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Airway Eosinophils: Allergic Inflammation Recruited Professional Antigen-Presenting Cells[1]

Hai-Bin Wang,* Ionita Ghiran,* Klaus Matthaei,† and Peter F. Weller[2]*

The capacity of airway eosinophils, potentially pertinent to allergic diseases of the upper and lower airways, to function as professional APCs, those specifically able to elicit responses from unprimed, Ag-naive $CD4^+$ T cells has been uncertain. We investigated whether airway eosinophils are capable of initiating naive T cell responses in vivo. Eosinophils, isolated free of other APCs from the spleens of IL-5 transgenic mice, following culture with GM-CSF expressed MHC class II and the costimulatory proteins, CD40, CD80, and CD86. Eosinophils, incubated with OVA Ag in vitro, were instilled intratracheally into wild-type recipient mice that adoptively received i.v. infusions of OVA Ag-specific $CD4^+$ T cells from OVA TCR transgenic mice. OVA-exposed eosinophils elicited activation (CD69 expression), proliferation (BrdU incorporation), and IL-4, but not IFN-γ, cytokine production by OVA-specific $CD4^+$ T cells in paratracheal lymph nodes (LN). Exposure of eosinophils to lysosomotropic NH_4Cl, which inhibits Ag processing, blocked each of these eosinophil-mediated activation responses of $CD4^+$ T cells. By three-color fluorescence microscopy, OVA Ag-loaded eosinophil APCs were physically interacting with naive OVA-specific $CD4^+$ T cells in paratracheal LN after eosinophil airway instillation. Thus, recruited luminal airway eosinophils are distinct allergic "inflammatory" professional APCs able to activate primary $CD4^+$ T cell responses in regional LNs. ***The Journal of Immunology,*** **2007, 179: 7585–7592.**

During inflammation and infection, "inflammatory" Ag-presenting dendritic cells (DCs)[3] active at mucosal sites may derive from $Gr1^+$ monocytes (1, 2). Eosinophils represent another $Gr1^+$ lineage that are prominent leukocytes in Th2-mediated immune responses that underlie reactions to infections with helminthic parasites (3) and notably contribute to allergic inflammation at airway mucosal sites (4). Traditionally, functions of eosinophils focused singularly on their roles as end-stage "effector" cells, e.g., releasing their four granule cationic proteins and generating paracrine mediators of inflammation, including eicosanoids. Based in part on recognition that eosinophils have distinct innate capacities to differentially secrete multiple preformed cytokines (5), additional roles for eosinophils in modulating host immune, and even lymphocyte-mediated responses are now being considered (6, 7). Eosinophil-associated allergic inflammatory diseases notably occur in the airways and include nasal polyposis, allergic rhinoconjunctivitis, and especially asthma (8). Eosinophils recruited into mucosal airway tissues and secretions are positioned to encounter aeroallergens.

With the increasing prevalence of asthma and related allergic disorders (9), attention has focused on cells that mediate or modulate ongoing Ag-dependent allergic airways inflammation in response to inhaled Ags. Thus, in addition to leukocytes that have terminal effector roles within airways tissues, APCs that direct lymphocyte-mediated inflammation are of increasing interest. In this regard, DCs are well established as APCs for their roles in initiating primary T cell-mediated immune responses (10). DCs exhibit the three requisite attributes of "professional" APCs in that DCs can: 1) process and present MHC class II restricted Ags, 2) provide required second-signal costimulation of T cells, and 3) initiate T lymphocyte responses among Ag-naive T cells (11). Airway DCs can capture inhaled Ag, migrate to regional paratracheal lymph nodes (pLNs), and present Ag to naive $CD4^+$ and $CD8^+$ T cells (10). Airway DCs are also implicated in sustaining allergic airways inflammation in murine models of asthma (12).

A potential role for eosinophils, notably those recruited within the airways in conjunction with allergic inflammation, as APCs is possible. In humans, blood eosinophils, that normally do not display MHC class II (MHC II) proteins, can be induced to express MHC class II proteins by stimulation with cytokines, including GM-CSF, IL-3, IL-4, IL-5, and IFN-γ (13–15). Moreover, human eosinophils recruited into the airways, as evidenced in the sputum of asthmatics (16) and in lung lavages after allergen challenges (17, 18), typically express MHC class II proteins. In murine models of asthma, endobronchial eosinophils migrate into regional pLNs (19–22). We previously demonstrated that murine airway eosinophils exposed to aerosolized Ag migrated from the airway lumina to elicit Ag-specific and CD80- and CD86-dependent $CD4^+$ T cell responses in the pLNs of previously Ag-sensitized mice (19). These findings established that eosinophils, as APCs, could both present Ag and costimulatory proteins to elicit responses of Ag-primed $CD4^+$ T cells, but did not assess whether eosinophils also exhibited the third requisite and critical defining attribute of professional APCs: their capacity to stimulate Ag-naive T cells.

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[3] Abbreviations used in this paper: DC, dendritic cell; pLN, paratracheal lymph node; MHC II, MHC class II; Tg, transgenic; BAL, bronchoalveolar lavage; i.t., intratracheal.

Studies in murine models of airway inflammation have suggested that eosinophils might function as professional APCs (21). Notably: 1) transgenic (Tg) eosinophil-ablated mice following OVA-airway challenge had lower bronchoalveolar lavage (BAL) levels of Th2 cytokines (23); 2) eosinophil-deficient IL-$5^{-/-}$/eotaxin$^{-/-}$ double knockout mice had diminished Ag-driven T cell IL-13 production that was corrected specifically by reconstitution with transferred eosinophils (24); and 3) eosinophil-deficient ΔdblGATA mice exhibited a profound defect in local lung Th2 cytokine production (25). Although these findings supported roles for eosinophils as potential professional APCs, one study reported that airway eosinophils were specifically incapable of activating naive T cells and hence were not professional APCs (20).

