Airway Eosinophils Accumulate in the Mediastinal Lymph Nodes but Lack Antigen-Presenting Potential for Naive T Cells[1]

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Asthma is characterized by infiltration of the airway wall with eosinophils. Although eosinophils are considered to be effector cells, recent studies have reported their ability to activate primed Th2 cells. In this study, we investigated whether eosinophils are capable of presenting Ag to unprimed T cells in draining lymph nodes (DLN) of the lung and compared this capacity with professional dendritic cells (DC). During development of eosinophilic airway inflammation in OVA-sensitized and challenged mice, $CCR3^+$ eosinophils accumulated in the DLN. To study their function, eosinophils were isolated from the bronchoalveolar lavage fluid of mice by sorting on $CCR3^+B220^-CD3^-CD11c^{dim}$ low autofluorescent cells, avoiding contamination with other APCs, and were intratracheally injected into mice that previously received CFSE-labeled OVA TCR-transgenic T cells. Eosinophils did not induce divisions of T cells in the DLN, whereas DC induced on average 3.7 divisions in 45.7% of T cells. To circumvent the need for Ag processing or migration in vivo, eosinophils were pulsed with OVA peptide and were still not able to induce T cell priming in vitro, whereas DC induced vigorous proliferation. This lack of Ag-presenting ability was explained by the very weak expression of MHC class II on fresh eosinophils, despite expression of the costimulatory molecules CD80 and ICAM-1. This investigation does not support any role for airway eosinophils as APCs to naive T cells, despite their migration to the DLN at times of allergen exposure. DC are clearly superior in activating T cells in the DLN of the lung. *The Journal of Immunology,* **2003, 171: 3372–3378.**

Airway mucosal eosinophilia is one of the hallmarks of allergic asthma. Airway eosinophilia is controlled by allergen-specific Th2 cells. In response to Ag presentation by dendritic cells (DCs),[3] Th2 cells release several inflammatory cytokines inducing adhesion molecules on endothelium and recruiting eosinophils to the inflamed airways (1). Eosinophils are considered to be terminal effector cells (1–3). By releasing numerous proinflammatory mediators and cytokines as well as cationic proteins, they damage lung epithelium and account for many of the histopathologic abnormalities of asthma (4). However, studies showing expression of MHC class II (MHCII) and the costimulatory molecules CD80 and CD86 by eosinophils (5–10) suggested a possible function as APCs. Ag presentation by eosinophils is getting more and more consideration. Murine eosinophils derived from bronchoalveolar lavage (BAL) fluid of sensitized and challenged mice were reported to stimulate sensitized Th2 cells in vivo, although stimulation was read out ex vivo (7). Eosinophils derived from the peritoneal cavity of IL-5-transgenic (Tg) mice were capable of sensitizing mice after repeated i.p. injections, although the mechanism of T cell priming was not investigated in a direct manner (8).

As yet no studies have addressed the question whether eosinophils obtained from the bronchoalveolar compartment of inflamed lungs are able to directly activate Ag-specific naive T cells. Therefore, we investigated the Ag-presenting potential of eosinophils for naive T cells in vitro and in vivo and compared it with Ag presentation by professional Ag-presenting DCs. Bronchoalveolar lavage fluid (BALF) eosinophils from allergic lungs were isolated from the lungs of mice with experimental murine asthma using a new flow cytometric sorting method, based on scatter characteristics and staining for the eotaxin receptor CCR3, at the same time avoiding contamination with other BALF APCs such as macrophages, DCs, and B cells (11, 12). To detect T cell priming in vivo and in vitro, T cell proliferation was studied in OVA TCR Tg T cells (DO11.10 T cells) labeled with the mitosis-sensitive dye CFSE. Our data show that although eosinophils accumulate in the draining lymph nodes during eosinophilic airway inflammation and are able to induce some proliferation in effector T cells in vitro, they are not capable of inducing T cell proliferation in OVA-specific naive T cells.

Materials and Methods

Animals

All experiments were performed with 8- to 10-wk-old female BALB/c ($H\text{-}2^d$) mice (Harlan, Zeist, The Netherlands) and DO11.10 mice (Erasmus Medical Center, Rotterdam, The Netherlands). Mice were housed under specific pathogen-free conditions at the animal care facility at the Erasmus University Rotterdam. All of the experimental procedures used in this study were approved by the Erasmus University Committee of Animal Experiments.

Generation of eosinophilic airway inflammation

To induce sensitization to inhaled OVA, bone marrow-derived DCs were pulsed with OVA in vitro and subsequently injected into the airways of naive mice (11). In short, bone marrow cells were cultured for 10 days in tissue culture medium (5% FCS; Biocell Laboratories, Rancho Dominguez, CA), RPMI 1640, gentamicin, 2-ME (all from Life Technologies, Paisley, U.K.) supplemented with 20 ng/ml recombinant murine GM-CSF (13). After 9 days of culture, cells were pulsed overnight with 100 μg/ml OVA (OVA-DC) (OVA, Worthington Biochemical, Lakewood, NJ). On day 10 of culture, cells were collected, washed, and 1×10^6 DCs were injected

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[3] Abbreviations used in this paper: Dc, dendritic cell; MHCII, MHC class II; BAL, bronchoalveolar lavage; BALF, BAL fluid; i.t., intratracheal; Tg, transgenic; PI, propidium iodide.

