

ORIGINAL ARTICLE

Evidence for a regulatory role of $\alpha 4$ – integrins in the maturation of eosinophils generated from the bone marrow in the presence of dexamethasone

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Summary

Background Although eosinophils co-express multiple integrin receptors, the contributions of integrins to eosinophil development have not been explored. We previously described extensive aggregation and cytological immaturity in eosinophils developing in bone-marrow (BM) cultures exposed to dexamethasone. Here we examined the relationship of $\alpha 4$ integrins with these effects of dexamethasone.

Objectives We evaluated: (a) the effects of exposure to dexamethasone in BM culture on eosinophil expression of $\alpha 4$ integrin receptors and ligands; (b) the contribution of $\alpha 4$ integrins to eosinophil aggregation and maturation.

Methods Cultures were established with IL-5 (alone or with dexamethasone) for up to 7 days, and eosinophil production, $\alpha 4$ integrin receptor/ligand expression, aggregation and morphology were evaluated before and after targeting $\alpha 4$ integrin-dependent adhesions.

Because prostaglandin E2 (PGE2) modifies the effects of dexamethasone on eosinophilopoiesis, PGE2 effects on $\alpha 4$ integrin expression and function were also evaluated.

Results Dexamethasone increased the yield of eosinophils up to day 7. The frequency of eosinophils expressing $\alpha 4$, $\beta 1$ and $\beta 7$ integrin receptors at day 7 was also increased by dexamethasone. Eosinophils also expressed the $\alpha 4\beta 1$ ligand, VCAM-1. Dexamethasone increased the expression of $\alpha 4$ integrin and VCAM-1 in aggregates containing eosinophils as early as day 3. PGE2, added up to day 3, modified the effects of dexamethasone to suppress the expression of $\alpha 4$ integrin, decrease aggregation and promote cytological maturation of eosinophils recovered at day 7. Dissociation of immature eosinophils from clusters present at day 3 by reagents targeting $\alpha 4$ or $\beta 1$ integrins or VCAM-1 also induced cytological maturation. The concordant effects of targeting $\alpha 4$ integrins with drugs and antibodies support a relationship between $\alpha 4$ -mediated aggregation and maturational arrest.

Conclusions These observations support a novel role for $\alpha 4$ integrin receptors and ligands in eosinophilopoiesis. In addition, increased $\alpha 4$ expression following glucocorticoid exposure may contribute to the retention and accumulation of eosinophils in haemopoietic tissue.

Keywords adhesion molecules, bone marrow, eosinophils, glucocorticoids, prostaglandin

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Introduction

Mature eosinophils, prominent in allergic inflammation [1], are highly sensitive to glucocorticoids, which induce eosinopenia and accelerate apoptosis [2, 3]. However, the effects of glucocorticoids on earlier stages of eosinophil

development are more complex, as they decrease IL-5 production [4], but paradoxically enhance the responses of eosinophil precursors to IL-5 [5, 6]. As a result, eosinophils developing in bone-marrow (BM) culture are protected by dexamethasone [7] from apoptosis [both spontaneous and induced by exogenous ligands such as

prostaglandin E2 (PGE2)]. In stress experimentally induced by surgical trauma in mice, BM presented increased eosinophilia *in vivo*, and BM cells displayed enhanced responses to IL-5 *ex vivo*; both phenomena were entirely dependent on glucocorticoid hormones [6]. Furthermore, stress states, characterized by elevated glucocorticoid hormone levels, paradoxically predispose to allergic episodes [8, 9]: for instance, in atopic dermatitis, which is characterized by chronic eosinophil infiltration, decreased eosinophil apoptosis and tissue injury induced by release of eosinophil major basic protein, chronic stress plays a major role in the maintenance of active disease [10].

The cellular targets regulated by dexamethasone during murine eosinophil development are currently being investigated. Adhesion molecules are one likely target, because eosinophils that have developed in the presence of both IL-5 and dexamethasone are highly aggregated [5]. Interestingly, these aggregated eosinophils are also cytologically immature [5]. This unusual phenotype prompted us to further evaluate the effects of dexamethasone on the receptors and ligands involved in eosinophil aggregation, and to examine their role, if any, in the maintenance of an immature phenotype.

In this study, we focused on $\alpha 4$ integrins, the best-characterized eosinophil integrins [11], because they were previously shown to influence the development of several haemopoietic lineages other than eosinophils, as follows: (a) $\alpha 4\beta 1$ integrin mediates haemopoietic progenitor adhesion to VCAM-1- and fibronectin-expressing BM stroma [12]; (b) haemopoietic progenitors constitutively express $\alpha 4\beta 1$ with high affinity [13]; (c) anti- $\alpha 4\beta 1$ integrin antibodies block development of lymphoid and myeloid cells [12, 14]; and (d) haemopoietic progenitor retention inside BM is $\alpha 4$ integrin-dependent [15]. Even though their role in eosinophil development remains unexplored, $\alpha 4$ integrin heterodimers are differentially regulated during terminal differentiation [16, 17]. Here, we show that, in developing eosinophils, dexamethasone increases $\alpha 4$ integrin expression, and that treatments targeting $\alpha 4$ integrins induce dissociation and cytological maturation, supporting a regulatory role for $\alpha 4$ integrins during terminal differentiation.

Methods

Animal procedures

Six to eight-week-old BALB/c mice (CECAL-FIOCRUZ, Rio de Janeiro, Brazil) were housed and handled following institutionally approved procedures (protocol CEUA #PO107-02).

Reagents

Fetal calf serum (FCS) was from Hyclone (Logan, UT, USA), culture media from Gibco (Life Technologies SARL, Cergy-Pontoise, France); Dexamethasone (21-phosphate),

Cy3-conjugated streptavidin (ref. S-6402), Fast Red kit and secondary antibodies from Sigma-Aldrich (St Louis, MO, USA); PGE2 from Cayman Chemical Company (Ann Arbor, MI, USA); ovalbumin (5 $\times$, crystallized) from ICN Biomedicals Inc. (Costa Mesa, CA, USA), Al(OH)₃ from Merck (Darmstadt, Germany); Anti-LFA-1 (FITC, CD11a/LFA α_L chain, clone 121/7), anti- $\alpha 4$ (rat IgG1 anti-mouse CD49d, unlabelled, biotinylated or PE-conjugated, clone PS/2, refs. 1520-01, 1520-08 and 1520-09), anti-VCAM-1 (rat IgG1 anti-mouse VCAM-1, both biotinylated and unlabelled, Clone M/K-2, Refs. 1510-01 and 1510-08) and isotype control (Rat IgG1 Isotype Control, both biotinylated and unlabelled, Clone KLH/G1-2-2) antibodies from Southern Biotechnology Associates Inc. (Birmingham, AL, USA); Recombinant rmIL-5, anti- $\beta 7$ integrin (FITC, clone M293), anti-MADCAM-1 (biotin, anti-mouse, clone MECA-89), anti- $\alpha 5$ integrin (purified anti-mouse CD49e; ref. 01740D) from Pharmingen (San Diego, CA, USA); anti- $\beta 1$ integrin (purified anti-mouse CD29, ref. 550531) from BD Biosciences (Franklin Lakes, NJ, USA); anti-mouse CCR3 MAb (Clone 83103; ref. MAB1551), recombinant mouse VCAM-1/Fc chimera fusion protein (ref. 643-VM); Streptavidin-PE from R&D Systems (Minneapolis, MN, USA); Alexa Fluor[®] 488-conjugated goat anti-rat IgG (H+L) Cat A-11006, from Invitrogen (Carlsbad, CA, USA); and To-Pro[®]-3 iodide ref. T3605 from Molecular Probes Inc. (Eugene, OR, USA).

