

Cells isolated from bone-marrow and lungs of allergic BALB/C mice and cultured in the presence of IL-5 are respectively resistant and susceptible to apoptosis induced by dexamethasone

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Abstract

We have previously reported that, in IL-5-stimulated bone-marrow cultures, dexamethasone upregulates eosinophil differentiation and protects developing eosinophils from apoptosis induced by a variety of agents. Recently developed procedures for the isolation of hemopoietic cells from allergic murine lungs have enabled us to evaluate how these cells respond to dexamethasone in IL-5-stimulated cultures, when compared with bone-marrow-derived cells isolated from the same donors, and whether differences in response patterns were linked to apoptosis. Ovalbumin challenge of sensitized mice increased significantly the numbers of mature leukocytes as well as hemopoietic cells recovered from digested lung fragments, relative to saline-challenged, sensitized controls. Both mature eosinophils and cells capable of differentiating into eosinophils in the presence of IL-5 were present in lungs from sensitized mice 24 h after airway challenge. Dexamethasone strongly inhibited eosinophil differentiation in IL-5-stimulated cultures of lung hemopoietic cells. By contrast, dexamethasone enhanced eosinophil differentiation in cultures of allergic bone-marrow cells, in identical conditions. Hemopoietic cells from lungs and bone-marrow were respectively susceptible and resistant to induction of apoptosis by dexamethasone. The dexamethasone-sensitive step was the response to IL-5 in culture, while accumulation of IL-5 responsive cells in allergen-challenged lungs was dexamethasone-resistant. Cells from lungs and bone-marrow, cultured for 3 days with IL-5 in the absence of dexamethasone, did not respond to a subsequent exposure to dexamethasone in the presence of IL-5. These findings confirm that IL-5-responsive hemopoietic cells found in challenged, sensitized murine

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lungs differ from those in bone-marrow, with respect to the cellular responses induced by dexamethasone, including apoptosis.

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1. Introduction

Interleukin-5 (IL-5), a hemopoietic cytokine secreted by Th2 lymphocytes, mast cells, and eosinophils [1] plays an important role in asthma [2,3]. In several models of asthma, IL-5 is required for the development of bronchopulmonary hyperreactivity [4–7], and transgenic mice that overexpress IL-5 in the lung epithelium present pulmonary changes pathognomonic of asthma [8]. Furthermore, allergen sensitization followed by airway challenge increases circulating IL-5 levels [9], production of IL-5 in the bone-marrow [10,11], and the responsiveness of bone-marrow cells to IL-5 [9,12,13]. Both circulating IL-5 and IL-5 locally secreted in the lungs contribute to antigen-induced airway eosinophilia, even though the relative magnitude of their respective contributions remains to be established [14,15]. Traditionally, targets for IL-5 comprise hemopoietic cells in bone-marrow as well as mature, differentiated leukocytes in blood and tissues. However, more recently, intrapulmonary hemopoietic targets responsive to IL-5 (such as the CD34+/IL-5R α mRNA+ cells that accumulate in allergen-challenged lung, as reported in Robinson et al. [16]) have also been proposed to contribute to the eosinophilia of asthmatic lung, by differentiating in situ [4,17], despite the lack of direct evidence for the presence of functionally intact hemopoietic progenitors in the lung, due to the technical difficulties involved in their isolation. These technical problems were overcome by a novel procedure for the quantitative recovery of fully functional hemopoietic cells from allergic murine lung [18], which comprise both colony-forming cells responsive to a combination of IL-5, IL-3 and GM-CSF, and cells which respond to IL-5 alone, by differentiating into mature eosinophils in liquid culture. These so-called eosinophil precursors could not be quantified directly, because of the lack of a stage-specific phenotypic marker. Instead, their presence was assessed by the ability of the cultures to give

rise to mature eosinophils in the presence of IL-5. Hence, in the present study, “eosinophil precursors” are always defined by their ability to differentiate into eosinophils, which can be objectively quantified. Other studies [19] have confirmed the presence of hemopoietic cells in lung tissue.

One important aspect to consider in evaluating the role of IL-5 in eosinophilopoiesis is its potential for inhibition of the effects of glucocorticoids, which are probably the most effective, as well as the most widely employed therapeutic agents for asthma [20]. Even though glucocorticoids can downregulate production of IL-5, they can also interact with preformed IL-5 [21] to synergistically enhance eosinophil production from precursors found in murine bone-marrow [22]. Glucocorticoids are known as inducers of eosinophil apoptosis [23], but this effect is affected by the presence of cytokines. The outcome differs according to the exact experimental conditions: there are reports of induction of apoptosis [24], while others report no effect [25], or even inhibition of apoptosis [26]. In murine bone-marrow culture, dexamethasone completely protects developing eosinophils from apoptosis induced by prostaglandin E₂, by blocking induction of NO synthase and thereby preventing activation of the CD95-CD95L death receptor pathway [27]. We have reported that the response of lung-derived eosinophil precursors to IL-5 was suppressed by dexamethasone, unlike the response of bone-marrow cells [18]. However, these initial observations were limited in scope, because different purification procedures had been used for lung and bone-marrow, and because the possible role of apoptosis was not evaluated. In this study, we have evaluated the effects of dexamethasone on lung and bone-marrow eosinophil precursors isolated and cultured in identical conditions. Here we report that these target populations differ strikingly in their patterns of response to dexamethasone, and these differences are linked to differences in susceptibility to the direct apoptosis-inducing effects of dexamethasone.