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intratracheal (i.t.) in naive mice (11), followed 10 days later by a booster of 10 μg OVA absorbed in 1 mg alum adjuvant i.p. injection. Ten days after boosting, mice were challenged four times with a daily 30-min OVA aerosol (grade III, 1% w/v in PBS; Sigma-Aldrich, St. Louis, MO).

Flow cytometric analysis on BALF and lymph node eosinophils

Twenty-four hours after the last aerosol, mice were sacrificed by avertin overdose followed by exsanguination. BAL was performed with 3×1 ml of Ca^{2+}- and Mg^{2+}-free PBS supplemented with 0.1 mM EDTA. RBCs were lysed using ammonium chloride lysis buffer. BALF cells and cell suspensions of lung draining and nondraining lymph nodes were stained with the following monoclonals: MHCII-FITC (2G9), allophycocyanin-labeled CD11c (HL3), and CyChrome-labeled Abs against CD3 (145-2C11) and B220 (RA3-6B2) and PE-labeled CD80, CD86, ICAM-1 (BD PharMingen, San Diego, CA), or CCR3 (83101.111; R&D Systems, Abingdon, U.K.). To prevent a specific binding to FcR, 2.4.G2 blocking reagent was used. Eosinophils were recognized as nonautofluorescent highly granular (SSC^{high}) cells expressing the eotaxin receptor CCR3, intermediate levels of CD11c, and very low or lacking expression of MHCII, B220, and CD3. In indicated experiments, propidium iodide (PI; Sigma-Aldrich) was used to determine viability of the cells.

Purification and Ag pulsing of airway eosinophils

Eosinophils were purified from BALF under sterile conditions on a FACS DIVA flow cytometer (BD Biosciences, Mountain View, CA). BALF cells were stained as described above and eosinophils were recognized and sorted as nonautofluorescent highly granular (SSC^{high}) cells expressing the eotaxin receptor CCR3, intermediate levels of CD11c, and lacking expression of B220 and CD3. Purity of >96% was determined by H&E staining on cytospins of sorted eosinophils. Four hundred cells were counted per cytospin. In some experiments, eosinophils were pulsed ex vivo with 10 μg/ml $OVA_{323–339}$ peptide (Ansynth Service, Roosendaal, The Netherlands) for 45 min at 37°C or cultured overnight with or without GM-CSF (2 ng/ml) in the presence of $OVA_{323–339}$ peptide. The Ag-presenting capacity of eosinophils was compared with that of well-known professional APCs, bone marrow-derived OVA-pulsed DCs grown in GM-CSF as previously described (11–13).

Ag presentation assay to OVA TCR Tg T cells in vivo

Cell suspensions were made of pooled peripheral lymph nodes (cervical, mediastinal, brachial, axillary, inguinal, and mesenteric) from DO11.10 mice and were labeled with the mitosis-sensitive dye CFSE (Molecular Probes, Eugene, OR) as previously described (14). $CFSE^+$ T cells (10×10^6) were transferred i.v. into naive mice. Twenty-four hours after T cell adoptive transfer, either 1×10^6 purified eosinophils, 1×10^6 OVA peptide-pulsed eosinophils, or 1×10^6 OVA-pulsed DCs (11, 12) were transferred i.t. or 2×10^6 OVA peptide-pulsed eosinophils i.p. Seventy-two hours after immunization, lung draining lymph nodes of recipient mice were analyzed for the proliferation of CFSE-labeled OVA TCR Tg T cells on a FACSCalibur flow cytometer using FlowJo software (Treestar, Costa Mesa, CA). As a control, brachial lymph nodes were also harvested to compare division of T cells in lymph nodes not draining the lung. Transferred OVA TCR Tg $CD4^+$ T cells were recognized as $CFSE^+$/$Kj1.26^+$(MM7504; Caltag Laboratories, Burlingame CA)/$CD4^+$ cells; PI (Sigma-Aldrich) was used to exclude dead cells. To prevent a specific binding to FcR, 2.4.G2 blocking reagent was used.

Ag presentation assay to OVA TCR Tg T cells in vitro

Eosinophils, bone marrow DCs, and OVA TCR Tg T cell suspensions were prepared as described above. To obtain unstimulated T cells, DO11.10 lymph node cell suspensions were depleted from MHCII, B220, CD11b-positive cells by Dynal beads (Dynal, Etten-Leur, The Netherlands) to prevent stimulation by endogenous APCs. To obtain effector T cells, DO11.10 lymph node suspensions were cocultured with OVA-pulsed DCs for 2 wk; fresh DCs were added after 1 wk. After 2 wk, $CD4^+$ cells were isolated using a negative CD4 T cell isolation kit for the auto-MACS (Miltenyi Biotec, Gladbach, Germany) with a purity of 99%, of which 75% had an effector T cell phenotype ($CD62L^{low}$/$CD44^{high}$). The kit was used according to the manufacturer's instructions. Cells were rested overnight in 50 U/ml recombinant human IL-2 (PeproTech, Rocky Hill, NJ) before coculture with eosinophils. Fifty thousand $CFSE^+$ unstimulated or effector T cells were cocultured with 1×10^3, 1×10^4, or 1×10^5 either unpulsed or peptide-pulsed eosinophils in comparison to 1×10^3, 1×10^4, or 1×10^5 bone marrow-derived OVA-pulsed or unpulsed DCs. Seventy-two hours later, stimulation of naive T cells or effector T cells was analyzed by staining cocultures with Kj1.26-PE and CD4-APC; PI was used to exclude dead cells. To prevent a specific binding to FcR, 2.4.G2 blocking reagent was used.