BM cell procedures

BM cells were collected from both femurs of naïve mice, washed, counted in a haemocytometer, seeded at 10^6 in 1 mL of RPMI 1640 medium, 10% FCS and rmIL-5 (1 ng/mL; optimal dose defined in refs. Gaspar-Elsas et al.) in 24-well clusters and incubated at 37 °C, 5% CO₂/95% air for 7 days [5]. Cultured cells were resuspended, collected, counted, cytocentrifuged and stained for eosinophil peroxidase (EPO) [20]. EPO⁺ cells were counted at $\times 400$ under oil. Where indicated, cultures received dexamethasone, or PGE2, or both, at 10^{-7} M (final concentration; for detailed dose-response relationships for both agents, see Gaspar-Elsas et al. [18, 19]). Where indicated, anti- $\alpha 4$ integrin, anti-VCAM-1, VCAM/Fc fusion protein, anti-MadCAM-1, anti- $\beta 1$ integrin or anti- $\alpha 5$ integrin (at 1 μ g/mL in all cases, optimal dose previously determined in titration experiments) were added at days 0 or 3. Cultures were collected at day 7 and evaluated for the numbers, morphology and aggregation of eosinophils. Where indicated, the distribution of eosinophils among clusters of different size ranges was determined by counting > 500 cells. Where indicated, cultures at days 0, 1, 3, 5 and 7 were resuspended, counted, cytocentrifuged, fixed in methanol and stained in Wright-Giemsa, and differential counts of neutrophils and eosinophils were performed ($\times 1000$, under oil) on at least 300 cells. Where indicated, nuclear morphology as

exemplified in Fig. 1 (To-Pro[®]-3 staining) and 5 (haematoxylin staining) was used to classify eosinophils as mature or immature, as follows: cells were resuspended from day 7 cultures, counted, cytocentrifuged, fixed in phosphate-buffered saline (PBS) 10% formalin and stained for EPO⁺. Nuclear morphology was examined in at least 300 EPO⁺ cells. Absolute numbers of eosinophils (mature or immature), neutrophils or total cells in resuspended cultures were calculated from total and differential counts.

Immunostaining and microscopical studies

Unless otherwise indicated, immunocytochemical studies were carried out with cells gently resuspended from day 7 cultures, fixed in PBS 1% paraformaldehyde, blocked for FcR binding in PBS containing 2% normal mouse serum for 10 min, washed in PBS 1% FCS, incubated with primary antibodies against $\alpha 4$, $\beta 1$ or $\beta 7$ integrins or VCAM-1, or with an isotype control antibody, washed four times with PBS 1% FCS and further incubated with alkaline phosphatase-conjugated anti-rat IgG biotinylated secondary antibodies. After washing four times with PBS 1% FCS, antibody-coated cells were stained using the Fast Red kit. Because these samples could be counterstained with haematoxylin (nuclear stain), but not with EPO (granular stain) or Wright-Giemsa (nuclear and cytoplasmic stain), which interfere with visualization of the red immunocytochemical stain, we used nuclear morphology to confirm that the stained cells were eosinophils, the only granulocyte population present in 7 day cultures (see 'Results', Figs 1a and b). Three hundred cells were examined under a Leica DMLS microscope (Leica Microsystems GmbH, Wetzlar, Germany) for each experimental point, and photographed with a Leica DC 300F digital camera using the IM50 software. Where indicated, cultures were established on LabTek chambers (Lab-Tek Chamber Slide System, 16 well, NUNC, New York, NY, USA) for 3 days; non-attached cells (representing 84% and 86% of all cells in control and dexamethasone-exposed cultures, respectively) were then carefully removed and used for flow cytometry studies. The cells attached to the substratum (amounting to 16% and 14% of all cells, respectively, as evidenced by counting cells recovered after mild trypsinization with a cell scraper, from separate cultures). For day 3, neutrophils are found in the cultures, and the presence of eosinophils was confirmed by separately staining attached cells for EPO (see Fig. 3a). Where indicated, colocalization of adhesion proteins and eosinophil lineage markers was carried out under a Zeiss LSM-510 Meta confocal microscope (Carl Zeiss, Jena, Germany), as follows: cells were resuspended from 7 day cultures, cytocentrifuged and fixed in 1% paraformaldehyde for 10 min, before blocking FcR, and incubation with primary antibodies to CCR3 (1 : 500 dilution, 1 μ g/mL final, 30 min). Slides were washed and

incubated with Alexa Fluor[®] 488-conjugated goat anti-rat IgG (H+L) secondary antibodies (1 : 2000, 1 μ g/mL final, 30 min). After washing, slides were further stained with primary biotinylated antibody to $\alpha 4$, to VCAM-1 or isotype control (0.5 μ g/mL, 30 min) before washing and incubation with Cy3-streptavidin and To-Pro[®]-3. Slides were immersed in anti-fading solution, covered and sealed, and maintained at 4 °C until use. Excitation wavelengths and respective emission filters were 488 nm and BP515-530 for Alexa fluor 488; 543 nm and BP560-615 for Cy3; and 633 nm and LP650 for To-Pro[®]-3.

Flow cytometry

After FcR blocking, 10⁶ cells were stained with primary antibodies to: VCAM-1 (biotinylated); MadCAM-1 (biotinylated); or $\alpha 4$ integrin chain (PE-conjugated) and isotype control antibodies (biotinylated or PE-conjugated, respectively) (15 min on ice), washed in cold PBS. Biotinylated antibody-covered cells were further incubated with PE-conjugated streptavidin (15 min on ice). Cells were washed in PBS, fixed in PBS with 2% formaldehyde and analysed in a FACScalibur (B&D PharMingen) using the Cell Quest software (B&D PharMingen).

Statistical analysis

Data were analysed by factorial analysis of variance using Systat for Windows version 4 software from Systat Inc. (Evanston, IL, USA). Because multiple comparisons were used throughout the study, the Tukey HSD correction was used as recommended [21]. This is a parametric method, and assumes that the data are normally distributed; the software automatically rejects data that do not conform to this model.