2. Methods

2.1. Animals and animal procedures

BALB/c mice bred at FIOCRUZ, Rio de Janeiro, were used at 6–8 weeks. Mice were sensitized by two s.c. 0.4 ml injections of 100 µg ovalbumin mixed with 1.6 mg Al(OH)₃ (alum) in 0.9% NaCl (saline), at 7-day intervals, followed by intranasal challenge with 10 µg ovalbumin in saline, 1 week after the second injection. Control animals were either naive, or sensitized mice challenged with saline. Challenge was carried out by slow instillation of a total 50 µl volume, dispensed in both nostrils, with the help of a Gilson pipetman, over a period of 2–3 min. In these conditions, allergen is retained almost completely in the upper airways [28]. Sensitized animals were sacrificed 24 h after challenge. Where indicated, animals were injected i.p. with dexamethasone (5 mg/kg, 2 h before challenge for sensitized–challenged mice, or 24 h before bone-marrow harvest, for naive controls). Animal handling followed the standard procedures adopted at FIOCRUZ, and the study protocols were approved by the institutional Ethics Review Committee. The procedure for collecting allergic lung tissue has been detailed elsewhere [18]. Briefly, mice were anesthetized with thiopental and exsanguinated from the abdominal vena cava. After opening the thoracic cage, an incision was made in the right atrium, and lungs were thoroughly washed by retrogradely pumping sterile saline from the left atrium. We have ruled out, in control experiments, the presence of any marginated hemopoietic cells in the pulmonary microcirculation, following this washing step. Lungs were excised and used for preparing single cell suspensions in Iscove's modified Dulbecco's medium (IMDM).

2.2. Separation, staining and counting of hemopoietic cells

The procedure for purification of hemopoietic cells from lungs has been detailed elsewhere [18]. Pooled lungs from groups of up to 8 mice were minced, and digested with 24-mg collagenase and 0.125-mg DNase I (20 min, 37 °C). Digestion was interrupted by adding fetal calf serum (FCS) up to 20%. Single cell suspensions were prepared by filtration through a

Cell Strainer (Falcon 2360, 100 µm, nylon) and separated on discontinuous gradients (density = 1.130 ± 0.005 g/ml; 20 min, $100 \times g$, 20 °C, 2×10^7 cells per gradient), prepared as (from bottom to top) 75%, 60% and 40% Percoll in IMDM [18]. Viability was assessed by Trypan blue exclusion. Total and differential cell counts were carried out after staining with Turk's solution and Panoptic, respectively. Cells from the eosinophil lineage were identified by staining for eosinophil peroxidase (EPO), a lineage-specific marker, detectable from promyelocytes to mature eosinophils [29,30]. Mature and immature eosinophils were differentiated on morphological criteria [9]. Collection and handling of bone-marrow followed procedures detailed in Ref. [23]. For both lung [18] and bone-marrow [9], hemopoietic cells are found only in the middle layer (40%–60% Percoll interface). To allow comparison with cells from lung, bone-marrow cells were also separated on Percoll.

2.3. Progenitor assays

Conditions for assaying colony formation have been detailed elsewhere, for progenitors from both lung [18] and bone-marrow [22]. For bone-marrow, cultures were established from 2×10^5 cells [9]; for lung-derived cells, we have previously established that optimal colony formation by lung-derived hemopoietic cells requires a inoculum of 4×10^5 cells [18]. Cells were cultured in methylcellulose, in IMDM with 20% FCS, in the presence of a synergistic combination of GM-CSF (2 ng/ml), IL-3 (2 ng/ml) and IL-5 (1 ng/ml) [31]. Colonies were scored at day 10 in all cases. For lung-derived hemopoietic cells, results are presented as Mean $\pm$ SEM of colony-forming cell numbers per animal, as calculated from the number of colonies formed by 4×10^5 cultured cells and from the total number of mononuclear cells recovered per animal in the middle layer of the Percoll gradients.

2.4. Precursor assays

For lung and bone-marrow eosinophil precursors, liquid cultures were established from 10^6 cells as described in Ref. [9], in the presence of IL-5 (1 ng/ml). Precursor differentiation assays evaluate a more mature target than colony formation assays [22,32] and do not require GM-CSF and IL-3. The frequency

of EPO+ cells was determined at day 7. We have previously shown that increases in the %EPO+ cells expressed are paralleled by increases in the absolute numbers of EPO+ cells in culture, and are to be preferred for statistical analysis [9,22]. Where indicated, dexamethasone was added at the beginning of the culture to a final 10^{-7} to 10^{-14} M concentration, and was not replaced during the entire duration of the culture. Where indicated, dexamethasone at a final 10^{-7} M concentration was added at day 3 to cultures that had been seeded in the presence of IL-5 alone. We have previously shown that dexamethasone addition at day 3 has no effect on eosinophil production from bone-marrow cells [22]. Where indicated, RU 486 (mifepristone) was added to the IL-5-stimulated cultures at the same time as dexamethasone, to a final 10^{-6} M concentration. Control cultures received IL-5 and RU 486, but no dexamethasone. For both lung and bone-marrow, cultures established in the absence of IL-5 present no cells of the eosinophil lineage by day 7 [9]. Dexamethasone in the absence of IL-5 does not support differentiation or survival of eosinophils [22].

2.5. Apoptosis assays

TUNEL labeling was detected with an apoptosis detection kit from R&D Systems (Minneapolis, MN; Ref. TA4625). Cytoentrifuged samples were stained in parallel for TUNEL labeling (counterstained with Methyl Green), or EPO (for quantification of EPO+ cells bearing pyknotic nuclei). 300 Cells were counted per slide.

2.6. Reagents

FCS, L-glutamine and culture media were from Gibco (Life Technologies SARL, Cergy Pontoise, France); Panoptic stain was from DADE Diagnostika GmbH (Unterschleissheim, Germany); Harris' hematoxylin was from Réactifs RAL (Paris, France); rmIL-3, rmIL-5 and rmGM-CSF were from Pharmingen (San Diego, California); methylcellulose was from Stem Cell Technologies (Methocult M3134); Percoll, DNase I (Ref. DN-25), and dexamethasone 21-phosphate were from Sigma (St. Louis, MO); ovalbumin (5× crystallized) was from ICN Biomedicals, Inc. (Costa Mesa, CA); Al(OH)₃ was from Merck (Darm-

stadt, Germany); collagenase (Type 4, ref. 4188) was from Worthington Biochemical Corporation. IMDM was from Hyclone (Logan, Utah); mifepristone (RU486) was from Sigma Chemical Co. (St. Louis, MO, USA).