Results

OVA exposure induces accumulation of eosinophils in BALF and lung draining lymph nodes of OVA DC-immunized mice

Sensitization was induced by i.t. injection of 1×10^6 OVA-pulsed DCs, followed by a booster of OVA/alum i.p. 10 days later. Sensitized mice were challenged with four OVA aerosols. Mice were sacrificed 24 h after the last challenge. Differential analysis of BALF cells showed a distinct increase in eosinophils. Using flow cytometry, several markers were used to determine eosinophils in BALF. Eosinophils were characterized as nonautofluorescent highly granular (SSC^{high}) cells expressing intermediate levels of CD11c and lacking expression of B220 and CD3, as described previously (12) (see also Fig. 3). These highly granular cells also expressed the eotaxin receptor CCR3 (15). Average percentage of eosinophils in BALF after challenge was 40–70%. In addition to the induced eosinophilic airway inflammation, also other hallmarks were induced as goblet hyperplasia and Th2 cytokine production by draining lymph nodes (data not shown).

To investigate whether airway inflammation increased the number of eosinophils in the draining lymph nodes of the lung, where APCs present Ag to naive T cells, lymph nodes were collected, homogenized, and analyzed for the presence of $CCR3^+$ eosinophils. Draining lymph nodes of the inflamed lungs showed an increased number of eosinophils compared with lymph nodes not draining the lungs and to lung draining lymph nodes from naive animals (3.6×10^4 vs, respectively, 246 ($p = 0.008$) and 52 ($p = 0.008$; Fig. 1).

To determine whether eosinophils in the bronchoalveolar compartment are expressing the molecules needed to prime T cells,

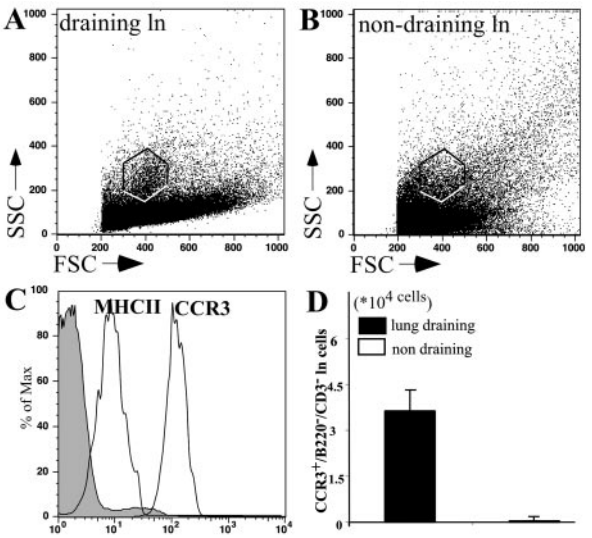

FIGURE 1. Accumulation of $CCR3^+$ eosinophils in draining lymph nodes of the lung. Mice were immunized on day 0 with 1×10^6 OVA-pulsed DCs and received an OVA/alum booster injection at day 10. At days 20–23, they were challenged daily for 30 min with OVA aerosols. *A* and *B*, Ungated forward scatter (FSC)/side scatter (SSC) plots of cell suspensions of draining or nondraining lung lymph nodes of OVA-sensitized and challenged mice showing the presence of highly granular (SSC^{high}) cells. A gate was set on these cells (*C*). Within the same gate, these granular cells expressed CCR3 and very weakly MHCII (open histograms). Staining by isotype control IgG2a is indicated by the filled histogram. *D*, $CCR3^+MHCII^{low}$ eosinophils accumulated in draining lymph nodes of the lung and not in nondraining lymph nodes.

eosinophils from allergically inflamed lungs were examined for the expression of MHCII, costimulatory molecules CD80 and CD86, and ICAM-1. Eosinophils showed a very weak expression of MHCII and a distinct expression of CD80 and ICAM-1, but had a minimal expression of CD86 (Fig. 2)

Intratracheal injection of BALF eosinophils does not prime naive OVA TCR Tg T cells in vivo

As eosinophils accumulated in the draining lymph nodes at times of eosinophilic inflammation and expressed the molecules necessary for T cell interaction, we speculated that they might be presenting Ag to OVA-specific T cells. To investigate this further, eosinophils were purified from BALF of sensitized and challenged mice based on characteristics described above. Using this method, a distinction could be made between eosinophils and other (possible) APCs in BALF (Fig. 3) e.g., B cells (Fig. 3*E*, $B220^{high}$ $MHCII^{high}$), macrophages (Fig. 3*F*, large autofluorescent cells), and DCs (Fig. 3*D*, nonautofluorescent $CD11c^{high}MHCII^{high}$ cells) (12). Purity of sort was determined by immunocytochemical staining on cytospins of sorted eosinophils identifying eosinophils by their donut-shaped nucleus and eosin staining of cytoplasm (Fig. 4*B*). After sorting, a purity of >96% was achieved based on flow cytometry and differential cell counts on cytospins (Fig. 4). Purified in vivo-pulsed eosinophils were injected into the trachea of naive mice. Viability of these transferred cells was determined by flow cytometry 3 days after instillation. A distinct $CCR3^+$ eosinophil population could be observed in the BAL compartment, which was PI negative, indicative of viable cells. (20.4% eosinophils of total BAL cells vs 0.7% after OVA-pulsed DC instillation and 0.5% after unpulsed DC instillation) (Fig. 5). Mice received a cohort of CFSE plus OVA TCR Tg T cells by i.v. injection 1 day before i.t. injection of purified in vivo-pulsed eosinophils. BALF eosinophils from allergic mice did not induce divisions of naive T cells in contrast to OVA-pulsed DCs, which induced vigorous T cell division (Fig. 6*A*).