Results

Dexamethasone effects on $\alpha 4$ integrin receptor/ligand expression

Initially, we evaluated the effects of dexamethasone on cultured BM cells, and identified the cells that show increased $\alpha 4$ integrin expression following dexamethasone exposure. As shown in Fig. 1a, neutrophils are numerous in uncultured BM (day 0). However, neutrophil numbers declined drastically before day 5 of culture, and neutrophils were <1% of all cells at day 7, as evidenced by Wright-Giemsa staining. By contrast, as shown in Fig. 1b, eosinophils accumulated over the same period, and were significantly increased by day 7 in dexamethasone-exposed cultures. Counts of EPO-stained cells were in close agreement with eosinophil counts by Wright-Giemsa: as shown in Fig. 1c, dexamethasone (10⁻⁷ M), associated with IL-5, increased the frequency of EPO⁺ cells at day 7, relative to IL-5 control cultures ($P < 0.001$)

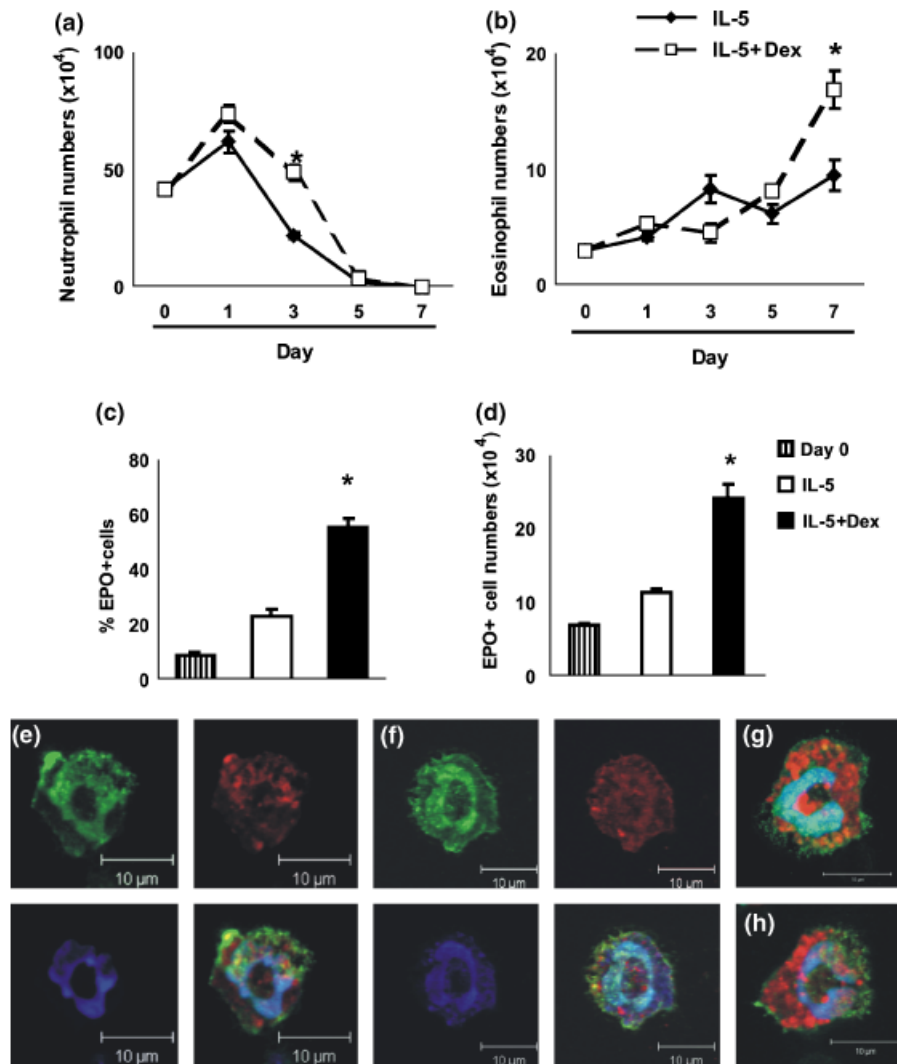


Fig. 1. Cells from control and dexamethasone-stimulated cultures expressing $\alpha 4$ integrin and VCAM-1. BM cells were examined directly (day 0) or after culture in the presence of IL-5 alone (IL-5) or IL-5-plus-dexamethasone (IL-5+Dex) for 7 days. (a–b), data (mean $\pm$ SEM) are numbers of Wright–Giemsa-stained neutrophils (a) or eosinophils (b) at the indicated time-points. * $P=0.002$ (a) and $P=0.026$ (b) relative to IL-5 controls. (c–d), data (mean $\pm$ SEM) are frequency (a) and numbers (b) of EPO⁺ cells at days 0 and 7; * $(P<0.001)$ relative to IL-5 controls. (e–h), confocal fluorescence microscopy images of cells at day 7, from IL-5 (e–g) or IL-5+Dex (h) cultures, labelled with anti-CCR3 antibody (green), DNA marker To-Pro³ (blue), and anti- $\alpha 4$ (e–h) or anti-VCAM-1 (f) antibodies (red). Data for (a–d) are derived from 3, 3, 12 and 5 experiments, respectively. EPO, eosinophil peroxidase; BM, bone marrow.

and uncultured BM (day 0; $P<0.001$) [5]. This was paralleled by an increase in the absolute numbers of EPO⁺ present in culture (Fig. 1d), relative to the same controls ($P<0.001$ in both cases).

Dexamethasone-exposed eosinophils showed extensive homotypic aggregation [5]. This suggests that eosinophils express both the receptors and the ligands required for aggregation. We therefore examined by confocal fluorescence microscopy the expression of $\alpha 4$ integrin or the $\alpha 4\beta 1$ ligand, VCAM-1, in cells co-expressing CCR3, an eosinophil-lineage marker. Surface staining for both $\alpha 4$ integrin and CCR3 was found in cells with the donut-shaped nucleus typical of mature eosinophils, present in IL-5-stimulated cultures (Fig. 1e). VCAM-1 and CCR3

were also co-expressed by these cells (Fig. 1f). Immature eosinophils, presenting a C-shaped nucleus, also co-expressed $\alpha 4$ and CCR3 (Fig. 1g). Finally, eosinophils from dexamethasone-exposed cultures also co-expressed $\alpha 4$ and CCR3 (Fig. 1h). Staining for $\alpha 4$ and VCAM-1 was specific, as shown by the absence of staining with an IgG1 isotype control antibody (not shown). We further evaluated by immunocytochemistry the expression of $\alpha 4$ integrin, and of $\beta 1$ and $\beta 7$ integrins, which associate with $\alpha 4$, by mononuclear cells and eosinophils in 7 day cultures. As shown in Fig. 2, the frequency of eosinophils expressing $\alpha 4$ (Fig. 2a), $\beta 1$ (Fig. 2b) and $\beta 7$ (Fig. 2c) was significantly increased in cultures stimulated by dexamethasone and IL-5 (respectively, $P<0.001$, $P=0.002$ and