2.7. Statistical analysis

Factorial analysis of variance with the Tukey HSD correction was used for multiple comparisons. Two-tailed *t* tests were used for pairwise comparisons. Use of pooled cells from groups of syngeneic donors treated identically was necessary because hemopoietic cells are a minor subpopulation in lungs, and therefore difficult to quantify in individual lungs. The same was done with bone-marrow to allow comparison. For this reason, the experimental unit subjected to statistical analysis is the cell population, and *n* is the number of independent experiments carried out with pooled cells, not the number of animals used as cell donors.

3. Results

3.1. Characterization of the cell populations accumulating in ovalbumin-challenged murine lungs

Previous studies showed accumulation of hemopoietic progenitors in allergen-challenged, sensitized lungs [18], but did not evaluate how this relates to changes in other cell populations that might be expected to accumulate within allergen-exposed lungs. To do so, we performed total and differential cell counts in single cell suspensions prepared from saline-challenged and ovalbumin-challenged sensitized lungs, as well as in the three layers recovered after separation on Percoll gradients. As shown in Fig. 1, in both groups, the major cell subpopulations, before and after separation, were mononuclear cells (interstitial and alveolar macrophages, as well as lymphocytes) and various subtypes of structural cells of the lung, grouped together as "nonleukocytes." By contrast, eosinophils, both mature and immature, were only contaminants in the saline-challenged group, and neutrophils were present in small numbers. Total cell numbers before gradient separation were significantly increased in ovalbumin-challenged, sensitized animals (panel B) relative to sensitized/saline challenged

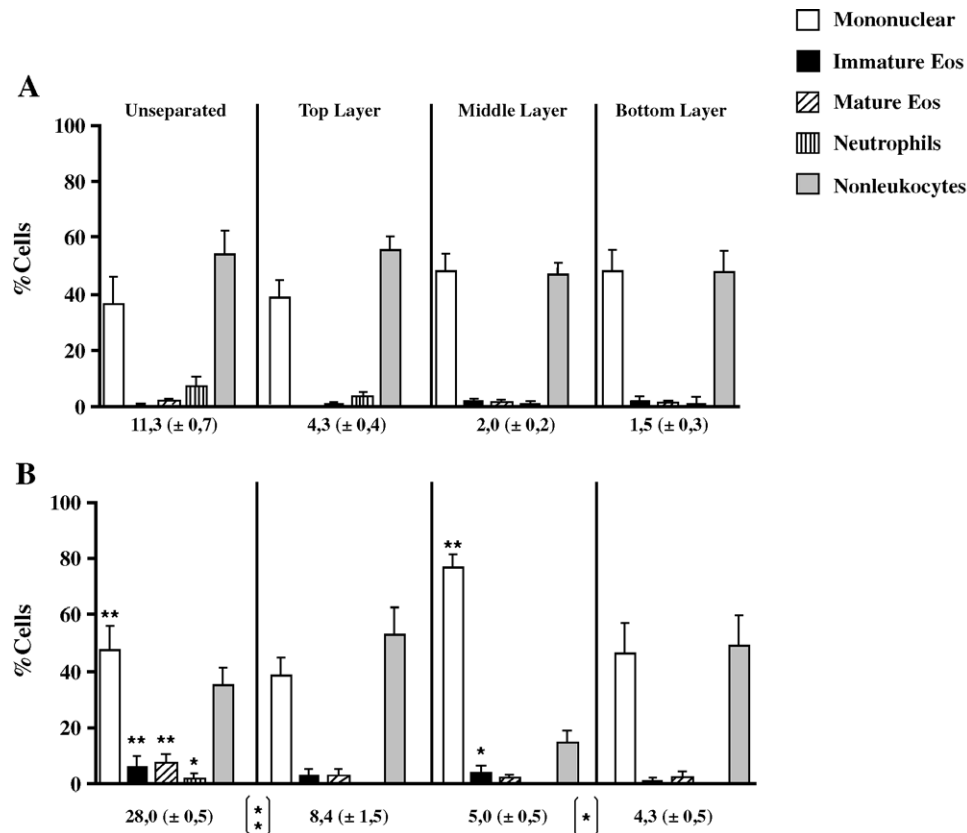


Fig. 1. Relationship between allergen challenge and the recovery of different cell populations from sensitized murine lungs. Lung cells were isolated from ovalbumin-sensitized mice 24 h after challenge with saline (panel A) or ovalbumin (panel B) by separation on Percoll gradients as described in Methods. Cells initially loaded ("unseparated") and those recovered from the top, middle, and bottom gradient layers were submitted to total and differential counts. Data underneath each distribution profile are mean $\pm$ SEM of the cell numbers (expressed as millions of cells) recovered per animal in the corresponding fraction. Data within each distribution profile are mean $\pm$ SEM of the frequency of the leukocyte subtypes, expressed as percent of the cells recovered, and classified as mononuclear cells, immature eosinophils, mature eosinophils, neutrophils, and nonleukocytes. For the sake of clarity, statistical significance symbols are displayed only for comparisons involving hemopoietic cells and granulocytes, as detailed in Results. * $P < 0.05$ and ** $P < 0.001$ relative to the respective control (saline-challenged) groups. Data were obtained from 5 to 8 experiments.

controls (panel A). This difference was accounted for by mononuclear cells, as well as mature and immature eosinophils, which were all significantly increased. By contrast, neutrophils were significantly decreased in ovalbumin-challenged mice. After separation, the middle layer in both groups, which was used for hemopoietic assays, contained neutrophils and mature eosinophils only as a contaminant (as evaluated morphologically). This layer was significantly enriched in mononuclear cells and significantly depleted of nonleukocytes in ovalbumin-challenged mice relative to saline-challenged controls. Contamination of the middle layer by mature eosinophils and

neutrophils was comparable in both groups ($P = 0.225$ and $P = 0.282$, respectively, for the differences between groups in the frequency of eosinophils and neutrophils in the middle layer). On the other hand, immature eosinophils were significantly increased in the middle layer in ovalbumin-challenged mice relative to saline-challenged controls ($P = 0.016$). Overall losses (numbers recovered in all three layers divided by numbers before separation) were comparable between ovalbumin- and saline-challenged mice for (a) all cell populations taken together (63% and 69%, respectively); (b) mononuclear cells, which include hemopoietic cells (87% and 77%, respectively).