The lack of T cell division after injecting eosinophils obtained from OVA-induced airway inflammation could be due to insufficient uptake of OVA by eosinophils in vivo. To investigate whether eosinophils were able to present processed OVA peptide to naive OVA TCR Tg T cells in vivo, bypassing the need for Ag uptake and processing, eosinophils were pulsed in vitro with OVA peptide after sorting. Intratracheal injection of in vitro-pulsed eosinophils in mice that received $CFSE^+$ T cells i.v. did not induce divisions (Fig. 6*B*). The absence of T cell division was not due to a lack of responsiveness of our T cells as i.t. injection of DCs induced an average of 3.7 divisions in 45.7% of the T cells.

To exclude the possibility that eosinophils from the BALF were incapable of priming naive T cells because of their inability to migrate to the draining lymph nodes, 2×10^6 peptide-pulsed eosinophils were injected i.p. because i.p. injection of cells leads to localization of cells in the thoracic lymph nodes by peritoneal drainage (16). Eosinophils still did not induce divisions of T cells when injected i.p. (Fig. 6*C*).

BALF eosinophils do not prime naive OVA TCR Tg T cells in vitro in contrast to effector T cells

To investigate the Ag-presenting capacity of eosinophils in direct contact with OVA TCR Tg T cells, 5×10^4 $CFSE^+$ $MHCII^-$ $B220^-CD11b^-$ unstimulated T cells were cocultured with 1×10^3, 1×10^4, or 1×10^5 fresh in vivo-pulsed BALF eosinophils or in vitro OVA-pulsed eosinophils (Fig. 7*B*, *i-vi*). After 72 h, no

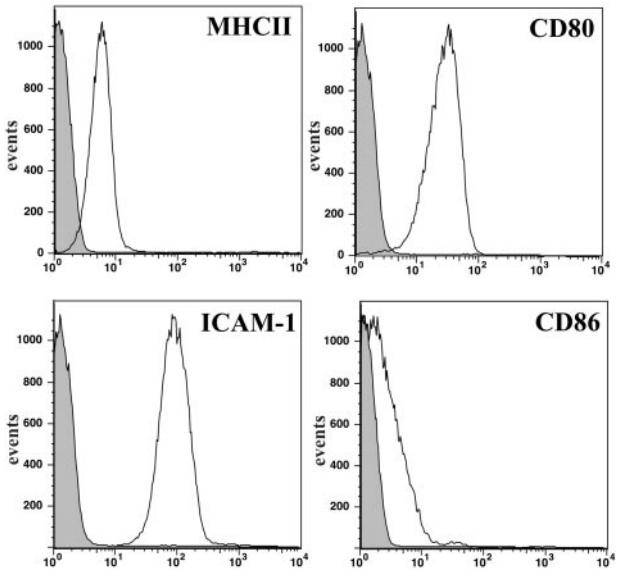

FIGURE 2. Expression of molecules associated with Ag presentation on BALF eosinophils. Eosinophils were recognized as highly granular, low autofluorescent cells that lacked expression of CD3 and B220. Staining revealed a very low expression of MHCII, a distinct expression of CD80 and ICAM-1, and a minimal CD86 expression. Staining by isotype control Abs is expressed as filled histograms.

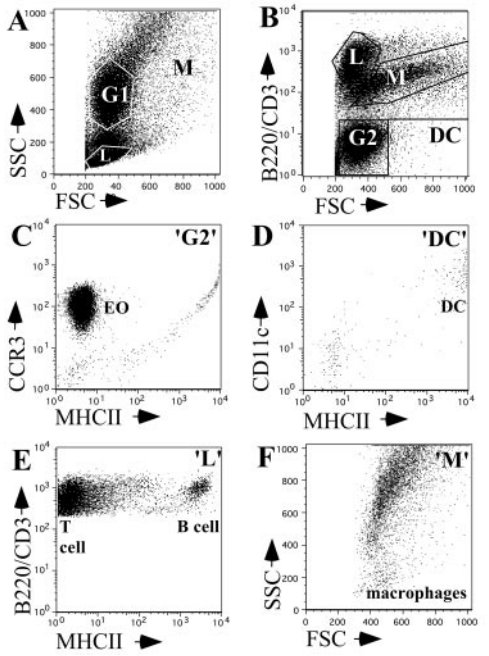

FIGURE 3. Isolation of BALF eosinophils without contamination of other APCs. BALF cells of OVA-sensitized and challenged mice were stained to isolate eosinophils (EO) using flow cytometry. *A*, The forward scatter (FSC)/side scatter (SSC) plot of ungated BALF cells contains granulocytes (G1) and lymphocytes (L) and macrophages (M). *B*, By staining with B220 and CD3, several BALF cell populations could be discriminated. Gates were set on these populations and analyzed further by additional staining or by scatter characteristics. Used gates are displayed in the *upper right corner*. *C*, To isolate eosinophils, a gate was set on low autofluorescent cells that lacked expression of CD3 and B220 (G2). Within this gate, CCR3 expression made it possible to distinguish eosinophils. BALF eosinophils expressed MHCII very weakly. *D*, Other APCs could be identified as $MHCII^{high}CD11c^{high}$ cells representing DCs (*B* and *D*), whereas B cells can be identified in the L gate from T cells as $MHCII^{high}$ (*B* and *E*), and alveolar macrophages (M) as large and spontaneously autofluorescent cells (*B* and *F*).