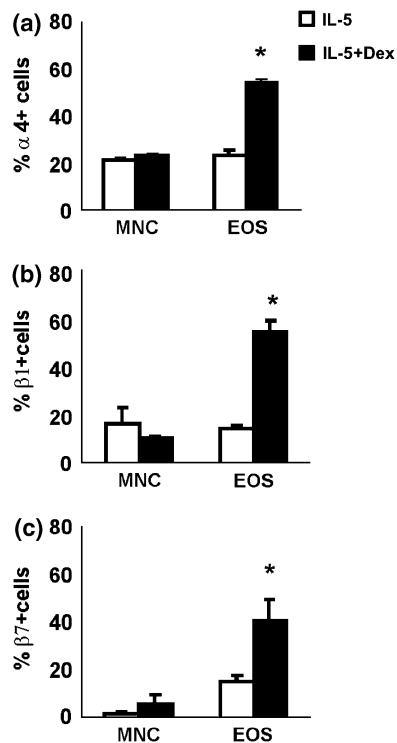


Fig. 2. Effect of dexamethasone on $\alpha 4$ and $\beta 1$ integrin expression. BM cells were examined after culture in the presence of IL-5 alone (IL-5) or IL-5-plus-dexamethasone (IL-5+Dex) for 7 days. Data (mean $\pm$ SEM) are frequency of cells expressing $\alpha 4$ (a), $\beta 1$ (b) and $\beta 7$ (c) integrin chains, for both mononuclear cells (MNC) and eosinophils (EOS). * $P < 0.001$ (a), $P = 0.002$ (b) and $P = 0.007$ (c) relative to IL-5 controls. Data for (a–c) are derived from three experiments. BM, bone marrow.

0.007 relative to IL-5 controls, in each panel). No difference was observed in mononuclear cells between experimental and control cultures. Furthermore, dexamethasone had no effect on the expression of $\alpha_L\beta 2$ (LFA-1) in eosinophils ($6 \pm 0.9\%$, in comparison with $7.9 \pm 1.1\%$ in controls).

Because dexamethasone enhances eosinophil production if added until day 3, but not at later times [5], we next examined the presence of eosinophils and the expression of $\alpha 4$ integrin receptors/ligands in dexamethasone-exposed BM cultures at day 3. Single cells were examined by flow cytometry; by contrast, cells attached to the substratum, which were not amenable to flow cytometry, were examined by immunocytochemistry. Figure 3a, shows representative views of stroma and stroma-attached cells (eosinophils, mononuclear cells and neutrophils) from both control and dexamethasone-exposed cultures. As shown in (Fig. 3b), no surface immunostaining was detectable with isotype control antibodies. By contrast, cells contained in aggregates, in both control and dexamethasone-treated cultures, showed diffuse surface staining for $\alpha 4$ -integrin or VCAM-1. Again, staining for both markers was specific, as shown by the absence of staining with an isotype control antibody. Strong staining

of individual cells for $\alpha 4$ integrin was prominent in dexamethasone-exposed cultures. MadCAM-1 was weakly expressed (not shown).

Figure 4a, shows the flow cytometry profiles of single, unattached cells either present in uncultured BM (day 0) or harvested from day 3 cultures, gated on R1 (granulocytes), R2 (lymphoid cells) and R3 (monocytes). Granulocytes were increased following culture, and further increased in dexamethasone-treated cultures (Fig. 4b). By contrast, monocytes and lymphocytes were decreased by culture; lymphocytes were markedly depleted by dexamethasone exposure. Single granulocytes staining positive for $\alpha 4$ integrin and VCAM-1 were found under both culture conditions, in comparable proportions (Fig. 4c); no increase in fluorescence intensity per cell for either marker was induced by dexamethasone. However, because R1 included 58% of all events in dexamethasone-treated cultures, as opposed to 34% in IL-5 controls, dexamethasone-exposed cultures contained larger numbers of $\alpha 4^+$ granulocytes. In all samples, expression of MadCAM-1 was undetectable in single granulocytes.

Drug effects on integrin expression and eosinophil aggregation. Because dexamethasone and PGE2 interact in BM culture [7], we examined their effects on eosinophil production, aggregation and $\alpha 4$ -integrin expression as observed in day 7 cultures. As shown in Fig. 5a, PGE2 decreased ($P < 0.001$), and dexamethasone increased ($P < 0.001$), eosinophil numbers relative to IL-5 controls. When added together, PGE2 and dexamethasone interacted, so that eosinophil production was brought back to the level in IL-5 control cultures ($P = 0.68$). As shown in Fig. 5b, the frequency of eosinophils expressing $\alpha 4$ integrins, as detected by immunocytochemistry, was also increased by dexamethasone ($P < 0.001$) and decreased by PGE2 ($P < 0.01$), relative to the IL-5 controls; importantly, when added together, PGE2 and dexamethasone decreased significantly ($P = 0.02$) the frequency of eosinophils expressing $\alpha 4$ integrin, relative to the positive controls (exposed to dexamethasone alone), reverting to the levels found in the negative (IL-5) controls ($P = 0.15$). When added together, PGE2 and dexamethasone also markedly reduced EPO^+ cell aggregation, relative to controls exposed to IL-5 and dexamethasone (Fig. 5c, upper row). These effects on cell aggregation were paralleled by changes in the intensity of staining for $\alpha 4$ integrin (Fig. 5c, lower row), which was strikingly diminished in cultures exposed to the association of PGE2 and dexamethasone, relative to dexamethasone controls.

We further quantified the effects of dexamethasone and PGE2 on eosinophil maturation, classifying EPO^+ cells on the basis of nuclear morphology, as illustrated in Fig. 5d for mature (left) and immature (right) eosinophils. As shown in Fig. 5e, relative to IL-5 controls, the effects of dexamethasone alone on eosinophil numbers at day 7