Eosinophils are not common in the normal lungs or airways (26, 27), so that in the absence of allergic inflammation, several cell types in the airways and parenchyma function as professional APCs resident within the lungs (28–30). In the context of allergic upper and lower airways diseases in which eosinophils are characteristically elicited, the capacity of eosinophils to serve as additional recruited "inflammatory" full-function APCs could be pertinent to Ag-elicited immune responses in the airways of those with often chronic, eosinophilic allergic diseases. To evaluate the capabilities of airway eosinophils as APCs, we ascertained the ability of airway eosinophils to initiate primary immune responses relevant to allergic airways diseases. We used a murine model in which eosinophils, exposed to OVA Ag, were introduced into the airways of mice that had received adoptively transferred unsensitized OVA-specific TCR Tg DO11.10 $CD4^+$ T cells, to evaluate whether airway eosinophils could function as professional APCs to stimulate naive OVA-specific $CD4^+$ T cells. We report that eosinophil APC presentation of OVA Ag stimulated naive OVA-specific $CD4^+$ T cells in regional pLNs to: 1) become activated, 2) proliferate, and 3) exhibit an IL-4 dominant, likely Th2-biased, cytokine response. Each of these naive T cell stimulatory responses was inhibited by exposing eosinophils to NH_4Cl that inhibits lysosomal Ag processing and MHC class II-mediated antigenic peptide presentation by APCs (31, 32). Moreover, we directly visualized cellular interactions of OVA Ag-loaded eosinophils with naive OVA-specific $CD4^+$ T cells in pLNs. Thus, airways eosinophils, as allergic disease recruited airway cells, fully function as distinct, allergic "inflammatory" APCs capable of initiating primary responses among Ag-naive $CD4^+$ T cells pertinent to allergic immune reactions.

Materials and Methods

Mice

BALB/c mice and DO11.10 OVA TCR Tg BALB/c mice were purchased from Charles River Laboratories. IL-5 BALB/c Tg mice (21) provided by Drs. Alison A. Humbles and Craig Gerard (Children's Hospital Medical Center, Boston, MA) and IL-5 Tg C3H/HeN mice provided by Dr. Akira Tominaga (Kochi Medical School, Kochi, Japan) (33) were both obtained pathogen-free from Charles River Laboratories. GFP Tg BALB/c mice were generated by Dr. Klaus Matthaei (The John Curtin School of Medical Research, Canberra, Australia). IL-5 and GFP double Tg mice were generated by crossing IL-5 Tg C3H/HeN mice and GFP Tg BALB/c mice. All double Tg mice used were fully backcrossed to a BALB/c background (>10 generations). Mice, housed in a pathogen-free facility, were used for experiments at 6–8 wk of age. Experimental protocols were approved by Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Isolation of cells

To isolate eosinophils, spleens of IL-5 Tg or IL-5 and GFP double Tg mice, a rich source of non-Ag-elicited or exposed eosinophils, were made into single cell suspensions by cutting spleens into small pieces, followed by mechanical disruption. In pelleted cells, RBCs were lysed by adding 5 ml 0.2% hypotonic NaCl for 15 s followed by an equal volume of 1.5% NaCl or adding 3 ml NH_4Cl lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 3 min. Eosinophils were purified as described previously (19) with additional modifications to assure the depletion of $CD11c^{high}$ DCs. In brief, single-cell suspensions of splenocytes from IL-5 Tg mice in PBS containing 1% FCS (PBS/FCS) were layered onto four-step discontinuous Percoll density gradients (1.085, 1.080, 1.075, and 1.070 g/ml) and centrifuged for 25 min at 1,500 × *g* at room temperature. Cells between the 1.085 and 1.080 g/ml layers were recovered and washed twice in PBS/FBS. After any remaining macrophages were removed by 1 h adherence on plastic petri dishes in RPMI 1640 supplemented with 5% FCS, eosinophils were isolated using magnetic-activated cell-sorting columns (Miltenyi Biotec). Specifically, B cells, T cells, and $CD11c^+$ DCs were removed by positive selection with mAb-conjugated micromagnetic beads specific for CD45-R (B220) B cells, CD90 (Thy 1.2) T cells, and CD11c (Miltenyi Biotec). Because the quantity of anti-CD11c beads suggested by the manufacturer substantially depleted eosinophils, one-tenth the amount of anti-CD11c beads was used to preferentially deplete $CD11c^+$ DCs. By negative selection, eosinophils were >99.8% pure as assessed by microscopy and flow cytometry. Viability by trypan blue exclusion of purified eosinophils was >98%. Before being used as APCs, eosinophils were cultured for 24 h with 10 ng/ml GM-CSF in RPMI 1640 supplemented with penicillin (200 U/ml), streptomycin (200 μg/ml), L-glutamine (2 mM), HEPES (10 mM), and 10% FCS. Ten μg of OVA or OVA-beads was added during the last 1 h of culture to expose eosinophils to Ag.

Naive $CD4^+$ T cells were purified from single cell suspensions of pLNs and spleens of DO11.10 OVA TCR Tg mice using the $CD4^+$ T cell isolation kit from Miltenyi Biotec, according to the manufacturer's instructions. Purity of isolated $CD4^+$ T cells was >96% as determined by flow cytometry.

Conjugation of OVA with fluorescent beads

Per a previously described protocol (2, 34), carboxylate-modified 0.431 μm diameter blue fluorescent beads (Sigma-Aldrich) were washed three times in sterile PBS by centrifugation at 14,000 × *g* for 15 min and resuspended in sterile PBS. Conjugation of beads to OVA was conducted in 25 mM MES (pH 6.1), 1 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, with beads at 7.5×10^{10} beads/ml, and 2 mg/ml OVA (all from Sigma-Aldrich). Conjugation reactions were rotated overnight in the dark at room temperature followed by two washes in 25 mM MES. Immediately before use, beads were washed twice in sterile PBS and resuspended at 7.5×10^{10} particles/ml. After conjugation, beads were stained with mouse anti-OVA mAb (OVA-14; Sigma-Aldrich) and then with a FITC-conjugated rat anti-mouse IgG1 (A85–1, BD Pharmingen) to assess the fraction of beads conjugated to OVA by flow cytometry. Approximately 80% of beads were positive for OVA as assessed by flow cytometry.