In order to confirm that the middle layer contained hemopoietic progenitors, we carried out colony formation assays with cells from both naive and sensitized (either saline-challenged or ovalbumin-challenged) mice. As shown in Fig. 2, panel (A), virtually no colonies were generated by lung mononuclear cells from naive mice. The numbers of progenitors present in the lungs of sensitized/ovalbu-

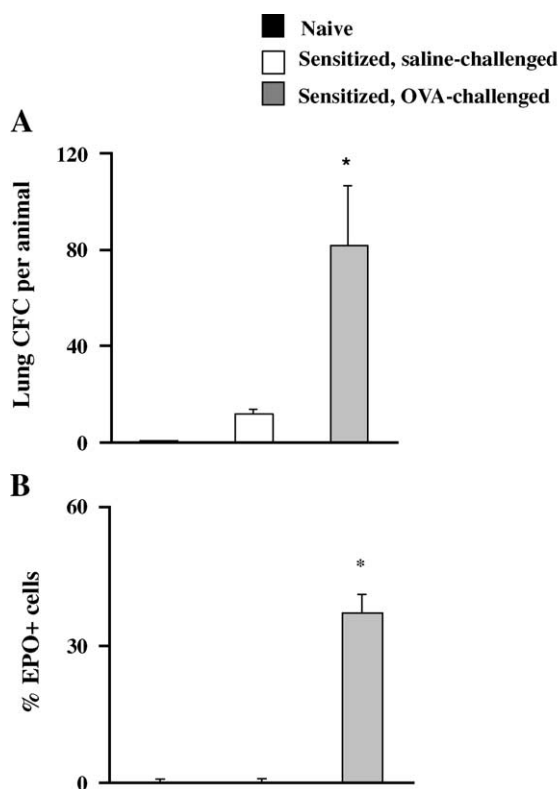


Fig. 2. Accumulation of hemopoietic progenitors and precursors in allergen-exposed, sensitized murine lungs. Lung cells were isolated from BALB/c mice of the indicated groups, by separation on Percoll gradients. Cells from the middle layer were cultured. (panel A) Data are the numbers (Mean ± SEM) of colony-forming cells per animal in 10-day semi-solid cultures of hemopoietic cells. Asterisk (*) indicates significant differences relative to both naive and sensitized, saline challenged mice. Data are derived from 4 to 17 experiments. (panel B) Data are mean ± SEM of the %EPO+ cells in 7-day liquid cultures of cells in the middle layer isolated from lungs of naive and sensitized BALB/c mice. Sensitized mice were challenged with either saline or ovalbumin. EPO+ cells before culture represented 0.6% and 3% of the inoculum, in naive and sensitized mice, respectively. Asterisk (*) indicates significant differences ($P < 0.001$) relative to both naive mice and saline-challenged, sensitized mice. Data are derived from 4 experiments.

min-challenged mice were significantly different from both naive ($P = 0.003$) and from sensitized/saline-challenged ($P = 0.047$) controls. By contrast, despite a trend towards higher colony counts, sensitized/saline-challenged mice did not significantly differ from naive controls. Cells from the middle layer were also examined for the presence of IL-5-responsive eosinophil precursors. As shown in Fig. 2, panel (B), no significant response to IL-5 was observed in cultures from naive or from sensitized/saline-challenged mice. By contrast, a vigorous response was seen in cultures from sensitized/ovalbumin-challenged.

Taken together, these findings indicate that hemopoietic progenitors and precursors accumulated in murine lungs after allergen sensitization and challenge, along with mature and immature eosinophils. The presence of hemopoietic cells in the middle layer was not associated with an increased number of mature granulocytes in that fraction, relative to unchallenged controls.

3.2. Contrasting patterns of response to dexamethasone in bone-marrow and lung eosinophil precursors

We next evaluated whether eosinophil precursors isolated from bone-marrow and lung responded differently to dexamethasone, and whether any observed differences depended on (a) the procedure used for isolation of the hemopoietic cells; (b) the target cells being exposed to dexamethasone in vivo or in vitro; (c) the timing of addition of dexamethasone. As shown in Fig. 3, panel (A), bone-marrow cells isolated from ovalbumin-challenged, sensitized mice and directly cultured without further separation (used as controls for the effect of separation on Percoll) displayed a pattern of response to dexamethasone in culture that was nearly identical to that previously reported for bone-marrow cells from naive mice of this strain [22]. Dexamethasone (10^{-7} and 10^{-9} M) significantly enhanced eosinophil production when added to IL-5-stimulated cultures ($P < 0.001$ and $P = 0.004$, respectively). By contrast, dexamethasone was ineffective at 10^{-12} M and 10^{-14} M concentrations ($P = 0.994$ and $P = 0.562$ respectively). As previously reported, dexamethasone by itself did not induce eosinophil differentiation at any of the concentrations tested (not shown). In agreement with