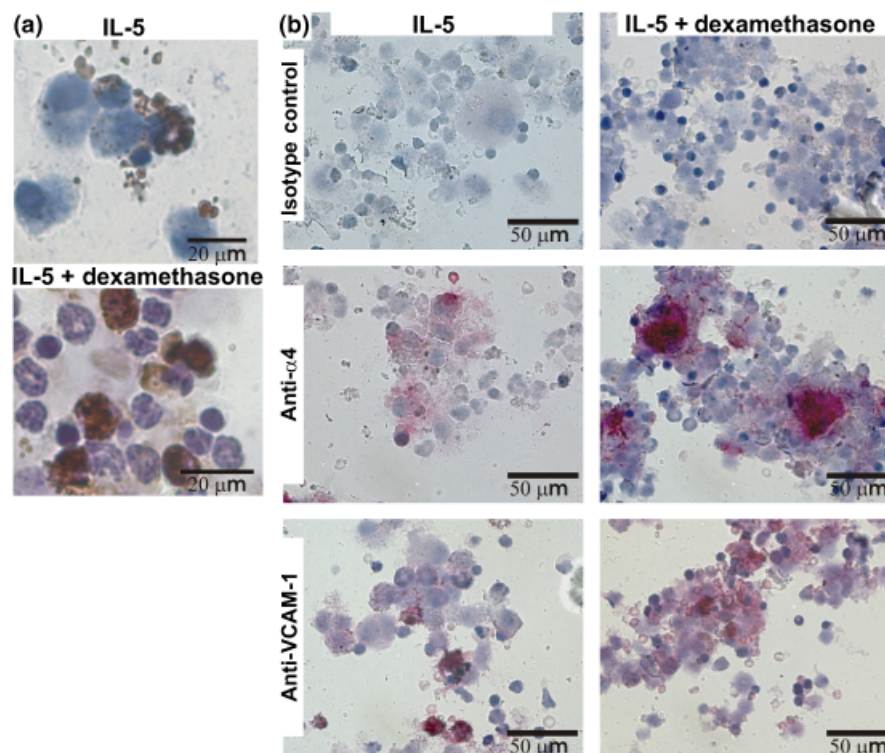


Fig. 3. Expression of $\alpha 4$ integrin and VCAM-1 in BM cellular aggregates. BM cells were cultured for 3 days in LabTek slides with IL-5 alone, or IL-5-plus-dexamethasone. Non-attached cells were removed, attached cells were stained for EPO⁺ (a) or immunocytochemically evaluated for $\alpha 4$ integrin and VCAM-1 expression (b). (a), representative views of IL-5 (top) or IL-5-plus-dexamethasone (bottom) cultures showing eosinophils with dark brown-stained cytoplasmic granules, mononuclear cells and neutrophils. (b), Representative views ($\times 1000$ under oil) of cells stained with isotype control, anti- $\alpha 4$ or anti-VCAM-1 antibodies. Scales are included to facilitate comparisons after photographic reduction. Images are from one experiment representative of 3. EPO, eosinophil peroxidase; BM, bone marrow.

could be accounted for by a significant increase in immature (but not in mature) eosinophils. By contrast, the effects of PGE₂ alone were associated with a significant reduction in mature eosinophils. With respect to the numbers of immature eosinophils in PGE₂-exposed cultures, differences relative to the IL-5 controls were not significant. Importantly, when both agents were added together, a striking reduction was observed in immature eosinophil numbers, relative to IL-5 plus dexamethasone controls. Hence, in dexamethasone-exposed cultures, PGE₂ acted on immature (*not* on mature) eosinophils, and this effect alone accounted for the reduction of eosinophil numbers down to the level of IL-5 controls. Together, these observations show that the effects of PGE₂ on eosinophils are profoundly modified by the presence of dexamethasone.

Because pre-incubation with dexamethasone alone, for a short period, was previously shown to prepare ('prime') BM cultures for increased eosinophil production upon subsequent addition of IL-5 [5], we further examined whether the presence of dexamethasone, for different intervals before addition of PGE₂ to the cultures, would modify the effects of the latter agent, as compared with cultures in which both agents were introduced together. In

IL-5-stimulated (control) cultures, PGE₂ significantly reduced EPO⁺ cell numbers, if added from days 0 to 1 (Fig. 5f). By contrast, if added later, it had no significant effect (day 2, $P=0.221$, day 3, $P=0.944$). In IL-5- and dexamethasone-stimulated cultures, PGE₂ reduced significantly the numbers of EPO⁺ cells if added from days 0 to 1 ($P<0.05$ at both time-points, relative to the controls with dexamethasone but no PGE₂). However, it had no effect on eosinophil numbers if added later (day 2, $P=0.117$; day 3, $P=0.740$). Hence, the suppressive effect of PGE₂ on eosinophil numbers was progressively lost as the interval between dexamethasone exposure and PGE₂ addition increased. Importantly, at all times, PGE₂ addition induced dissociation and cytological maturation of eosinophils (not shown). As the result of pre-treatment with dexamethasone for 2 days, true synergism was achieved, as the total numbers of eosinophils were increased, and maturation was promoted.

Direct targeting of $\alpha 4$ integrin receptors/ligands. We further evaluated whether aggregation and maturation of eosinophils could be affected by directly targeting $\alpha 4$ integrins and their ligands. Because $\alpha 4$, VCAM-1 and MadCAM-1 were present in aggregated EPO⁺ cells by