Adoptive transfer and immunofluorescent microscopy

Purified DO11.10 $CD4^+$ T cells were labeled with the red fluorescent dye, $DiIC_{16}$ (3), according to the manufacturer's protocol (Molecular Probes). Fluorescently labeled $CD4^+$ T cells (8×10^6) were washed twice in sterile PBS and adoptively transferred i.v. into naive WT BALB/c mice. Twenty-four hours after $CD4^+$ T cell transfer, GFP-labeled eosinophils (1×10^6) from IL-5/GFP double Tg mice loaded with OVA-beads or Ag free, were transferred intratracheal (i.t.) into recipient BALB/c mice. Mice were sacrificed at 12 or 24 h after eosinophil transfer and pLNs were harvested, frozen with liquid nitrogen, embedded in OCT compound, and stored at −70°C. Frozen tissues were cryosectioned, and sections were observed with an Olympus BX62 fluorescence microscope equipped with an internal rotary encoder. Separate blue, green, and red images for each section (8 μm) or serial sections (0.5 μm intervals) of selected areas were collected and overlaid by using IPLab 3.7 program (Scanalytics). Numbers of green eosinophils within a 100-μm diameter around each red $CD4^+$ T cell on lymph node sections were measured and counted by IPLab. Interactions between red DO11.10 $CD4^+$ T cells and green eosinophils bearing OVA-blue beads were quantified by using the colocalization module of IPLab. Results are expressed as mean percentage of red $CD4^+$ T cells (± SD) that overlap with green eosinophils. For each time-point, 36 red $CD4^+$ T cells were counted. Images were imported into Volocity 3.6 (Improvision) software and deconvoluted using theoretical Point Spread Function for each individual channel. Following deconvolution, images were rendered either in 2-D or 3-D.

Flow cytometry

For cell surface staining, cells were stained at 4°C for 30 min with saturating quantities of following mAbs or isotypic IgG: FITC-anti-MHC-II (MCA1501F), FITC-anti-CD40 (MCA1143F), FITC-anti-CD80 (MCA1586F), FITC-anti-CD86 (MCA1587F), and PE-anti-CD11c (N418)

(each from Serotec), and FITC-anti-CD11c (HL-3), FITC-anti-CD69 (H1.2F3), PerCP-Cy5.5-anti-CD4 (RM4–5), and PE-conjugated anti-DO 11.10 TCR (KJ1–26) (each from BD Pharmingen). The 2.4G2 mAb (BD Pharmingen) was used to block FcR-mediated nonspecific binding. To exclude contamination of eosinophils with DCs, purified eosinophils were stained with FITC hamster anti-mouse CD11c and analyzed by flow cytometry. The CD11c^{high} cells were flow cytometrically sorted (Mo-Flo; Dako Cytomation), stained with Hema 3 and observed by microscopy.

For intracellular staining, after surface staining, pLN cells were fixed and permeabilized in Cytofix/Cytoperm and washed twice in Perm/Wash solution (Cytofix/Perm kit; BD Biosciences). For intracellular staining, the following mAbs were used: FITC-anti-BrdU, FITC-IFN-γ (XMG1.2), and FITC-anti-IL-4 (11B11) (BD Pharmingen). Stained cells were analyzed on a BD Biosciences FACScan with CellQuest software (BD Biosciences).

DO11.10 CD4$^+$ T cell activation and proliferation assays

Eosinophils, treated with either hypotonic saline or NH_4Cl lysis buffer and pulsed with or without Ag OVA, were injected i.t. into naive BALB/c mice that had received i.v. transfusions of DO11.10 CD4$^+$ T cells (8×10^6) 24 h earlier. Seventy-two hours after eosinophil injection, pLNs were recovered and made into single cell suspensions. Transferred DO11.10 CD4$^+$ T cells were uniquely recognized by PE-anti-KJ1–26 mAb. For DO11.10 CD4$^+$ T cell activation, pLN cells were stained with FITC-anti-CD69 and analyzed by two-color flow cytometry. For DO11.10 CD4$^+$ T cell proliferation, mice were injected twice i.p. with 2 mg BrdU in 0.2 ml sterile PBS at 2 h intervals before harvest of pLNs. DO11.10 CD4$^+$ T cell proliferation was measured by BrdU incorporation using a BrdU Flow Kit from BD Biosciences.

Cytokine production by DO11.10 CD4$^+$ T cells

The pLN cells from recipient mice were stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) for 4 h in the presence of monensin (2 μM). LN cells were first surface stained with PerCP-Cy5.5-anti-CD4 and PE-anti-DO 11.10 TCR (KJ1–26). After fixation and permeabilization with the Cytofix/Cytoperm buffer, cells were stained with either FITC-anti-IFN-γ or FITC-anti-IL-4. Intracellular expression of cytokines within gated CD4$^+$ T cells was analyzed by three-color flow cytometry.

Statistics

Paired one-tailed (for NH_4Cl inhibition studies) or unpaired *t* tests were used to compare data (Excel, Microsoft).

Results

Exclusion of other contaminating APCs from purified eosinophils

Critical to evaluating the functions of eosinophils as APCs was ascertaining that purified eosinophils were not contaminated with other APCs. Eosinophils, isolated from spleens of IL-5 Tg mice by sequential density gradient centrifugation, adherent macrophage depletion, and immunomagnetic removal of contaminating B and T lymphocytes and CD11c^{high} DCs, were >99.8% pure, as determined by both flow cytometry and Hema 3 differential leukocyte staining. Eosinophils were identified by their donut-shaped nuclei and cytoplasmic staining with eosin (Fig. 1*A*). By flow cytometry, isolated eosinophils appeared in varying proportions as both CD11$^-$ and CD11c^{low} populations (Fig. 1*B*). To exclude contamination with CD11c$^+$ APCs, i.e., CD11c^{high} DCs, purified eosinophils were stained with FITC-labeled anti-mouse CD11c (HL-3), and CD11c^{high} cells were isolated by flow cytometric cell sorting. Among the purified eosinophils, even those cells exhibiting higher CD11c expression were morphologically and tinctorially eosinophils (Fig. 1*B*). The constitutive expression of CD11c on some isolated eosinophils was confirmed by staining with another PE-labeled anti-mouse CD11c mAb (N418) (Fig. 1*B*, *inset*), compatible with the recognized expression of CD11c on murine eosinophils (35, 36). Thus, contamination of purified eosinophils with DCs was excluded.