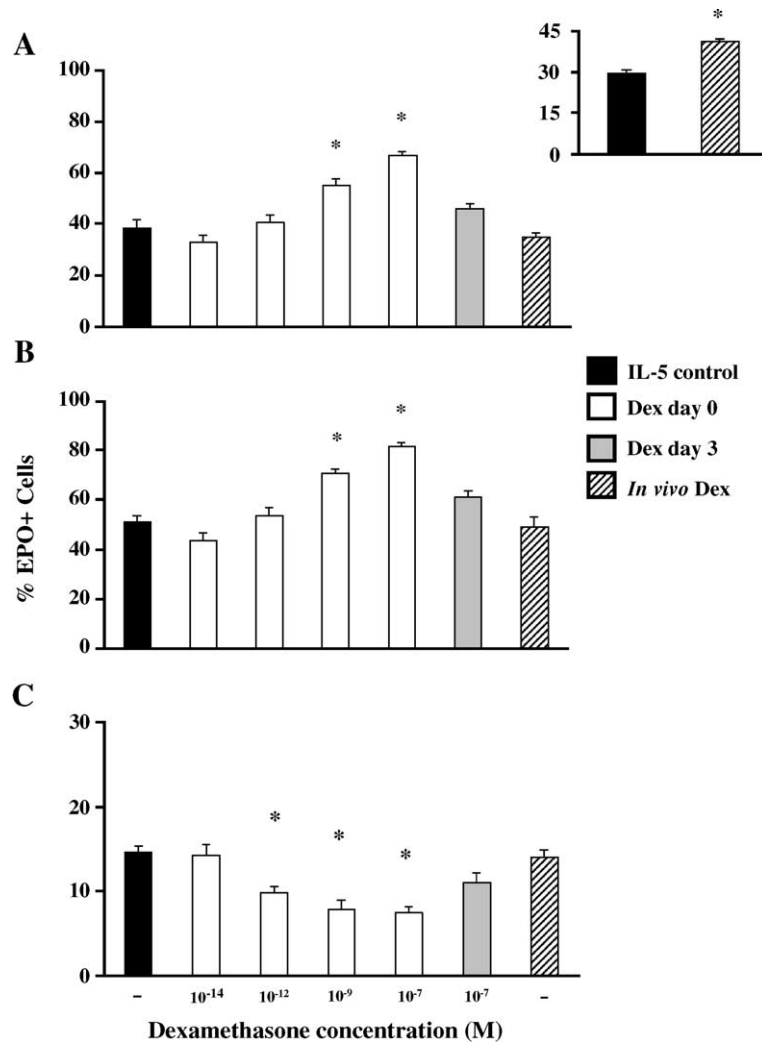


Fig. 3. Effect of dexamethasone on eosinophil precursors isolated from the bone-marrow and lung. Sensitized/challenged BALB/c (panels A, B, and C) or naive mice (insert) were used as donors of lung (panel C) and bone-marrow cells (panels A and B, and insert) as indicated. Bone-marrow cells were either unseparated (panel A) or separated on Percoll gradients (panel B) exactly as were lung cells (panel C). Cells from the middle layer were cultured after separation (panels B and C). Data are mean $\pm$ SEM of the %EPO+ cells in 7-day liquid cultures. Where indicated, donor mice, either sensitized/challenged (panels A, B, and C) or naive (insert) were injected with dexamethasone 5 mg/kg as described in Methods (in vivo Dex). Where indicated, dexamethasone was added at day 3 to cultures that had been established with IL-5 alone. Asterisk (*) indicates significant differences relative to the respective IL-5 controls ($P<0.005$ in all cases as detailed in Results). Data are derived from 6 to 12 experiments for dexamethasone in vitro and 3 to 5 experiments for dexamethasone in vivo. For bone-marrow cells separated on Percoll gradients, the middle layer contained (mean $\pm$ SEM, data derived from 4 experiments) $76.4\pm4.4\%$ mononuclear cells, $14.0\pm3.8\%$ immature eosinophils and $7.17\pm1.6\%$ mature eosinophils and $2.4\pm0.23\%$ neutrophils. Cells recovered in the middle layer represented 8% of the total bone-marrow nucleated cell inoculum.

previous observations on naive cells, dexamethasone (10^{-7} M) was ineffective if added at day 3 of culture ($P=0.343$). As further shown in Fig. 3, panel (B), when bone-marrow cells from ovalbumin-challenged, sensitized donors were separated on Percoll gradients,

following the same procedure used for purification of hemopoietic cells from lung, and cells from the middle layer were cultured as above, this pattern was unchanged. Dexamethasone (10^{-7} and 10^{-9} M) significantly enhanced eosinophil production when

added to IL-5-stimulated cultures ($P<0.001$ and $P=0.003$, respectively). No inhibitory effect of dexamethasone was observed in any of the concentrations tested. As before, dexamethasone at 10^{-12} M and 10^{-14} M were ineffective ($P=0.997$ and $P=0.717$, respectively), as was dexamethasone (10^{-7} M) if added at day 3 ($P=0.538$). By contrast, as shown in Fig. 3, panel (C), for IL-5-stimulated cultures of lung hemopoietic cells, dexamethasone had no enhancing effect on eosinophil differentiation. Quite the opposite, it strongly inhibited responses to IL-5 at 10^{-7} and 10^{-9} M ($P<0.001$ for both concentrations). Significant inhibition could still be detected with dexamethasone concentrations as low as 10^{-12} M ($P=0.002$), which were totally ineffective on bone-marrow cells. However, no effect was observed at an even lower dexamethasone concentration (10^{-14} M). Again, timing was critical for the effectiveness of dexamethasone: if added at day 3 of culture, dexamethasone 10^{-7} M was ineffective ($P=0.116$), in contrast to its strong inhibitory effect when added at the beginning of the culture.

In the same experiment, a separate group of sensitized mice were injected with dexamethasone, 5 mg/kg, 2 h before intranasal ovalbumin challenge, and hemopoietic cells from their lungs and bone-marrow were isolated and cultured 24 h after challenge in the presence of IL-5, without further addition of dexamethasone to the cultures. As shown in Fig. 3, panel (A), in sensitized/challenged BALB/c mice, in vivo administration of dexamethasone did not increase the subsequent responses of bone-marrow eosinophil precursors to IL-5 in culture ($P=0.965$). Similar results were found in bone-marrow separated on Percoll gradients (Fig. 3, panel (B); $P=0.617$). By contrast, bone-marrow of naive mice, injected at the same time with dexamethasone as positive controls (Fig. 3, insert), responded to this pretreatment ($P=0.011$), confirming previous observations [22]. These data indicate that bone-marrow cells from sensitized/challenged mice remain responsive to the enhancing effects of dexamethasone in vitro, but differ from those of naive mice in their ability to be primed by in vivo exposure to dexamethasone. On the other hand, as shown in Fig. 3, panel (C), following the same in vivo exposure to dexamethasone, no significant decrease was observed in the subsequent response of eosinophil precursors isolated from the

lungs to IL-5 in culture. Together, these data show that the dexamethasone-sensitive step is placed (a) *before* day 3 of culture in the presence of IL-5, for both lung and bone-marrow cells; (b) *after* their initial accumulation in allergen-challenged lungs, for lung cells.