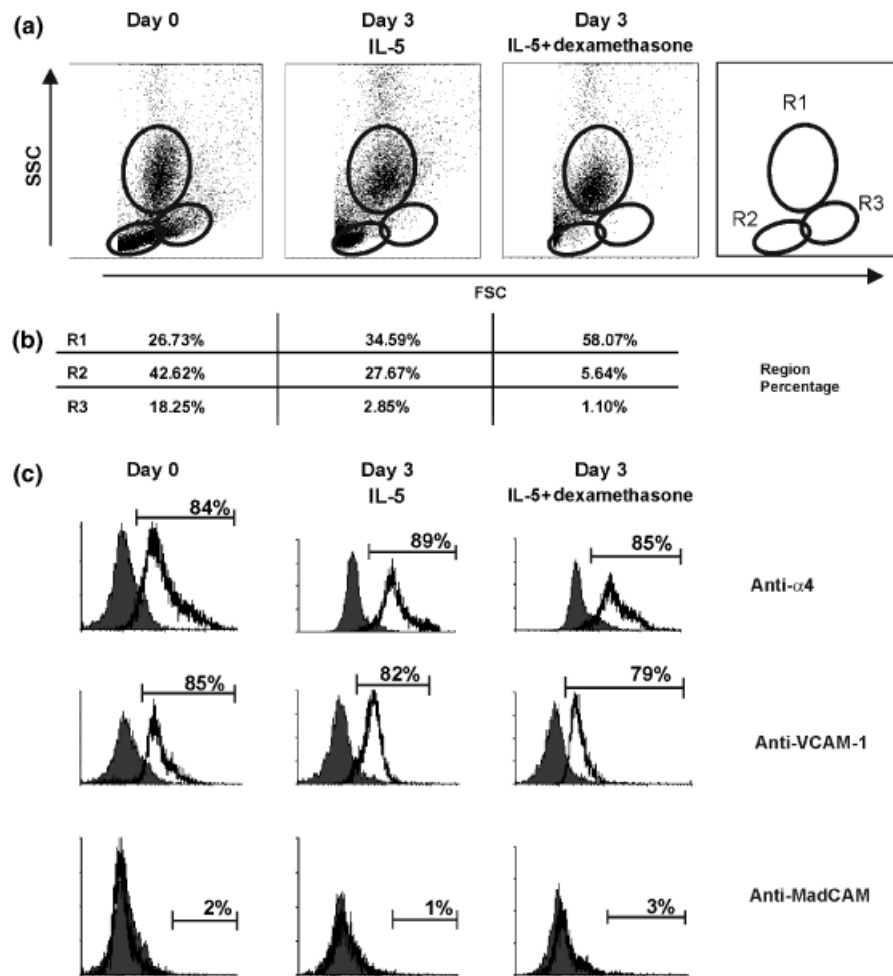
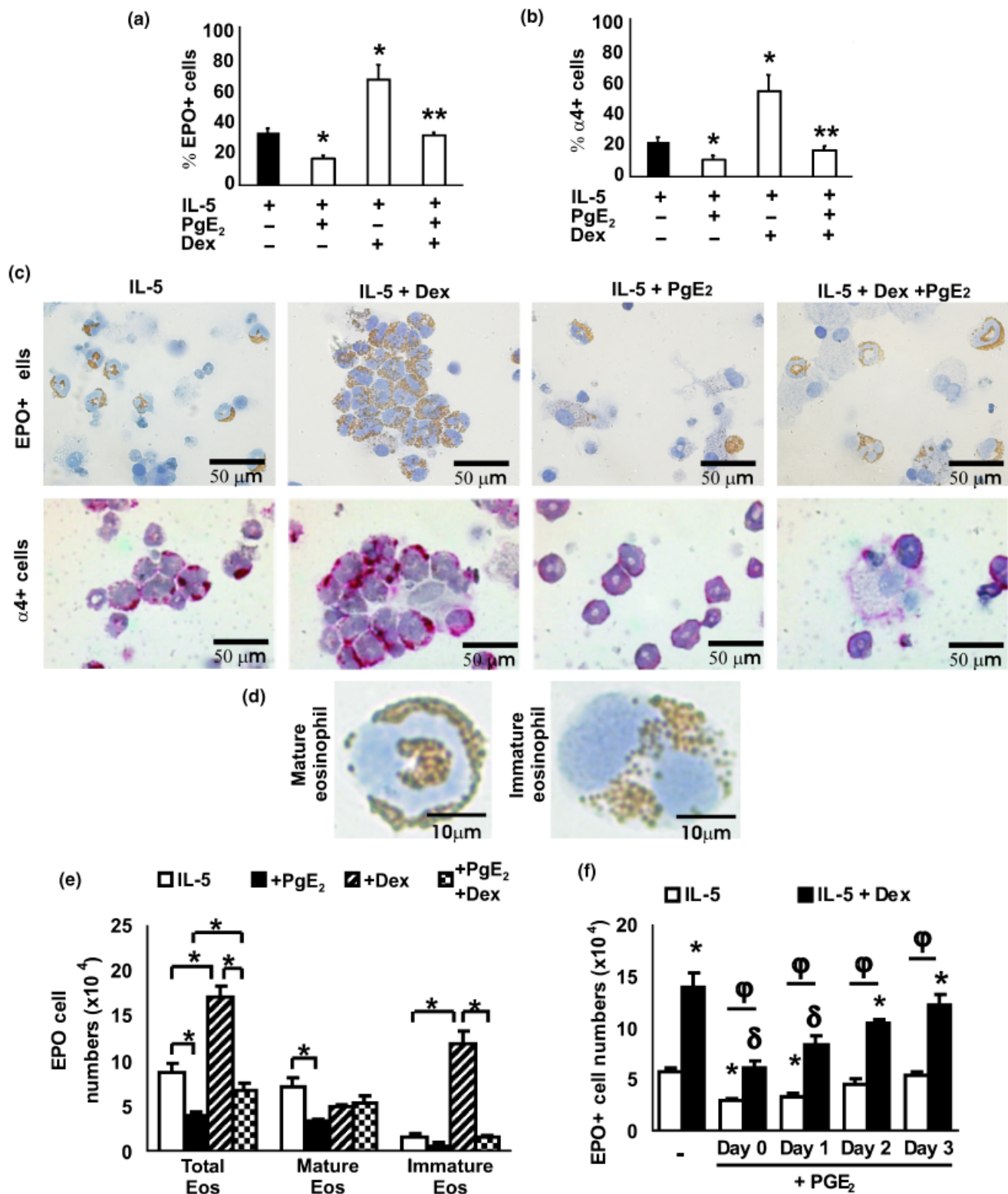


Fig. 4. Expression of $\alpha 4$ integrin and VCAM-1 in single cells. BM cells were either studied directly (day 0) or cultured for 3 days in the presence of IL-5 alone or IL-5-plus-dexamethasone. Single, unattached cells were gently resuspended, stained, fixed and analysed by flow cytometry. Data are: (a) SSC $\times$ FSC plots, outlining the regions (R1–R3) of comparison; (b) % of all events falling within each of the indicated regions; (c) fluorescence intensity profiles of cells in R1 for the indicated anti- $\alpha 4$, anti-VCAM-1 or anti-MadCAM-1 (open curves) or isotype control (biotinylated or PE-conjugated) antibodies (full curves). Data are representative of three experiments. BM, bone marrow.

day 3, and because dexamethasone exerts all its effects from day 0 to 3 [5], intervention would most likely be effective during this interval. BM cultures were established in the presence of IL-5, alone or in association with dexamethasone, and antibodies to $\alpha 4$ as well as other integrin receptors/ligands were added at days 0 or 3 of

culture, with further incubation up to day 7, to allow for completion of eosinophil maturation. As shown in Fig. 6a, anti- $\alpha 4$ antibody, added at day 0, very effectively inhibited eosinophil development in culture, even if dexamethasone was present for the entire culture period. Therefore, the effect of this treatment on aggregation

Fig. 5. Effects of dexamethasone and PGE₂, alone and in combination. BM cultures were established with IL-5 alone, or associated with dexamethasone (Dex), PGE₂ or both, for 7 days. (a), % EPO⁺ cells, (b), % $\alpha 4$ ⁺ eosinophils (mean $\pm$ SEM). Asterisks indicate significant differences ($P \leq 0.05$) relative to IL-5 controls (*) or IL-5-plus-dexamethasone controls (**). (c), representative images of EPO⁺ (upper row) and $\alpha 4$ ⁺ (lower row) cells in cultures subjected to the indicated treatments. (d), representative images of mature (left) and immature (right) eosinophils. (e), counts (mean $\pm$ SEM) of total, mature and immature eosinophils in cultures subjected to the indicated treatments. * Pairwise comparisons showing significant differences ($P = 0.033$ or smaller, in all cases). (f), counts (mean $\pm$ SEM) in cells cultured in the absence (IL-5) or presence (IL-5+Dex) of dexamethasone and exposed to PGE₂ from the indicated time-points. * $P < 0.05$ relative to the IL-5 control (first open bar). $\delta P < 0.05$ relative to the IL-5-plus-dexamethasone control (first black bar). * $P < 0.05$ between the indicated dexamethasone-exposed and control cultures. Data for (a, b, e and f) are derived from 5, 5, 3 and 3 experiments, respectively. EPO, eosinophil peroxidase; BM, bone marrow; PGE₂, prostaglandin E₂.