Up-regulation of eosinophil MHC II and costimulatory molecules by GM-CSF

In IL-5 Tg mice, splenic, in contrast to lung, eosinophils do not exhibit evidence of activation (37). We examined the expression of MHC II and candidate lymphocyte costimulatory proteins, CD40, CD80, and CD86, on splenic-derived eosinophils by flow cytometry. Unlike recruited airway eosinophils isolated from the BAL of OVA-sensitized and challenged mice (19), eosinophils from the spleens of IL-5 Tg mice showed lower level surface MHC II expression (Fig. 2). However, after incubation with GM-CSF for

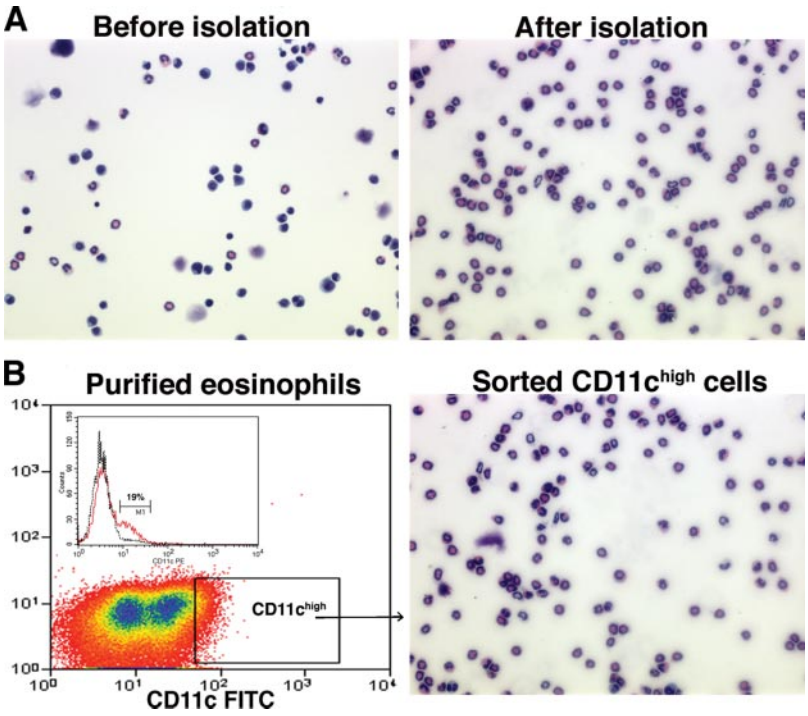

FIGURE 1. Isolated eosinophils are pure and free of CD11c^{high} DCs. *A*, Splenocytes from IL-5 Tg mice were stained with Hema 3 before and after isolation and purification of esoinophils, as described in *Materials and Methods*. Eosinophils were identified by their donut-shaped nucleus and eosinophilic cytoplasmic staining. *B*, To exclude contamination with CD11c$^+$ DCs, purified eosinophils, stained with FITC-labeled (HL-3) or PE-labeled (N418) (*inset*) anti-mouse CD11c, were analyzed by flow cytometry and CD11c^{high} cells were isolated by flow cytometric cell sorting. The sorted CD11c^{high} population stained with Hema 3 was morphologically and tinctorially eosinophils.

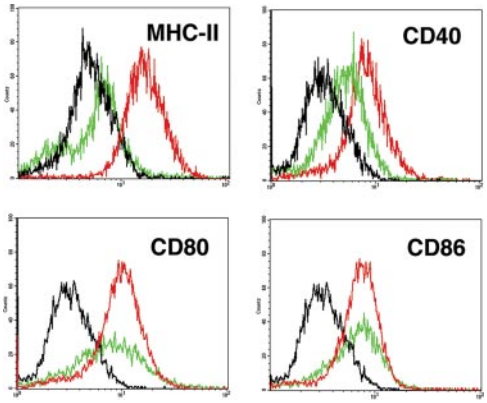

FIGURE 2. Effect of GM-CSF on expression of MHC II and costimulatory molecules by eosinophils isolated from spleens of IL-5 Tg mice. Fresh eosinophils (green) or eosinophils incubated with GM-CSF (10 ng/ml) for 24 h (red) were stained with FITC-labeled anti-MHC II, anti-CD40, anti-CD80 anti-CD86, or isotype control IgG (gray) mAbs. Flow cytometric data, with cell counts on the *y*-axis and fluorescence intensity on the *x*-axis, are representative of three experiments.