We have further evaluated whether the effect of dexamethasone was mediated through the glucocorticoid receptor or not. Liquid cultures were established with cells from the middle layer after separation of cells from bone-marrow and lung on Percoll gradients. Cultures were established in the presence of IL-5 alone, or in association with dexamethasone (10^{-7} M) and/or RU486 (10^{-6} M). As shown in Fig. 4, the effects of dexamethasone on both bone-marrow (panel A) and lung (panel B) cells were completely blocked by RU486. RU486 had no effect on either cell population in the absence of dexamethasone. This

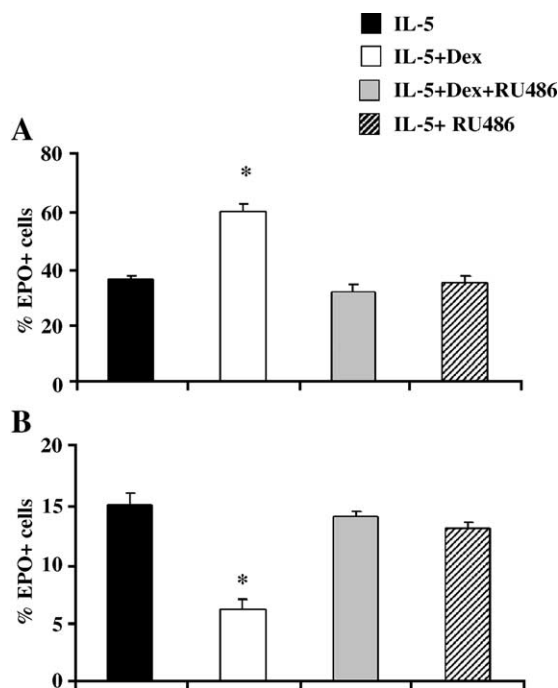


Fig. 4. RU486 blocks the effects of dexamethasone on eosinophil precursors isolated from bone-marrow and lung. Eosinophil precursors isolated from bone-marrow (panel A) and lung (panel B), after separation on Percoll gradients, were cultured in the presence of IL-5. Dexamethasone (10^{-7} M) and RU486 (10^{-6} M), alone or in combination, were added as indicated. Data are mean $\pm$ SEM of the %EPO+ cells in 7-day liquid cultures. Asterisk (*) indicates significant differences relative to the respective IL-5 controls ($P<0.005$ in all cases as detailed in Results). Data are derived from 3 experiments.

indicates that both the enhancing effects on bone-marrow and the inhibitory effects on lung cells are mediated through the glucocorticoid receptor.

3.3. Susceptibility to induction of apoptosis by dexamethasone in bone-marrow and lung eosinophil precursors

We next examined whether differences in response to dexamethasone in IL-5-stimulated culture were associated with differences in susceptibility to induction of apoptosis by dexamethasone. Apoptosis was evaluated at days 3 and 5, because (a) the effects of dexamethasone in vitro reflect its activity during the initial 72 h of culture (see Fig. 3), and (b) apoptosis

induction is expected to occur before any significant decrease in EPO+ cell counts. As shown in Fig. 5, panel (A), for day 3, dexamethasone (10^{-7} M) did not induce apoptosis in cultures from bone-marrow eosinophil precursors, established from the middle layer after Percoll separation (left; $P=0.315$ for the difference between dexamethasone-exposed cultures and their controls). By contrast, dexamethasone significantly increased the frequency of TUNEL-labeled (ApoptagTM+ cells) in cultures established from lung precursors ($P=0.033$). Comparable results were obtained at day 5 (not shown) for both cell types. TUNEL labeling, indicative of DNA fragmentation, was found only in cells bearing pyknotic nuclei. To confirm that the apoptotic cells belonged to the

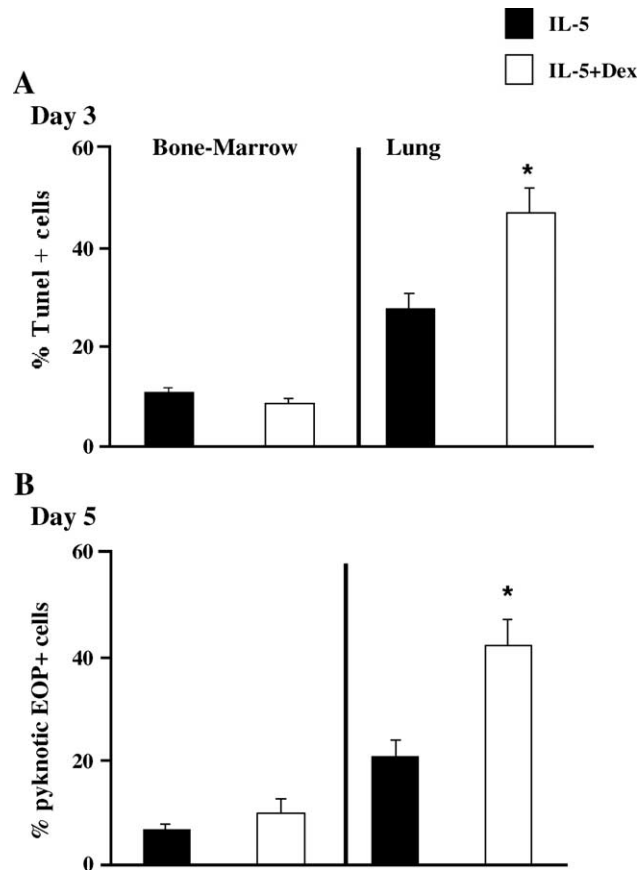


Fig. 5. Differential induction of apoptosis by dexamethasone in cultures of eosinophil precursors from bone-marrow and lungs. Eosinophil precursors isolated from bone-marrow and lung, after separation on Percoll gradients, were cultured for 3 days with IL-5, with or without dexamethasone, 10^{-7} M, and the percent of apoptotic cells were determined from cytocentrifuge preparations by TUNEL staining (panel A) or by counting EPO+ cells with pyknotic nuclei (panel B). Data are mean $\pm$ SEM. * $P=0.033$ (panel A) and $P=0.012$ (panel B) for the differences between lung cells exposed to dexamethasone and the respective controls. Data are derived from 4 experiments.