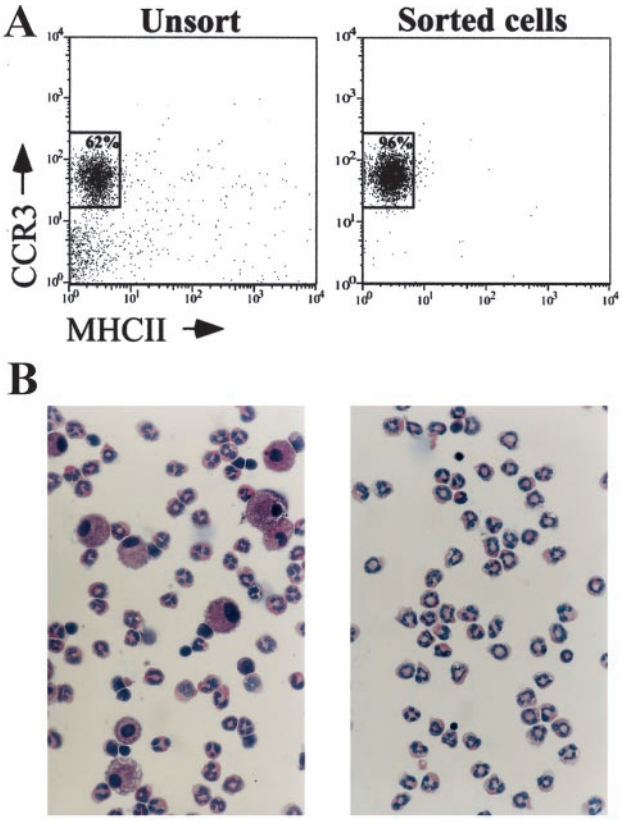

FIGURE 4. Sorting eosinophils from BALF. Cells were sorted as $CCR3^+$, low autofluorescent $CD3^-B220^-$ cells as described in Fig. 3. *A, Left,* MHCII vs CCR3 on ungated cells before sorting. *Right,* MHCII vs CCR3 on ungated cells after sorting. *B,* Cytospins of unsorted and sorted populations were analyzed for the number of eosinophils identified by an eosinophilic cytoplasm and a donut-shaped nucleus. *Left,* Sixty-five percent of cells were identified as eosinophils before sort; *right,* 96% of cells were identified as eosinophils after sort.

divisions of T cells were induced. In contrast, OVA-pulsed DCs induced a vigorous proliferation of 80% of T cells with an average of 3.8 divisions. (Fig. 7*A*).

Eosinophils are reported to present Ag to already sensitized T cells (7, 8). To investigate whether BALF eosinophils do have the capacity of stimulating sensitized T cells, 1×10^3, 1×10^4, or 1×10^5 fresh in vivo-pulsed BALF eosinophils or in vitro OVA-pulsed eosinophils were cocultured with effector $CD62L^{low}CD44^{high}$ OVA TCR Tg T cells that were obtained by in vitro stimulation by

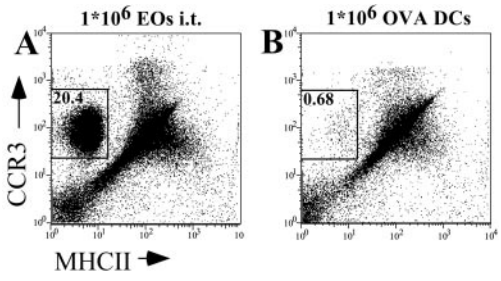

FIGURE 5. Viability of eosinophils from BALF after i.t. injection. Sorted eosinophils were injected i.t. in naive mice and 3 days later, lack of PI uptake by BALF eosinophils was determined as a marker for viability. *A,* Eosinophils were identified as $CCR3^{high}MHCII^{low}$ cells in the granulocyte gate and were all low in PI uptake, indicative of viable cells. By contrast, mice that received 1×10^6 OVA-DCs had no eosinophils in the BALF (*B*) EO, eosinophil.

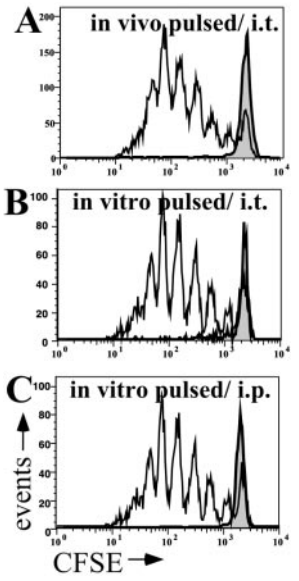

FIGURE 6. Ag presentation assay in vivo. Ag-presenting capacity of eosinophils was investigated by injecting eosinophils i.t. in mice that received CFSE-labeled OVA TCR Tg T cells i.v. 1 day earlier. Division of T cells induced by eosinophils was determined by FACS analysis (filled histograms). OVA-pulsed bone marrow-derived DCs were used as control APCs (open histograms). *A,* Eosinophils exposed to OVA in vivo by aerosols were not capable of inducing proliferation of naive T cells. *B,* Exposing eosinophils in vitro to OVA peptide, avoiding the need for Ag capture and processing, still did not induce priming capability for naive T cells in eosinophils. *C,* Intraperitoneal injection of OVA peptide-exposed eosinophils did not prime naive T cells. Plots shown are representative of multiple experiments with four to eight mice per group.