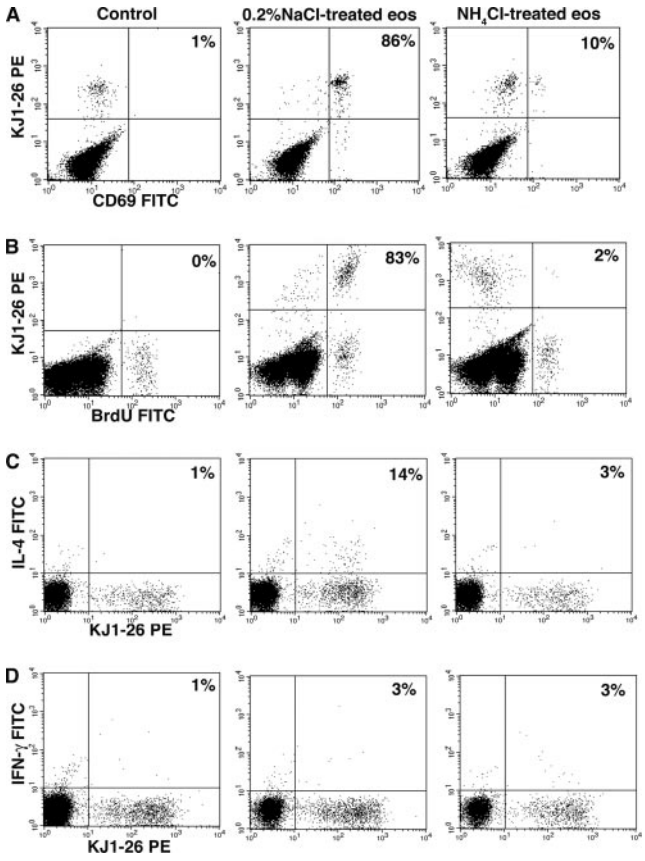

FIGURE 3. Airway eosinophils present OVA Ag to naive OVA-specific $CD4^+$ T cells. Eosinophils, exposed to 0.2% NaCl or NH_4Cl during RBC lysis, incubated with GM-CSF for 24 h, pulsed with OVA (*middle* and *right rows*) or without OVA (*left row*) for 1 h, were instilled i.t. into WT mice that received an i.v. infusion of OVA-specific TCR Tg $CD4^+$ T cells 24 h earlier. Seventy-two hours after eosinophil transfer, pLN cells were analyzed and stained with PE-KJ1–26 mAb specific for the OVA TCR. Responses of KJ1–26^+ OVA-specific T cells were evaluated for activation (CD69 expression) (*A*), proliferation (BrdU incorporation) (*B*), and intracellular cytokine expression for IL-4 (*C*) and IFN-γ (*D*), as detailed in *Materials and Methods*. In contrast to OVA-free control eosinophils (*left column*), airway eosinophils exposed to OVA Ag (*middle column*) elicited in OVA-specific naive T cells: activation (*A*), proliferation (*B*), and IL-4 (*C*) but not IFN-γ production (*D*). OVA-pulsed eosinophils exposed to NH_4Cl (*right column*) failed to elicit activation (*A*), proliferation (*B*), or IL-4 production by OVA-specific T cells (*C*). Noted percentages indicate percentages of KJ1–26^+ OVA TCR-specific T cells responding. Histograms represent analyses of pooled cells obtained from four mice per group. Data are representative of three (*A, C, D*) and two (*B*) experiments.

24 h, surface expression of MHC II on eosinophils increased (Fig. 2), and by microscopy and flow cytometry, eosinophils were the only cells present. In contrast, eosinophils isolated from spleens of IL-5 Tg mice constitutively expressed costimulatory CD40, CD80, and CD86 proteins, which for CD40 and CD80 were further upregulated by incubation with GM-CSF (Fig. 2). Thus, just as DCs are conventionally activated in vitro by culture with GM-CSF (20), in the following in vivo experiments, purified, spleen-derived eosinophils were cultured for 24 h with 10 ng/ml GM-CSF and during the last hour of culture were exposed to 10 μg of OVA or OVA-beads before being adoptively transferred as APCs.

In vivo priming of naive DO11.10 $CD4^+$ T cells by eosinophil APCs

Eosinophils purified from the spleens of IL-5 Tg mice were used to examine whether interactions between OVA-exposed eosinophils and DO11.10 OVA-specific TCR Tg $CD4^+$ T cells in pLNs lead to stimulation of Ag-naive $CD4^+$ T cells. Eosinophils, pulsed with OVA for 1 h in vitro, were instilled i.t. into normal recipient mice that had received i.v. adoptive transfers of DO11.10 $CD4^+$ T cells 24 h earlier. Three days after i.t. administration of eosinophils, isolated regional pLN cells from recipient mice were stained with PE-KJ1–26 mAb, a clonotypic mAb that specifically recognizes the OVA-specific TCR on DO11.10 $CD4^+$ T cells. Activation of DO11.10 $CD4^+$ T cells was evaluated by their expression of the early T cell activation marker, CD69. Data in Fig. 3 represent one of three replicate experiments. As exemplified in one experiment in Fig. 3*A*, overall 50.3 ± 31.4% (mean ± SD, $n = 3$) of OVA-specific naive DO11.10 $CD4^+$ T cells in pLNs of recipient mice were activated ($CD69^+$) by OVA-exposed eosinophils, whereas only 0.6 ± 0.5% (mean ± SD, $n = 3$; $p = 0.026$) of DO11.10 $CD4^+$ T cells from mice that received control Ag-free eosinophils expressed CD69. OVA-pulsed eosinophils, exposed to NH_4Cl RBC lysis buffer, failed to stimulate DO11.10 $CD4^+$ T cell activation ($CD69^+$) (3.7 ± 3.5%, $n = 3$; $p = 0.032$).

In addition to eliciting the activation of OVA-specific DO11.10 $CD4^+$ T cells, OVA Ag presentation by airway eosinophils stimulated proliferation of OVA-specific DO11.10 $CD4^+$ T cells in pLNs. As evaluated by flow cytometric analyses of in vivo BrdU incorporation (as demonstrated in Fig. 3*B* for one of two replicate experiments), overall 70.8 ± 17.7% (mean ± SD, $n = 2$) of OVA-specific naive DO11.10 $CD4^+$ T cells in pLNs of recipient mice were BrdU positive, whereas only 2.9 ± 0.9% (mean ± SD, $n = 2$; $p = 0.016$) of DO11.10 $CD4^+$ T cells from mice that received OVA-pulsed eosinophils pretreated with NH_4Cl.

Eosinophil APC-mediated biasing of Th2 responses by primed DO11.10 $CD4^+$ T cells

Allergic asthma is characterized by Th2 cytokine-driven chronic lung inflammation. To assess whether eosinophils, such as OVA Ag APCs, might contribute to biasing of OVA-specific DO11.10 $CD4^+$ T cell responses toward a Th2 phenotype, we evaluated intracellular IL-4 and IFN-γ cytokine production by DO11.10 $CD4^+$ T cells by three-color flow cytometry. Compared with control mice that received OVA Ag-free eosinophils (as shown in Fig. 3*C* for one of three experiments), i.t. instillation of OVA-exposed

eosinophils significantly increased IL-4 production by pLN DO11.10 $CD4^+$ T cells (22.0 ± 12.5% vs 1.9 ± 1.4%, mean ± SD, $n = 3$, $p = 0.024$). In contrast, eosinophils pretreated with NH_4Cl did not elicit IL-4 by DO11.10 $CD4^+$ T cells (1.9 ± 1.4% vs 1.3 ± 0.9%, mean ± SD, $n = 3$, $p = 0.469$). For a candidate Th1 cytokine IFN-γ, there was no augmented IFN-γ production above baseline by DO11.10 $CD4^+$ T cells with no differences among the groups of Ag-free and OVA-pulsed eosinophils with 0.2% NaCl or NH_4Cl RBC lysis (as exemplified in Fig. 3*D*). Thus, eosinophil OVA Ag APC function can stimulate the activation and proliferation of OVA Ag-specific naive $CD4^+$ T cells and promote an IL-4-biased Th2-like response in these responding $CD4^+$ T cells.