eosinophil lineage, we determined the frequency of EPO+ cells bearing pyknotic nuclei in cultures from both bone-marrow and lung. As shown in Fig. 5, panel (B), apoptotic EPO+ cells were present at low numbers in bone-marrow culture, and were not significantly increased in the presence of dexamethasone ($P=0.320$). By contrast, apoptotic EPO+ cells were numerous in cultures from lung progenitors and significantly increased by exposure to dexamethasone ($P=0.012$).

4. Discussion

Because recruitment of hemopoietic progenitors into asthmatic lung tissue has been postulated for a long time [4,17], but not directly demonstrated, we recently developed a method for the isolation and quantification of hemopoietic cells from lungs of ovalbumin-sensitized and -challenged mice [18]. In the present study, we used this procedure to evaluate the effects of dexamethasone, a powerful suppressor of allergic inflammation, on hemopoietic cells from lung and bone-marrow. By doing so, we confirmed that naive mice have virtually no hemopoietic cells in the lung, and that allergen exposure of sensitized mice is a very effective stimulus for the accumulation of both hemopoietic progenitors and eosinophil precursors in the lungs. We have further shown that these can be effectively separated from mature leukocytes recruited to the lungs after challenge. Our findings suggest that, if lung-derived eosinophil precursors contribute to in situ hemopoiesis in asthmatic lungs, they constitute one of the potential targets for the therapeutic action of steroids.

In evaluating these differences, a number of previously defined features of precursor culture have to be kept in mind. Culture is necessary to dissect the lineage-specific responses to cytokines and drugs free from interference from many other factors operative in vivo [33]. Eosinophils found at day 7 develop during this period from EPO-precursors; cultures established without IL-5 show no eosinophils at day 7, and contain only a few macrophages derived from the inoculum [9]. This requirement for exogenous IL-5 also applies to lung-derived precursors. This shows that other cell types present in the inoculum do not provide the cytokines required for eosinophil differ-

entiation. IL-5 alone is sufficient to support eosinophil development and therefore no addition of GM-CSF, IL-3 or other cytokines is necessary, unlike semi-solid culture of lung-derived hemopoietic progenitors [18]. Dexamethasone in the absence of IL-5 does not support any hemopoietic activity from the inoculum [22]. Hence, the effects of dexamethasone on developing eosinophils must be studied in the presence of IL-5, and defined by comparison with controls from the same source cultured in IL-5 alone.

Finally, it should be kept in mind that a number of studies evaluating the effects of glucocorticoids on eosinopoiesis differ widely in experimental conditions and observed outcomes. Bjornson et al. have shown that eosinophil colony formation was decreased and neutrophil colony formation was increased by hydrocortisone [34]. In that study, hydrocortisone was able to prevent production of colony-stimulating factors for both eosinophils and neutrophils. Butterfield et al. have shown that eosinophil colony formation from human bone-marrow was inhibited by large doses of dexamethasone, but not hydrocortisone [35]. However, these effects might reflect inhibition of the production of growth factors. Both studies differ from our own, where recombinant IL-5 was present. More recently, Kuo et al. have shown that dexamethasone decreases IL-5 mRNA and protein in human circulating CD34+ eosinophil progenitors, but does not decrease expression of IL-5R α on these cells [36]. In their study, dexamethasone only decreased eosinophil colony formation by CD34+ cells from asthmatic donors. It is clear, however, from their and other studies (reviewed in Refs. [21,33]) that the outcome depends greatly on the assay conditions (colony formation as opposed to liquid culture), on the origin of the cells (human or mouse) and on the exact source analysed (peripheral blood, cord blood or bone-marrow). In our study, only liquid culture was used to study responses to dexamethasone, and always in the presence of exogenous IL-5.

4.1. Two different patterns of eosinophil precursor response to dexamethasone can be identified

We observed two different patterns of response to dexamethasone in IL-5-stimulated culture of eosinophil precursors. In lung-derived cells, *inhibition* of eosinophil differentiation was associated with direct

induction of apoptosis by dexamethasone. This was not observed with bone-marrow cells, which are strongly *stimulated* to differentiate into eosinophils by dexamethasone in association with IL-5 [22] and can be protected by dexamethasone from apoptosis induced by other agents [27]. Therefore, the difference between lung- and bone-marrow-derived precursors is not quantitative, but qualitative, and cannot be accounted for by differences in the numbers of hemopoietic cells present in either source. It is likely that eosinophil differentiation in lung-derived cultures is decreased because the IL-5-stimulated cells die by apoptosis. However, dexamethasone has no effect on the frequency of apoptotic cells in bone-marrow cultures (Fig. 5 and Ref. [25]). Hence, the enhanced production of eosinophils from bone-marrow cells cultured in the presence of IL-5 and dexamethasone cannot be accounted for by a decreased apoptotic rate of eosinophil precursors. It is possible, on the other hand, that the mechanism of enhanced eosinopoiesis in bone-marrow culture involves adhesion proteins: we have shown that large clusters of cytologically immature eosinophils [33] develop in the presence of IL-5 and dexamethasone [22], and, in recent studies, observed complete inhibition of cluster formation, along with completion of cytological differentiation, when anti- $\alpha 4$ integrin antibodies were added to these cultures (Gaspar Elsas et al., unpublished observations). It is conceivable that dexamethasone increases the numbers of eosinophils produced from bone-marrow at the end of the 7-day culture period by delaying terminal differentiation and preserving proliferative capacity.