OVA-pulsed DCs. Both in vivo- and ex vivo-pulsed eosinophils were able to induce some proliferation in effector T cells but not as vigorous as that induced by OVA-pulsed DCs (Fig. 8*B*: resp. 1 APC: 5 T cells: 16.9 and 16.1% compared with 88.5% by DCs, 2 APC: 1 T cell: 31.4 and 37.8% compared with 70% by DCs). Experiments with or without adding 5 ng/ml GM-CSF to the culture medium yielded similar proliferation results (data not shown). T cells cultured without APCs did not divide (Fig. 8).

Expression of molecules associated with Ag presentation on BALF eosinophils after stimulation with GM-CSF

Since naive T cell priming was not properly induced by freshly sorted eosinophils in vitro or in vivo, eosinophils were incubated overnight with GM-CSF to enhance Ag-presenting capacities as shown by others. We sorted $CCR3^+$ eosinophils from lavage fluid of sensitized and challenged mice and incubated these cells for 1 day with GM-CSF before culture with CFSE-labeled OVA TCR Tg T cells in the presence or absence of in vitro OVA peptide pulsing. Eosinophils cultured in GM-CSF did not differ in MHCII staining compared with eosinophils cultured in medium and accordingly failed to induce T cell division readily observed following stimulation with DCs (data not shown). No difference in viability between eosinophils cultured with or without GM-CSF was observed, both conditions yielded ~99% PI^- viable eosinophils after culture with T cells (data not shown).

Discussion

For several years, it has been described that eosinophils are able to express MHCII and costimulatory molecules and are able to migrate to the draining lymph node T cell area, suggesting that they

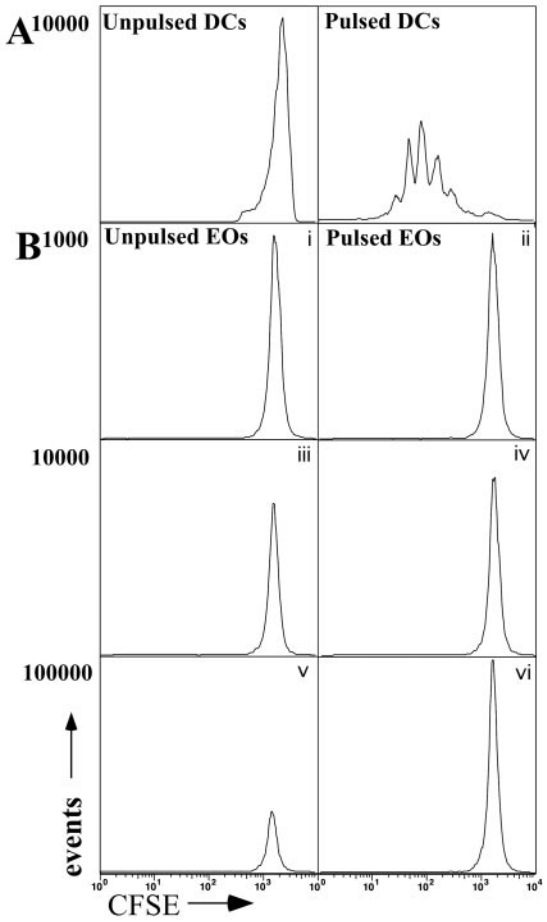

FIGURE 7. In vitro eosinophil Ag-presenting capacity to naive T cells (*B*). In vivo OVA aerosol exposed (indicated by unpulsed: *i*, *iii*, and *v*)- and in vitro OVA peptide-exposed eosinophils (indicated by pulsed: *ii*, *iv*, and *vi*) were cocultured with 50,000 naive T cells. Eosinophils (EOs) were added in different concentrations to naive T cells: 1×10^3,1×10^4, or 1×10^5. With up to two eosinophils per naive T cells, there was still no proliferation of naive T cells (*v* and *vi*). *A,* Bone marrow-derived DCs induced in 80% of T cells an average of 3.8 divisions only when DC were pulsed with OVA.