Eosinophil APC and $CD4^+$ T cell Ag-specific cellular interactions in vivo

Physical interactions between Ag-loaded APCs and Ag-specific $CD4^+$ T cells in secondary lymphoid organs are necessary for initiation of primary $CD4^+$ T cell responses (38). To evaluate eosinophil OVA APC interactions with OVA-specific $CD4^+$ T cells in vivo, we used a system in which fluorescent bead-conjugated OVA Ag (34), eosinophils, and OVA Ag-specific naive $CD4^+$ T cells could be monitored in pLNs after adoptive i.t. transfer into normal recipient mice. GFP-expressing eosinophils from the spleens of GFP and IL-5 double Tg mice, with and without exposure to OVA-conjugated blue fluorescent beads, were instilled i.t. into WT recipient mice that adoptively received i.v. transfusions of red fluorescent dye $DiIC_{16}$-labeled (3) OVA Ag-specific $CD4^+$ T cells from OVA TCR Tg DO11.10 mice 24 h earlier. Over 24 h following i.t. eosinophil instillation, GFP-green eosinophils and red T cells could be visualized in pLNs. In contrast to eosinophils not bearing OVA-beads (Fig. 4*A*), those containing OVA-blue beads were more frequently proximate to red OVA-specific T cells (Fig. 4*B*). Enumerating numbers of green eosinophils within a 100-μm diameter around red T cells revealed that there were 2.5 ± 2.4 (mean ± SD, $n = 22$) Ag-free eosinophils in proximity to OVA-specific $CD4^+$ T cells vs 6.3 ± 3.5 (mean ± SD, $n = 22$) OVA-blue/green eosinophils proximate to OVA-specific $CD4^+$ T cells. Moreover, as early as 12 h after eosinophil instillation, GFP-green eosinophils carrying fluorescent blue OVA-conjugated beads localized in clusters with red OVA-specific DO11.10 $CD4^+$ T cells (Fig. 4*C*). Yellow-colored clusters, reflecting overlap between red $CD4^+$ T cells and green/blue eosinophils, were even more prominent in pLNs 24 h after i.t. instillation of OVA-bead bearing eosinophils (Fig. 4*D*). At 12 h, 26 ± 9% (mean ± SD, $n = 36$) of red T cells overlapped with green eosinophils and, by 24 h, 42 ± 4% (mean ± SD, $n = 36$) of red T cells overlapped with green eosinophils. Ag-free green eosinophils did not form yellow clusters with red T cells (data not shown). Analyses of colocalized OVA-blue bead bearing green eosinophils with red OVA-specific DO11.10 $CD4^+$ T cells (Fig. 5) by deconvolution and rendering as 2-D (Fig. 5*C*) or 3-D (Fig. 5*D*) images confirmed the intimate cell-cell interactions of OVA-presenting eosinophil APCs with OVA-specific $CD4^+$ T cells.

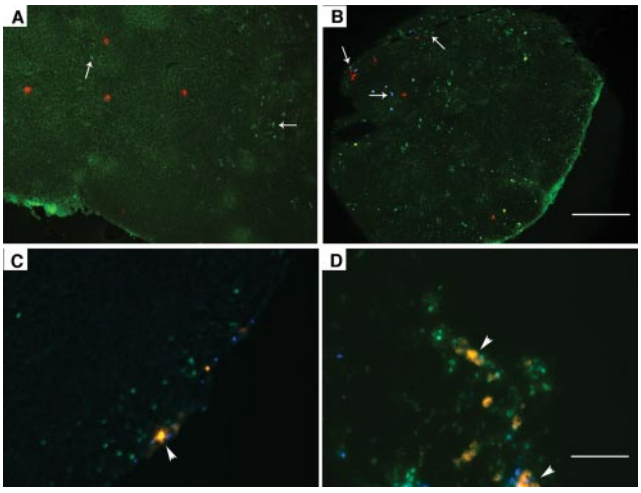

FIGURE 4. OVA Ag-loaded, but not Ag-free, eosinophils from the airways interact with naive OVA Ag-specific $CD4^+$ T cells in regional pLNs. *A*, OVA-free (green) or blue fluorescent bead OVA-loaded (*B*, green and blue) GFP Tg eosinophils, as indicated by arrows, were i.t. instilled into the mice that received i.v. red fluorescent dye loaded OVA TCR-specific $CD4^+$ T cells 24 h earlier. Twenty-four hours after eosinophil transfer, pLNs were harvested, frozen, sectioned, and observed by fluorescence microscopy. OVA-specific $CD4^+$ T cells exhibited greater proximity to OVA-bearing eosinophils (*B*) than to OVA-free (*A*) eosinophils in pLNs. In addition, physical interactions between OVA-loaded blue/green eosinophils and red OVA-specific $CD4^+$ T cells formed yellow-colored clusters of colocalized eosinophils and T cells by 12 h (*C*) and to a greater extent by 24 h (*D*) after i.t. transfer of eosinophils, as indicated by the arrowheads. Scale bars: *B*, 100 μm; *D*, 50 μm.

Discussion

In allergic diseases of the upper and lower airways, eosinophils are characteristically recruited from the bloodstream into the tissues and lumina of the airways and are present in respiratory secretions from the nose and lungs. As such, intraluminal and tissue eosinophils are exposed to inhaled allergens. In the present study, we assessed whether recruited airway eosinophils might serve as distinct "inflammatory" APCs for airway Ags in mediating T cell responses. Normal lungs contain several cell types, DCs, B cells, and macrophages, in their airways and parenchyma capable of acting as professional APCs (28–30). Native lung myeloid DCs, likely indicative of regulatory mechanisms that restrain local lung T cell activation, lack the full functional capabilities of Ag-processing APCs in that they can present antigenic peptides but not whole proteins to naive $CD4^+$ T cells (28). Lung myeloid DCs, however, acquire the full capabilities of professional APCs following in vitro exposures to cytokines, such as GM-CSF, or in vivo trafficking into regional LNs (28). Although the capacity of airway eosinophils to function specifically as professional APCs