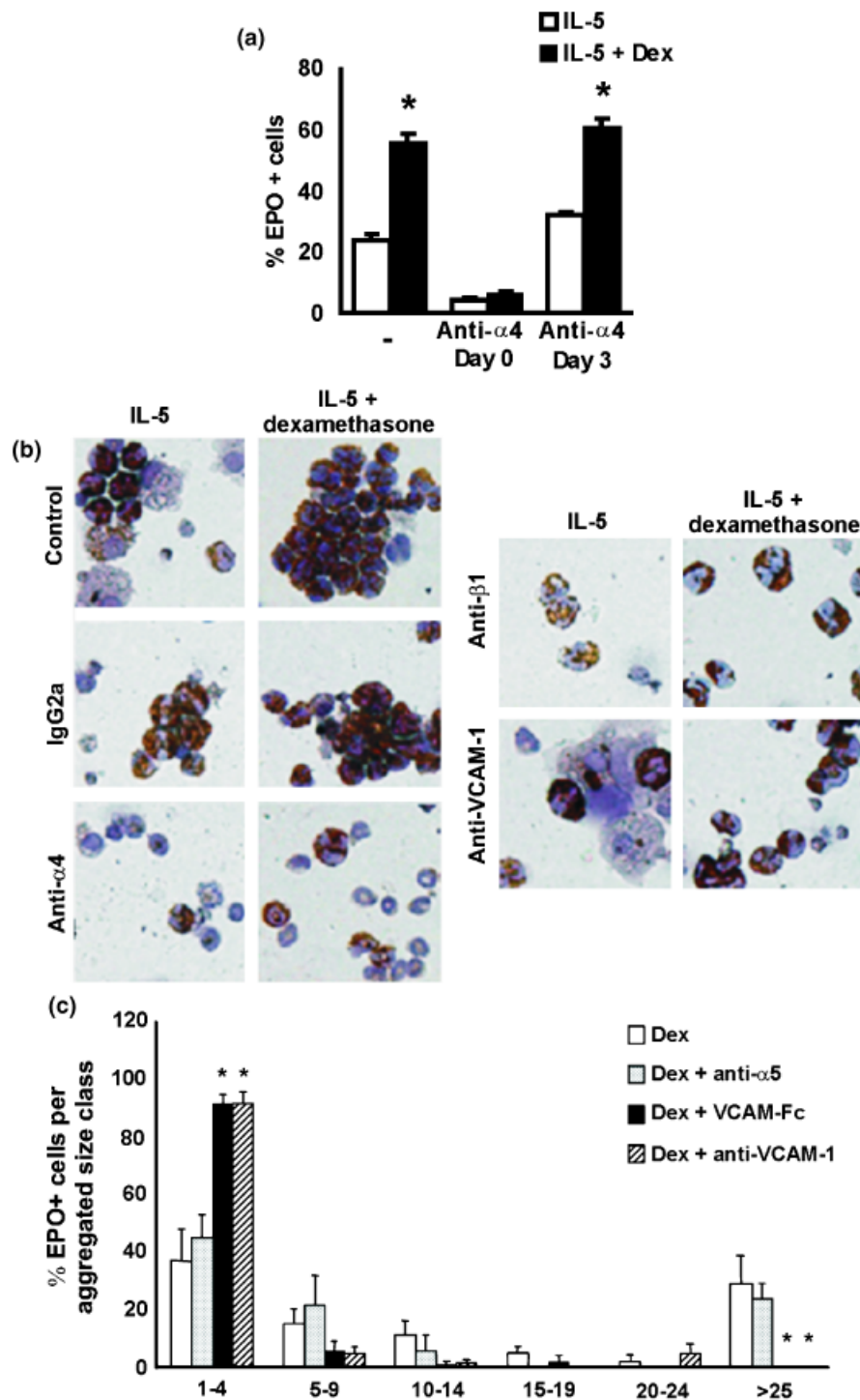


Fig. 6. Effect of disrupting $\alpha 4$ integrin-dependent interactions. BM cultures were established with IL-5 alone (IL-5) or IL-5-plus-dexamethasone (IL-5+Dex). (a), % EPO⁺ cells in untreated or antibody-treated cultures at day 7. Antibodies to $\alpha 4$ integrins were added at day 0 or 3. (b), effect of the indicated antibodies on the aggregation of EPO⁺ cells under both culture conditions at day 7. Antibodies to $\alpha 4$ and VCAM-1, or isotype controls, were added at day 3. Representative images of EPO⁺ cells in each experimental condition are shown (magnification: $\times 400$). (c), data are mean $\pm$ SEM of the % of all EPO⁺ cells that are found in clusters of each size class, shown as a class frequency distribution. VCAM-1- or $\alpha 5$ integrin-specific probes were added at day 3. Data from three experiments * $P < 0.05$ in each aggregate size range, relative to the untreated controls. EPO, eosinophil peroxidase; BM, bone marrow.

induced by dexamethasone could not be evaluated. By contrast, this antibody, added at day 3, had no effect on the numbers of EPO⁺ cells recovered at day 7 from IL-5-stimulated cultures, nor did it prevent the enhancing effects of dexamethasone. Nevertheless, as shown in Fig. 6b, following this treatment, eosinophil aggregation was markedly decreased (anti- α 4 relative to the isotype control, or to controls exposed to no antibody). A similar dissociation was achieved by targeting β 1 integrin. Finally, dissociation was also observed with antibodies to VCAM-1. Following any of these treatments, the dissociated cells were mature, despite the continued presence of dexamethasone. Finally, as shown in Fig. 6c, EPO⁺ cells cultured in IL-5 plus dexamethasone formed clusters of a wide size range, with approximately one-half of all EPO⁺ cells found inside large clusters (25 cells or more). This distribution was unaffected by control (anti- α 5) antibody, but shifted towards the small aggregate/single cell class (one to four cell) by two different reagents that target VCAM-1 through distinct mechanisms (ligand-binding anti-VCAM-1 antibodies and receptor-binding VCAM-1-Fc fusion protein).

Discussion

The following observations, taken together, support a role for α 4 integrins in eosinophil development: (a) α 4 integrins were strongly expressed by eosinophils that developed in the presence of both IL-5 and dexamethasone; (b) eosinophils also expressed the α 4 β 1 ligand, VCAM-1; (c) eosinophils in dexamethasone-exposed cultures were highly aggregated and included a majority of cytologically immature forms; (d) PGE2 counteracted the effects of dexamethasone on α 4 integrin expression by dexamethasone; (e) PGE2 interacted with dexamethasone to promote eosinophil dissociation and cytological maturation in culture; and (f) targeting either α 4 integrins or VCAM-1 with specific antibodies and soluble fusion proteins promoted eosinophil dissociation and cytological maturation. Because similar dissociative and maturation-promoting effects were achieved by distinct approaches, which interfered with α 4 integrin expression or function, our interpretation is that increased α 4 integrin expression not only holds developing eosinophils together, but delays or arrests their maturation. Consequently, dexamethasone or other treatments that up-regulate α 4 integrin during eosinophil maturation, if operative *in vivo*, might favour sequestration of eosinophils in the BM, mostly in an immature state.

There is no obvious reason why similar mechanisms should not operate *in vivo*. Eosinophils express α 4 integrins *in vivo* [11]. PGE2, a ubiquitous inflammatory mediator [22], has predominantly local actions, but its production can be systemically influenced by inducers such as IL-1 and TNF, and inhibitors including glucocorti-

coids. Indeed, the maturation-promoting effect of PGE2 described here resembles the effects of both PGE2 and TNF on dendritic cells: maturation-induced podosome dissolution and loss of α 5 β 1 integrin activity mark the transition from an adhesive to a highly migratory phenotype, a change rapidly induced by PGE2 and TNF and believed to play a major role in the initiation of the immune response *in vivo* [23, 24].

