecific Ag-Ab interactions. This higher molecular mass band was virtually replaced by the 42-kDa band following addition of IFN-γ. We interpreted these data as indicating the presence of both constitutive (higher molecular mass band) and IFN-γ-inducible (lower molecular mass) forms of IDO in peripheral blood Eos. Our result is consistent with a previous study (15) showing that macrophages and monocytes failed to express IDO mRNA transcripts until activated with IFN-γ, whereas CD8⁻ and CD8⁺ DCs constitutively expressed IDO. Thus, our study has identified Eos as the other hemopoietic cell type that may express IDO protein in the absence of IFN-γ treatment. A novel observation in our study is the apparent conservation of the constitutive form of IDO to the inducible form by IFN-γ. Other studies have suggested that this observation may be due to posttranslational modification of IDO (16).

Early IFN-γ-induced IDO expression in Eos is modulated by IL-3, IL-5, and GM-CSF

Eos are end-differentiated cells that may not survive in the absence of antiapoptotic cytokines (IL-3, IL-5, and GM-CSF) in culture during the initial experiments above. Since Eos from atopic individuals treated with IL-3, IL-5, and GM-CSF appeared to express the active form of IDO, we examined their potential modulatory effects on IFN-γ-induced IDO mRNA following a short-term (4-h) incubation. Using quantitative real-time RT-PCR, we confirmed that these cytokines modulate IFN-γ-induced IDO mRNA expression in Eos. GM-CSF enhanced IFN-γ-induced IDO mRNA expression. Conversely, IL-5 had no significant effect on IFN-γ-induced IDO expression while IL-3 pretreatment reduced IDO mRNA below constitutive baseline levels in Eos (Fig. 1B). It has previously been shown that IL-4, IL-10, and TGF-β down-regulate IDO induction by IFN-γ (17, 18) while IL-1 enhances IFN-γ-induced IDO in macrophages (19, 20). However, the effects of IL-3, IL-5, and GM-CSF, cytokines associated with allergic inflammation, have not been previously investigated. Our results showed that Eos from atopic patients appear to be primed for IDO...
expression and KYN-mediated T cell regulation by GM-CSF. A recent report also suggested the priming of monocytes and macrophages from atopic individuals for IDO expression (21).

Enzymatic activity of IDO from Eos
To investigate whether the induction of IDO in Eos had functional relevance, we measured the conversion of tryptophan to KYN. Eos cultured for 3 days in the presence of survival factors, IL-3, IL-5, and GM-CSF, produced KYN, confirming the constitutive expression of functional IDO activity in human Eos. Similarly, treatment of Eos with IFN-γ resulted in KYN production. Pretreatment of Eos with IL-3, IL-5, and GM-CSF enhanced KYN release into culture supernatant following IFN-γ treatment (Fig. 1D).

A novel observation in this study is the expression of IDO activity, measured as KYN release, following the ligation of CD28 on human Eos in the absence of IL-3, IL-5, or GM-CSF. Anti-CD28-induced IDO activity was almost completely blocked with a neutralizing anti-IFN-γ Ab (Fig. 1D) as a result of Eos apoptosis.

FIGURE 1. A, IDO gene expression in human Eos by RT-PCR analysis of IDO mRNA expression in cytokine-treated Eos. IDO expression was detectable in nonstimulated and cytokine-treated human Eos and monocytes, but not in the untreated human THP-1 cell line (macrophages). B, Western blot analysis of Eos treated (48 h) with IL-3 (lane 1), IL-5 (lane 2), GM-CSF (lane 3), or IFN-γ (lane 4). Treatment with IFN-γ up-regulated the 42-kDa form of IDO (lower band) and decreased the level of the upper band (~45 kDa). C, Real-time PCR analysis of IDO mRNA expression. Following pretreatment with IL-3, IL-5, and GM-CSF (10 ng/ml) or IFN-γ for 2 h, Eos were treated for an additional 2 h with IFN-γ (37°C). Following reverse transcription, PCR was performed with IDO-specific and 18S ribosomal RNA primers and quantified using the SYBR Green I dye. A standard curve was generated following the amplification of known starting copy numbers of IDO and 18S rRNA. Copy numbers of IDO mRNA were determined following normalization of the mRNA concentration with 18S rRNA (n = 4). D, KYN concentration in culture supernatants of cytokine-treated Eos and following cross-linking of CD28. Cells were treated as described in Materials and Methods, and KYN was measured colorimetrically using commercial KYN as standard. KYN concentrations (µM/10⁶ cells) are shown on the vertical axis (n = 13).