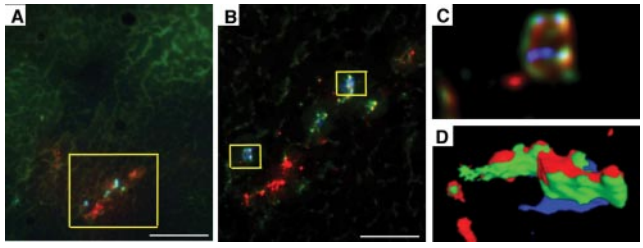

FIGURE 5. Imaging of OVA-Ag presenting eosinophil APCs with OVA Ag-specific $CD4^+$ T cell interactions in situ. pLNs were harvested from mice 24 h after i.t. administration of blue fluorescent bead OVA-loaded green GFP eosinophils and 48 h after i.v. injection of red fluorescent dye labeled OVA TCR $CD4^+$ T cells. *A*, Blue OVA Ag-loaded green eosinophils interacted with red OVA-specific $CD4^+$ T cells in pLNs. Selected areas in *A* were further sectioned at 0.5 μm intervals and *B* images were imported into software Volocity 3.6. Following deconvolution using theoretical Point Spread Function for each individual channel, images of the framed areas in *B* were rendered either in 2-D (*C*) or 3-D (*D*), demonstrating intimate cell-cell interactions between blue/green OVA-presenting eosinophils and red OVA-specific $CD4^+$ T cells.

was not demonstrable in a prior study (20), we considered mechanisms pertinent to eosinophil APC functions and re-evaluated the capabilities of airway eosinophils to function in vivo as professional APCs in activating unprimed, naive T cells.

Eosinophils, purified from spleens of IL-5 Tg mice using in part immunodepletion of $CD11c^+$ DCs with anti-CD11c mAb, were free of $CD11c^{high}$ DCs and exhibited $CD11c^-$ and $CD11c^{low}$ populations (Fig. 1). CD11c expression by murine eosinophils has been recognized on intrathymic eosinophils (36) and as a unimodal $CD11c^{low}$ staining pattern on blood eosinophils from helminth-infected mice (35) and on BAL eosinophils from OVA-sensitized and airway-challenged mice (39). Human blood eosinophils express low levels of CD11c (40). In our purified murine IL-5 Tg splenic eosinophils, eosinophils exhibited bimodal expression of $CD11c^-$ and $CD11c^{low}$ populations. The bimodal expression of CD11c was not an artifact of the purification protocol because splenic eosinophils, analyzed directly without further purification based on side scatter and $CCR3^+$ gating of IL-5 Tg splenocytes, likewise showed $CD11c^-$ and $CD11c^{low}$ populations (data not shown). Exposure of eosinophils to GM-CSF in vitro did not enhance CD11c expression (data not shown). Depletion of $CD11c^+$ DCs with the manufacturer's suggested quantity of anti-CD11c beads diminished the recovery of eosinophils (data not shown), raising the possibility that experimental strategies that aim to selectively deplete $CD11c^+$ DCs may also deplete $CD11c^+$ eosinophils.

Eosinophils from spleens of IL-5 Tg mice do not exhibit signs of activation ($CD62L^{low}$, Siglec-F^{high}) as present on eosinophils recruited into the lungs, thymus, and lymph nodes (37). Likewise, we found that MHC II expression was low on IL-5 Tg splenic eosinophils but increased after 24-h incubation with GM-CSF (Fig. 2). Increased levels of MHC II expression have been demonstrated on eosinophils recovered from the BAL of OVA-sensitized, airway challenged mice (19, 22), and eosinophil MHC II was further upregulated after eosinophil migration into draining pLNs (22). In humans, despite absent MHC II expression on blood eosinophils, eosinophils recruited into airways of subjects with asthma and other eosinophilic lung diseases (16) or following inhalational allergen challenges (17, 18) express HLA-DR. The airways may be a site where GM-CSF, and not IL-5, activates eosinophils (41, 42). Human lung microvascular endothelial cells express GM-CSF (43), and transendothelial migration of eosinophils, as occurs when bloodstream eosinophils enter tissues, increases eosinophil HLA-DR expression (44). Thus, both human and murine eosinophils from sites of airways allergic inflammation express MHC II. Isolated murine splenic eosinophils also expressed lymphocyte costimulatory proteins CD40, CD80, and CD86, which for CD40 and CD80 were further increased following incubation with GM-CSF (Fig. 2). Enhanced expression of CD80 and CD86 on Ag-elicited and airways recruited airway eosinophils has been previously documented (19). In addition, eosinophils recruited into pLNs following airway allergen challenge have exhibited increased MHC II, CD86, and CD80 expression and to a lesser extent CD40 expression and have shown a $CD62L^{low}$-activated phenotype (22). Thus, akin to maturation events needed to enhance the functions of pulmonary DCs as full APCs (28), eosinophils likewise, in their responses to cytokines, such as GM-CSF, and their recruitment into the airways lumina, tissues and regional LNs, manifest activated phenotypic responses that could enhance their functional roles as airway APCs.

To address the capacity of airway eosinophils to activate unprimed, Ag-naive $CD4^+$ T cells, we investigated whether eosinophils instilled i.t. could stimulate responses of naive OVA TCR Tg T cells that were adoptively transferred into recipient mice. In contrast to control eosinophils not exposed to OVA Ag, eosinophils pulsed with OVA for 1 h led to enhanced activation (CD69 expression) and proliferation (BrdU expression) of pLN OVA-specific $CD4^+$ T cells (Fig. 3, *A* and *B*). Treatment of eosinophils with NH_4Cl prevented OVA-pulsed eosinophils from stimulating the activation or proliferation of pLN OVA-specific $CD4^+$ T cells. NH_4Cl, like chloroquine, inhibits lysosomal acidification and blocks Ag processing and presentation by APCs, including eosinophils (31, 32, 45). That exposures of eosinophils to NH_4Cl early in their purification inhibited eosinophil APC function suggests that eosinophils were directly engaged in the intracellular processing of OVA protein and the presentation of OVA peptide and were not likely passively by crosspresentation carrying OVA protein to other host APCs in recipient mice for Ag presentation. In support of this interpretation, we have previously shown in a foot pad injection model that the capacity of OVA-pulsed eosinophils to elicit proliferative T cell responses in regional lymph nodes of sensitized mice required chloroquine-inhibitable lysosomal processing and was MHC-restricted (our unpublished data).

In addition to eliciting activation and proliferation of DO11.10 $CD4^+$ T cells, OVA-pulsed eosinophils adoptively transferred into the airways also stimulated OVA-specific DO11.10 pLN $CD4^+$ T cells to produce IL-4 and not IFN-γ following ex vivo stimulation with anti-CD3 and anti-CD28, suggesting that eosinophils as APCs presenting airway Ags can elicit a Th2-biased profile of cytokine formation by responding $CD4^+$ T cells. Again, NH_4Cl-exposed eosinophils were incapable of enhancing IL-4 production from stimulated OVA-specific $CD4^+$ T cells.