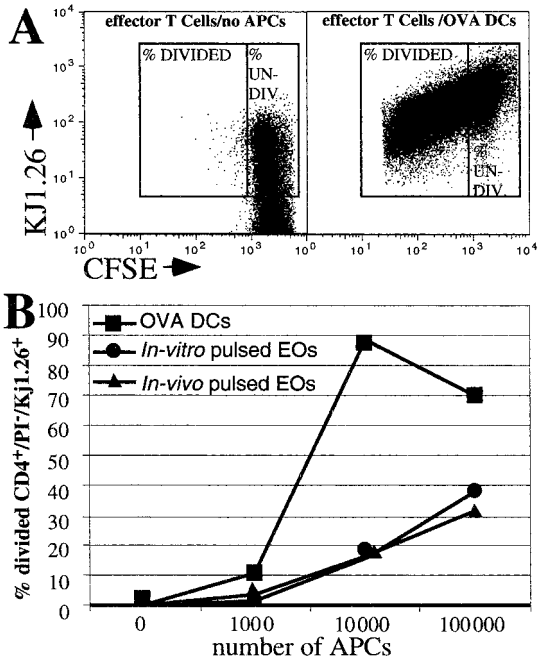

FIGURE 8. In vitro eosinophil Ag-presenting capacity to effector T cells Effector $CD62L^{low}CD44^{high}$ OVA TCR Tg T cells (5×10^4 cells/well) obtained in vitro by OVA DC stimulation of naive OVA-specific T cells were cocultured with 0, 1×10^3, 1×10^4, or 1×10^5 freshly isolated eosinophils (EOs) from BALF of OVA-sensitized and challenged mice. *A, Left,* A gate was set around $Kj1.26^+$ T cells cultured without APCs after gating on PI^-CD4^+cells to define undivided T cells. *Right,* Divided T cells were defined as $Kj1.26^+$ cells with low CFSE content. The limit to define undivided T cells was set on the CFSE content of unstimulated T cells. *B,* Eosinophils were either pulsed in vivo by OVA aerosols or ex vivo by OVA peptide pulsing followed by vigorous washing. Ex vivo- and in vivo-pulsed eosinophils were both able to induce some proliferation at a ratio of one or two eosinophils per effector T cells but induced proliferation in a lesser degree than OVA-pulsed DCs. Effector T cells cultured without any APCs did not divide.

might be endowed with Ag-presenting capacity. In our experiments, an accumulation of $CCR3^+$ eosinophils in draining lymph nodes of the lung was indeed observed at a time point when eosinophils were accumulating in the allergically inflamed lung, suggesting that eosinophils were migrating from the lung to the lymph nodes where T cells are recirculating. These findings are consistent with earlier reports by other groups and indeed suggest some Ag-presenting function (7–10). In our study, in addition to the presence of eosinophils at the site of T cell encounter, BALF eosinophils weakly expressed MHCII and expressed high levels of costimulatory molecules CD80 and ICAM-1. Functional studies have been performed to investigate Ag presentation of BALF eosinophils, but these studies focused mainly on the stimulation of polyclonal primed T cells and did not directly address T cell activation in vivo (7, 8).

This stimulation of primed T cells by eosinophils led us to investigate whether eosinophils obtained from an inflammatory site were capable of sensitizing naive Ag-specific T cells in vivo. We isolated eosinophils from the bronchoalveolar compartment of the inflamed lungs of OVA-sensitized and challenged mice (12, 14). Isolated eosinophils that were exposed to OVA Ag by aerosol exposures in vivo were introduced in the airways of naive mice that received CFSE-labeled OVA TCR Tg T cells i.v. 1 day earlier. Eosinophils injected into the airway lumen have been shown to reach the T cell area of the draining nodes of the lung (7). The adoptive transfer of CFSE-labeled Ag-specific T cells enabled us to investigate the Ag-presenting capacity of eosinophils to unprimed Ag-specific T cells in the draining lymph nodes of the lung by visualizing the number of T cell divisions in vivo (14). This system is extremely sensitive in detecting the presence of APCs. After 3 days, there was no induction of proliferation of T cells in the draining lymph nodes of the lung while OVA-pulsed DCs, injected i.t. as a positive control, induced proliferation in 45.7% of T cells, some cells reaching six to seven divisions. The absence of T cell priming was not due to the death of transferred eosinophils, as 20.4% of total BALF cells were viable eosinophils. One possible explanation for the lack of T cell priming could be that eosinophils from the BALF were not capable of capturing and processing sufficient OVA. To bypass the need for Ag uptake and processing, eosinophils were pulsed with OVA peptide in vitro. Intratracheally injecting in vitro-pulsed eosinophils still did not induce any divisions of OVA TCR Tg T cells. Still, these findings cannot exclude that eosinophils might have some Ag-presenting function. Isolated BALF eosinophils injected into the trachea had to migrate from the trachea through the epithelial barrier toward draining lymph nodes to sensitize naive T cells. Since the migration potential of eosinophils might be lower in noninflamed lungs,

an alternative route of injection was chosen in our studies. Material injected in the peritoneal cavity is drained nonspecifically to the thoracic lymph nodes (16). Therefore, we injected sorted OVA peptide-pulsed eosinophils i.p. to allow eosinophils to reach the draining lymph nodes, but still observed no divisions in Ag-specific T cells.

Finally, to exclude that the number of eosinophils encountering naive T cells was not sufficient to induce priming and to exclude that absence of migration was causing the absence of T cell activation, T cells were cultured in vitro with sorted eosinophils in a very high stimulator to the responder setting. In vitro data were in accordance with in vivo data, showing no Ag presentation despite these permissive conditions of naive T cell activation. In contrast, a proliferation of naive T cells by OVA-pulsed DCs was almost 80% of T cells in vitro (compared with 45.7% of T cells in vivo) at very low stimulator:responder ratios. To verify whether these eosinophils were capable of some Ag presentation, eosinophils were also cocultured with in vitro-obtained effector OVA-specific T cells. These eosinophils were capable of stimulating effector T cells, in accordance with other studies (7), although less strongly than proliferation induced by professional Ag-presenting DCs. Together these data suggest that freshly isolated eosinophils from the BAL compartment have no evident Ag-presenting capacity for naive T cells, whereas professional Ag-presenting DCs are clearly able to activate unstimulated T cells.