FIGURE 2. Intracellular cytokine staining of T cell lines and clones. A–H, Contour plots of either IFN-γ-PE (A–D) or IL-4-PE (E–H) as a function of CD4-FITC following ionomycin/PMA activation over 5 h. A, C, E, and G, Depict staining with isotype control-conjugated Abs while B, D, F, and H represent staining with test Abs for Th1 lines and Th2 clones, respectively. The data show IFN-γ production in ~20% of Th1 cells while almost all (92.4%) of the Th2 clones expressed IL-4.
We generated an IFN-γ-producing T cell line (Th1) and an IL-4-producing T cell clone (Th2) (Fig. 2). Coculture of KYN-producing Eos with the Th1 line activated with CD3 resulted in inhibition of proliferation (Fig. 3, A–C). In contrast, coculture of Eos with Th2 cells did not inhibit proliferation (Fig. 3, D–F). It is, therefore, conceivable that, during allergic inflammation, IDO-expressing Eos may create a tryptophan-depleted microenvironment in vivo and produce cytotoxic metabolites to inhibit the functions of bystander Th1 cells.

We further investigated whether Eos express IDO, in vivo. We determined the immunoreactivity of lung infiltrates with an anti-IDO Ab in a tissue section obtained from an asthmatic subject with a history of the Churg-Strauss syndrome. IDO immunoreactivity was observed in Eos and histiocytes. We also demonstrated extensive infiltration of lymphoid aggregates by IDO-positive Eos (Fig. 4, A–F). Following double staining for IDO and Eos-granule-specific major basic protein (MBP), we demonstrated that IDO was localized to lymphoid tissue-infiltrating Eos using a digital deconvolution restoration microscopic setup (DeltaVision; Applied Precision) (Fig. 4, G–I).

Previous studies have shown that Eos in allergic individuals express CD86 and interact with T cells (10). The studies of Grewe et al. (22) showed that ligation of CD86 on Eos induced the release of IL-12, which can act on Th1 cells, and also in an autocrine fashion on Eos to produce IFN-γ. Similarly, ligation of CD28 on Eos has been demonstrated to potentiate IFN-γ release from Eos (10), a finding confirmed in our present study. Therefore, an exciting conclusion stemming out of this study is that the presence of Eos in lymphoid tissue and their interaction with lymphocytes (through CD86) and APCs (through CD28) may provide optimal conditions for the release of IFN-γ, which, in turn, may exacerbate the immunomodulatory effects of cytokine-primed, IDO-expressing Eos on T cells. Our data led us to speculate that IL-4-producing cells (Th2) may be spared the inhibitory effects of such lymphoid tissue-infiltrating Eos, whereas IFN-γ-producing cells (Th1) are specifically inhibited through a paracrine effect of IFN-γ on Eos to enhance IDO production. This may also explain previous observations that administration of Eos to previously sensitized mice amplifies Th2 responses (9, 23). Thus, the finding of constitutively

**FIGURE 3.** Proliferation of CFSE-labeled T cell lines (A–C) and clones (D–F) following coculture with IDO-expressing activated Eos (producing KYN). A and D, Th1 (A) and Th2 (D) cells cocultured in the presence of the IDO inhibitor, 1-methyl tryptophan. T cells were cocultured with Eos and T cells at either a 1:2 ratio (B and E) or 2:1 ratio (C and F). Dead cells were gated out on the basis of forward/side scatter and propidium iodide staining. Data indicate that Eos-derived KYN inhibits proliferation of IFN-γ-producing cells (Th1), but not IL-4-producing T cell clones (Th2).

**FIGURE 4.** IDO in Eos infiltrating airway tissue in an asthmatic patient (with Churg-Strauss syndrome). A, IDO immunoreactivity in lymphoid aggregates and alveolar spaces (using a polyclonal rabbit anti-human IDO Ab; original magnification, ×100). B, The same section as A, but stained with Luna dye to identify Eos. C, Isotype control. D, A higher magnification of A showing IDO-expressing cells in lung lymphoid aggregates (original magnification, ×400). E, A higher magnification of B depicting Eos as the most prevalent IDO-expressing cells in lung lymphoid aggregates. F, A higher magnification of B showing Luna-staining Eos in lymphoid aggregates in the airway of the asthmatic subject. G, Restorative microscopy of human lymphoid tissue double stained for MBP (green), IDO (red), and nuclear counterstain with 4',6'-diamidino-2-phenylindole (blue): Three-channel fluorescent image, 400× original magnification. H and I, Image analysis and graph distribution of fluorescent intensities for MBP (Bodipy FL) and IDO (Rhodamine Red). Note the identical intensity scales for both fluorescence channels. This suggests colocalization of IDO with Eos-specific MBP in lymphoid tissue-infiltrating Eos, indicating that Eos specifically express intracellularly stored IDO.
functional IDO expression in Eos provides a novel paradigm to explain the Th2 polarization seen in diseases characterized by eosinophilic inflammation, including asthma. The abundance and persistence of IDO-expressing Eos in lymphoid tissue may accentuate or, at a minimum, potentiate the apoptotic effect on Th1 cells previously thought to be associated only with IDO-expressing tolerogenic DCs.

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