If airway eosinophils function as professional APCs to stimulate naive OVA-specific T cells in pLN, cell-cell interactions between OVA-presenting eosinophils and responding T cells should be demonstrable within regional LNs, as documented with other typical professional APCs (46, 47). By using GFP Tg eosinophils loaded with OVA Ag covalently conjugated to blue fluorescent beads and $CD4^+$ T cells from DO11.10 OVA TCR Tg mice tagged with a red fluorochrome, we visualized in situ the arrival of Ag-loaded airway eosinophils into pLNs and physical interactions between eosinophil APCs and naive OVA Ag-specific $CD4^+$ T cells. In contrast to control Ag unexposed GFP eosinophils, OVA-bearing eosinophils were increasingly, from 12 to 24 h, more proximate to OVA-specific T cells. Moreover, cell-cell interactions of green eosinophils bearing blue OVA Ag-bearing beads with red OVA-specific DO11.10 OVA TCR $CD4^+$ T cells were visualized as yellow clusters (Fig. 4) and following deconvolution as intimate eosinophil-T cell associations (Fig. 5).

Our findings not only demonstrate that airway eosinophils can function as unique "inflammatory" professional APCs, but also provide an explanation for why a prior study found that airway eosinophils that migrated well into regional pLNs failed to display professional APC function for naive T cells (20). In that study, eosinophils also failed to exhibit enhanced MHC II expression after culture with GM-CSF and failed when pulsed with $OVA_{323–339}$ peptide to elicit DO11.10 OVA TCR $CD4^+$ T cell proliferation in vitro (20). Differences between the prior findings and ours likely are based on methods of eosinophil isolation used in the prior study. In that study, eosinophils were exposed to NH_4Cl incidentally during RBC lysis. Lysosomotropic NH_4Cl blocks intracellular Ag processing and presentation (31) and has inhibited murine eosinophil APC function in vitro (45). In our comparisons of eosinophils isolated with alternative RBC lysis solutions, hypotonic saline or NH_4Cl, eosinophils exposed to NH_4Cl were specifically devoid of the three measures of professional APC

function that we evaluated (naive $CD4^+$ T cell activation, proliferation and cytokine synthesis) (Fig. 3). NH_4Cl also inhibits MHC II-mediated exogenous antigenic peptide presentation by APCs (32) and enhanced cell surface expression of MHC II proteins (48), perhaps helping to explain why the prior study (20) could not show eosinophil presentation of $OVA_{323–339}$ peptide or GM-CSF-elicited up-expression of MHC II on murine eosinophils as we (Fig. 2) and others (45) have found. Thus, exposures of eosinophils to NH_4Cl during RBC lysis could account for the inability of previously studied eosinophils (20) to exhibit professional APC function in stimulating unsensitized, naive OVA-specific $CD4^+$ T cells.

The lungs natively contain a range of cells capable of being induced to function as professional APCs, including plasmacytoid DCs, types of $CD11c^+$ myeloid DCs, B cells, and macrophages (28, 30). Unlike the gastrointestinal tract where eosinophils normally home and might be exposed to gut-derived Ags (26), eosinophils are not abundant in the normal lungs or airways (26, 27). In contrast, recruitment of eosinophils into the upper and lower airways is a frequent concomitant of allergic inflammation (4). It is in this setting of allergic airways diseases that recruited eosinophils might function not simply as effectors of local inflammation, but also as "inflammatory" full-function APCs in processing and presenting airway Ags. Information is lacking on eosinophil localization within regional LNs in humans with asthma or other allergic airways disorders, but in murine models (19–22) eosinophils migrate from the airways lumina into regional LNs, providing a mechanism for Ags inhaled into airways to be processed and transported into tissues for presentation to lymphocytes. Eosinophils recruited to the airways in concert with allergic inflammation, and especially as they traffic to regional LNs, exhibit enhanced MHC II and lymphocyte costimulatory protein (CD80, CD86, CD40) expression (19, 22). In addition to the previously demonstrated abilities of airway eosinophils to activate responses of sensitized $CD4^+$ T cells (19, 21), our studies now demonstrate that airway eosinophils act as professional APCs to fully activate and elicit proliferation of Ag-naive $CD4^+$ T cells and likely enhance their cytokine synthesis in favor of an IL-4 Th2-like response (Fig. 3).

Recognition that eosinophils can function as professional APCs to initiate T cell responses in Ag-naive recipients would help explain prior findings that: 1) Ag-loaded eosinophils, when transferred into naive recipient mice, primed for Th2 cell-driven allergic lung disease (21), 2) eosinophil-deficient IL-$5^{-/-}$/eotaxin$^{-/-}$ double knockout mice exhibited diminished Ag-driven T cell IL-13 production that was corrected specifically by reconstitution with adoptively transferred eosinophils before Ag sensitization (24), and 3) Ag-induced IL-4, IL-5, and IL-13 production by splenocytes was reduced in eosinophil deficient compared with WT mice (25).

Our findings do not diminish roles of natively resident lung and airway DCs and other cells as APCs (10, 28, 30), but rather establish that eosinophils, cells of the innate immune system, when recruited into sites of allergic inflammation may have additional roles as "inflammatory" APCs in modulating ongoing adaptive T cell-mediated immune responses. In the context of usually chronic, allergic inflammation that accompanies nasal polyposis, allergic rhinoconjunctivitis, and asthma, airway eosinophils have the capacity to provide additional functions as APCs. Recruited airway eosinophils exposed to inhaled allergens have been induced to express MHC II and lymphocyte costimulatory proteins, migrate facilely to regional LNs, cognitively interact with LN T cells, and stimulate even Ag-naive T cell activation, proliferation, and cytokine synthesis, likely with an IL-4 Th2-type bias. Moreover, eosinophils are sources of multiple immunomodulatory cytokines readily available for secretion (5). With their capacities to contribute to the initiation and amplification of immune responses to inhaled allergens, roles for eosinophils as APCs may be important in mediating allergen-induced Th2 responses in the lungs and upper airways, which provides novel insights into the capacity of an innate immune inflammatory cell to function as an inflammatory APC contributing to adaptive immune responses that may sustain or modulate the characteristically chronic nature of allergic diseases.

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Disclosures

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