On the other hand, glucocorticoids, at stress levels or at therapeutic concentrations, do not eliminate BM eosinophil production [6, 25]. This suggests that, after a brief exposure to elevated glucocorticoid concentrations, eosinophils inside BM remain potential targets for subsequently generated PGE2, and the possible impact of glucocorticoid-PGE2 interactions on eosinophil numbers and maturity inside BM should be evaluated.

We address below a number of aspects that require further consideration:

(a) *The choice of experimental conditions.* This study was conducted in liquid BM culture because IL-5 and modulators can be separately delivered to the target cells at precise times during development, an approach that cannot be adopted *in vivo*. There is nevertheless a good correlation between the *in vivo* and the *in vitro* effects of glucocorticoids on eosinophilopoiesis, and *in vivo* exposure prepares the BM for enhanced responses to IL-5 in culture [4–6, 19]. Differentiation was induced with IL-5 [26], the major eosinophilopoietic cytokine [27, 28]. These conditions were required, for in semisolid (clonal) culture cells are held together by the medium, and the effects of disrupting integrin-dependent adhesions cannot be evaluated. Finally, integrin expression was evaluated by immunocytochemistry, because aggregated cells were not amenable to flow cytometry.

(b) *The criteria used for eosinophil identification.* Confocal microscopy confirmed that both α 4 integrin and VCAM-1 were present in cells co-expressing CCR3, thereby positively identifying α 4⁺ and VCAM-1⁺ cells as eosinophils, not neutrophils. Furthermore, neutrophils represented <1% of the cells present at day 7, and could not possibly account for our observations. By contrast, eosinophils increased in numbers over the culture period [4–6]. Finally, the presence of EPO⁺ granules in murine eosinophils (*not* neutrophils) has been demonstrated from the earliest precursor to terminally differentiated eosinophils [29]. Although there was an excellent agreement between these three staining methods (CCR3, EPO and Wright–Giemsa), for practical reasons our experiments were conducted with EPO-stained samples, unless otherwise specified.

(c) *Role of integrin affinity and specificity.* The dissociation of eosinophil aggregates with reagents specific for α 4 integrins or VCAM-1 supports a role for α 4 integrins in dexamethasone-induced aggregation. Nevertheless, flow cytometry shows that integrin expression *per se* does not

necessarily lead to aggregation, as shown previously by others [11]. There may therefore exist differences in $\alpha 4$ integrin affinity (i.e., the degree of integrin activation, which cannot be determined immunocytochemically) between single and aggregated cells, which account for extensive aggregates. Consequently, modulation of $\alpha 4$ -dependent aggregation may involve changes in either expression or affinity. With respect to the effects of PGE2 exposure, decreases in expression were observed, which could in principle account for the resulting dissociation. Because we have not directly evaluated the effects of PGE2 on integrin affinity, however, a contribution of decreased affinity to the observed effect cannot be ruled out at present. Both $\alpha 4\beta 1$ and $\alpha 4\beta 7$ receptors were up-regulated by dexamethasone; $\alpha 4$ expression was down-modulated by PGE2.

(d) *Timing of critical events.* At day 3, aggregated cells expressed $\alpha 4$ and VCAM-1. $\alpha 4$ integrins were targeted at days 0 and 3, which encompass the critical period for dexamethasone regulation [5]. Anti- $\alpha 4$ antibody, added at day 3, could not prevent the enhancement of eosinophilopoiesis by dexamethasone. However, this treatment had a marked effect on aggregation, because it dissociated the aggregates and promoted terminal maturation. This suggests that, from day 3 onwards, the major contribution of increased $\alpha 4$ -dependent aggregation is to delay or arrest eosinophil maturation, not to promote an increase in cell numbers. Between days 0 and 3, however, $\alpha 4$ integrins might play a different role in eosinophilopoiesis, because the same antibody added at day 0 completely prevented eosinophil development. Interestingly, PGE2, present from day 0, did not completely suppress eosinophilopoiesis in dexamethasone-exposed cultures. This suggests that this early effect of anti- $\alpha 4$ antibodies involves mechanisms not shared by PGE2. It may be relevant, in this early-phase, that autocrine generation of granulocyte macrophage-colony stimulating factor (GM-CSF) in response to ligation of selected eosinophil surface receptors, including binding of fibronectin to $\alpha 4$ integrins, has been reported [2, 30], a mechanism likely to be affected by anti- $\alpha 4$ antibodies. On the other hand, GM-CSF has also been reported to induce $\beta 1$ integrin transcripts in blood eosinophils [31], and might conceivably contribute to the up-regulation of $\alpha 4$ integrins in dexamethasone-exposed BM eosinophils.

PGE2 had two distinct effects on dexamethasone-primed cultures, namely an effect on eosinophil numbers and an effect on eosinophil aggregation. If present during the initial 24 h, PGE2 brought eosinophil numbers back to the levels observed in IL-5 controls (i.e., without dexamethasone). Kinetic experiments showed, however, that addition of PGE2 at later times had a progressively smaller effect on eosinophil numbers, which became, for cultures receiving PGE2 at day 3, indistinguishable from those in positive control cultures without PGE2. The effect on

eosinophil yield depended therefore on the timing of PGE2 addition. By contrast, PGE2 was equally effective at dissociating eosinophils at the indicated time-points. These observations further support a contribution of $\alpha 4$ integrins to aggregation and delayed maturation, but not to cellular proliferation.

(e) *Limitations and implications of the study.* Our major finding is that $\alpha 4$ integrins play a role in the terminal differentiation of eosinophils, mediating the effects of glucocorticoids and PGE2 on immature eosinophils. This conclusion is drawn from the correlative results on differentiation, integrin/VCAM-1 expression and aggregation, not based on a direct examination of the role played by $\alpha 4$ integrins in terminal differentiation. Such a direct examination, however, should be prompted by the evidence described here, which is, to our knowledge, the first to implicate an eosinophil surface molecule in arrested maturation. This endeavour is likely to face important technical difficulties. While studies with an isolated BM, where eosinophils can be reliably generated and identified, minimize interference from effects on other cell types (a significant concern, given the ubiquitous distribution of integrins), the manipulation of integrin expression levels with currently available molecular approaches remains a challenging task in a non-synchronized, heterogeneous system such as the BM. Perhaps this investigation may benefit from the recent development of protocols in which sequential exposure to different cytokine combinations induces differentiation of homogeneous eosinophil populations [32]. A related finding is the expression of VCAM-1 by eosinophils, along with the dissociating and maturation-promoting effects of treatments targeting VCAM-1. Eosinophils express a wide variety of integrin receptors [11] that have been extensively studied in the context of cell migration and functional activation. To our knowledge, however, a role for integrin receptors and ligands in eosinophilopoiesis has not been established in previous studies. Furthermore, VCAM-1 expression by eosinophils has not, to our knowledge, been reported. These data provide, therefore, evidence for a hitherto undescribed function of these integrin receptors and ligands.

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