One further difference concerns the exquisite sensitivity of lung-derived eosinophil precursors to dexamethasone, since inhibition was caused by dexamethasone concentrations that no longer had any effect on bone-marrow cells. It remains to be determined whether sensitivity to very low concentrations of dexamethasone depends on the relatively long period of exposure (7 days in culture).

4.2. Nature of the dexamethasone-sensitive target in lung and bone-marrow cell populations

We favor the view that the dexamethasone-sensitive stage in lung cultures is the eosinophil precursor, rather than the mature eosinophil, because addition of dexamethasone to lung hemopoietic cell cultures at

day 3 no longer had a suppressive effect on the response to IL-5, indicating that the target of the inhibitory effects of the drug disappears at this time, while mature eosinophils persist. On the other hand, the presence of dexamethasone during this critical 3-day period prevented the appearance of the mature eosinophils, which are dexamethasone-resistant, indicating that these originate from dexamethasone-sensitive targets. Apoptotic cells were an early finding (day 3) in dexamethasone-exposed cultures from lung, where they persisted at least up to day 5. Since cells from lung are not inhibited by dexamethasone added after day 3, it is possible that the persistence of apoptotic cells in cultures by day 5 is due to slow or incomplete clearance of cells that entered apoptosis up to day 3.

The pattern of apoptosis susceptibility or resistance was consistent between morphological assessment of pyknosis in EPO+ cells and TUNEL labeling. However, TUNEL staining seemed more sensitive, which we interpret as a result of pyknosis being a relatively late event, compared to intranuclear DNA fragmentation.

Alternatively, however, it could be proposed that the targets of dexamethasone in lung cultures are among the mononuclear cells that constitute the majority of the cells in the cultured middle layer. It should be noted that eosinophil precursors themselves are believed to be mononuclear cells, just like precursors from other myeloid lineages [32]. Hence, these putative additional potential targets would necessarily be mononuclear cells other than precursors. One could hypothesize that these would be able to secrete IL-5, and that this IL-5 could be sufficient to counteract the effects of dexamethasone on lung-derived cells. This hypothesis could, in principle, explain why dexamethasone would seem to have no inhibitory effect on lung cell cultures if added at day 3, for this observation would simply reflect the antagonistic effect of increased IL-5 concentration [24]. We do not favor this view because (a) we have used a rather high concentration of IL-5, and endogenous secretion by mononuclear cells must be very intense to make any significant increment in the effective concentration; this possibility is clearly not supported by our observation that cultures established without exogenous IL-5, from either lung or bone-marrow, show no eosinophils and no eosinopoiesis, indicating that IL-5 is not being produced by the

mononuclear cells present in any functionally significant amount; (b) lung cells were exquisitely sensitive to the inhibitory effects of dexamethasone, in the presence of high concentrations of IL-5; hence, the view that IL-5 overcomes this sensitivity is not supported by our data.

It should be noted that dexamethasone was ineffective if added to the cultures from either bone-marrow or lung after day 3. This similarity in time course suggests that it is the *site of origin*, rather than the *developmental stage* of the target cells, that determines susceptibility to apoptosis [33, 37]. Such differences could be due to the target cells themselves, or to factors in their microenvironment which are yet to be identified. Candidate factors, as suggested by our recent studies (see above), would be ligands for $\alpha 4$ integrins.

Bloom and colleagues [26] reported that dexamethasone enhances survival of mature (blood) human eosinophils in the presence of relatively large concentrations of IL-5. In their study, susceptibility to apoptosis induced by dexamethasone was determined by suboptimal IL-5 concentrations. In our study, this factor has been ruled out, because bone-marrow- and lung-derived cells responded differently to the same concentrations of IL-5.

4.3. The role of IL-5

Because we collected cells from allergen-challenged, sensitized donors, which had been exposed *in vivo* to IL-5 [9], this cytokine might contribute in several different ways to the observed outcome, in addition to supporting eosinophil differentiation *in vitro*. Contrary to our expectations, exposure to dexamethasone *in vivo* did not decrease eosinophil differentiation from lung-derived precursors upon subsequent culture. On the other hand, also against expectations, exposure to dexamethasone *in vivo* did not enhance eosinophil differentiation from bone-marrow-derived precursors in culture. This apparent insensitivity to dexamethasone *in vivo* was observed only with cells from challenged, sensitized mice, because the same treatment enhanced responses to IL-5 in bone-marrow from naive donors (lung cells from naive donors could not be examined, for they lack detectable precursors). Our data suggest that the emergence of eosinophil precursors in the lungs is a

dexamethasone-resistant process. On the other hand, these findings also provide some evidence for a difference between bone-marrow precursors from naive and allergic mice in sensitivity to glucocorticoids *in vivo*, the mechanism of which is at present unclear.

4.4. Biochemical pathways linked to apoptosis versus protection from apoptosis in dexamethasone-exposed cultures

We could establish that the effects of dexamethasone on both lung and bone-marrow cells were glucocorticoid receptor-dependent. This suggests that the observed differences in outcome depend on the signalling pathways activated in either cell type by the same receptor. Our findings, taken together, are compatible with the hypothesis that eosinophil precursors isolated from two distinct sites in the same animals differ in their programmes of cellular response to the combination of IL-5 and dexamethasone. The nature of the biochemical differences underlying the opposite outcomes remains to be established. Recent studies have implicated mitochondrial apoptosis pathways, including the permeabilization of mitochondrial membrane, the depletion of an antiapoptotic protein, X-linked inhibitor of apoptosis, as well as the activation of c-Jun terminal kinase, as the critical determinants of dexamethasone-induced apoptosis in human eosinophils [38,39]. If transposable to our experimental system, these observations would imply that similar mechanisms are operative in lung-derived eosinophil precursors, but suppressed in bone-marrow-derived cells. On the other hand, dexamethasone is well-known as an inhibitor of human neutrophil apoptosis [23,40], and in other cell types induces the expression of a pro-survival gene, dexamethasone-induced gene-2 [41]. Again, if such findings can be transposed to murine hemopoietic cells, one would expect similar mechanisms to be operative in eosinophil precursors from murine bone-marrow, but not lung.

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