What then might be the explanation for this lack of APC function to naive T cells while at the same time eosinophils induced some proliferation in primed T cells? In accordance with other studies, the isolated BALF eosinophils used in this study expressed CD80 and ICAM-1 involved in T cell costimulation (7). However, they consistently expressed MHCII at very low levels in the bronchoalveolar compartment where the lung encounters inhaled Ag and did not up-regulate MHCII following their migration to the draining nodes or after overnight culture with or without GM-CSF. Naive T cells have more stringent requirements for signal 1 and signal 2 compared with effector T cells, which might explain the difference.

There are some conflicting data about MHCII expression by eosinophils. In humans, expression of the molecules for T and B cell interactions was only reported on eosinophils in allergen-challenged airways, but not on activated blood eosinophils (6, 17, 18). An explanation for this phenomenon could be that cytokines secreted by allergic lung cells could up-regulate MHCII expression on eosinophils. Several cytokines like IFN-γ, IL-3, and GM-CSF secreted by inflammatory cells are reported to induce MHCII expression in vitro (5, 17, 19). In support of this, eosinophils from the peritoneal cavity of IL-5 Tg mice that were purified using Percoll gradients and adherence steps were reported to have no MHCII expression, except when cultured with GM-CSF (19). Others showed that BALF eosinophils isolated using Percoll gradients and adherence culture weakly expressed MHCII, probably because of GM-CSF release in vivo (7). This is unlikely in our studies, as we have shown in previous experiments that GM-CSF levels in BAL are below detection limit (12). Similarly, eosinophils from BALF isolated based on forward and side scatter characteristics and light polarization were reported to have a low expression of MHCII, although at higher levels than on the eosinophils described in this study (8). In all of these studies, eosinophils were first purified using extensive protocols, before MHCII staining was performed, suggesting that MHCII might have been up-regulated ex vivo. In our hands, fresh eosinophils in lavage fluid, identified based on scatter characteristics and CCR3 staining, had a very weak MHCII expression that was only able to stimulate effector T cells in very high stimulator:responder ratios. Culture of highly purified BALF eosinophils, specifically in GM-CSF, did not enhance MHCII expression (data not shown). Perhaps the isolation method can attribute for the difference found in MHCII expression.

Others have demonstrated T cell stimulation of primed polyclonal T cells by peritoneal eosinophils and alveolar eosinophils in vitro. Proliferation of primed T cells in vitro was only induced by eosinophils stimulated with GM-CSF and not by directly isolated eosinophils (7, 19). In this study, we confirmed these findings using OVA-specific TCR Tg T cells and showed that freshly isolated BALF eosinophils were able to induce some proliferation in effector T cells but less strongly compared with professional DCs (Fig. 8).

We took great care to isolate eosinophils without contamination of other APCs. We developed a new more accurate purification method. By using flow cytometric scatter characteristics, multiple positive and negative markers, and taking advantage of the autofluorescence of macrophages, it was possible to sort $CCR3^+$ eosinophils while avoiding B cells, T cells, macrophages, and DCs, preventing contamination with these APCs (12, 15). In this study, Ag presentation of $CCR3^+$ purified eosinophils from the BALF to naive T cells was investigated directly in vitro and in vivo with a very sensitive readout, using naive OVA TCR Tg T cells labeled with CFSE, showing no direct Ag presentation. One other study has also investigated the potential of eosinophils to induce priming in a naive setting. In this study, eosinophils from the peritoneal cavity of IL-5 Tg mice were capable of sensitizing mice when injected repeatedly in the peritoneal cavity. After subsequent challenge with OVA aerosols, eosinophilia could be observed, and thus it was concluded that eosinophils induce T cell responses to OVA (8). These studies did not directly address when and how T cell priming occurred however. One possibility could be that repeated injection of Ag-carrying eosinophils led to uptake of Ag by endogenous professional APCs such as DCs. DCs have been shown to capture Ag from apoptotic cells, leading to Ag presentation to naive CD4 and CD8 T cells (20). Moreover, injected IL-5 Tg eosinophils in the peritoneum reach the thoracic lymph nodes by nonspecific drainage (16) or specific migration, introducing a source of IL-5 to the site of challenge. This higher level of IL-5 production in the lung could have lowered the threshold for development of eosinophilic airway inflammation induced by eosinophil immunization and/or subsequent OVA challenge, as Il-5 is known as an important chemoattractant and differentiation factor for eosinophils (1).

If eosinophils do not seem to activate naive T cells in the draining nodes, what then might be the role of eosinophil accumulation in the lung draining lymph nodes of allergic mice? One possibility would be that they stimulate already primed T cells (7, 8) and not naive T cells. It has been shown that the requirements for naive T cell activation are more stringent than for primed T cells, and indeed the very low MHCII expression level in our model did not induce proliferation in naive T cells but allowed induction of some proliferation of effector T cells, although not as strongly as professional DCs. Alternatively, eosinophils might indirectly effect Ag presentation by DCs by secreting cytokines and mediators. Studies that have used anti-IL-5 to eliminate eosinophils systemically from the body have however observed no obvious change in the levels of Th2 cytokine secretion in draining nodes or effector site, arguing against an absolute requirement for eosinophils in the stimulation of primed Th2 cells (21).

Together these data imply that although eosinophils accumulate in the draining nodes at times of eosinophilic airway inflammation and do express CD80, ICAM-1, and very low levels of MHCII,

they do not present Ag to naive T cells. These results do not diminish their evident role as effector cells in asthma. Further elucidation of the most important aspects of eosinophil biology in ongoing airway inflammation is necessary to develop an effective intervention therapy targeted to these cells.

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