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Differential effect of dexamethasone on cell death and STAT5 activation during *in vitro* eosinopoiesis

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Summary. Glucorticoids reduce eosinophilia through a direct effect on eosinophils or indirectly on cells producing cytokines and chemokines. Conflicting data have been previously reported concerning glucocorticoid effects on eosinopoiesis. To elucidate this point, dexamethasone was added during eosinophil differentiation of $CD34^+$ cells. Dexamethasone enhanced proliferation and differentiation through an early effect on immature cells. Dexamethasone inhibited apoptosis during early differentiation, whereas death of mature cells was increased. Signal transducer and activator of transcription 5 (STAT5) is a transcription factor involved in the proliferation, differentiation and survival of haematopoietic cells, which interacts with glucocorticoid receptor. Activation of STAT5 by interleukin-5 was investigated during eosinophil differentiation. Long isoforms of STAT5 were activated during the entire period in the culture as well as in blood eosinophils, while short isoforms were only activated during early differentiation. Short isoforms were less activated in the presence of dexamethasone. This suggests that dexamethasone could act on proliferation, differentiation and apoptosis during eosinophil differentiation through an association of STAT5 with the glucocorticoid receptor.

Keywords: apoptosis, CD34, eosinophils, differentiation, signalling.

Hypereosinophilia ($>1{\cdot}5 \times 10^9$/l) is mostly associated with a wide variety of diseases such as bronchial asthma, allergy, parasitic infection and malignant disorders. Anti-inflammatory glucocorticoids are usually administrated to rapidly reduce the number of circulating eosinophils. Glucocorticoids act by inhibiting the production of the cytokines or chemokines required for survival, migration and formation of eosinophils, such as interleukin-5 (IL-5), stem cell factor (SCF), eotaxin (Her *et al*, 1991; Finotto *et al*, 1997; Lilly *et al*, 1997). Glucocorticoids act directly on eosinophils by reducing their accumulation or sensitivity to chemotactic factors (Lantero *et al*, 1996; Kibe *et al*, 2003) or by inducing their apoptosis (Meagher *et al*, 1996). Controversial results have been previously reported regarding the effects of glucocorticoids on *in vitro* eosinopoiesis. Low concentration of hydrocortisone (10 nmol/l to 10 μmol/l) has an inhibitory effect on eosinophil colony formation from peripheral blood mononuclear cells (MNCs) (Bjornson *et al*, 1985). In human bone marrow cells, 3·3 μmol/l hydrocortisone increased eosinophil colony numbers, but high concentrations of hydrocortisone (up to 330 μmol/l) did not decrease the numbers of eosinophil colonies. On the same cells, dexamethasone (≤330 μmol/l) reduced eosinopoiesis (Butterfield *et al*, 1986). In contrast, low concentrations of dexamethasone (0·1 nmol/l to 0·1 μmol/l) enhanced bone marrow eosinopoiesis in mice (Gaspar Elsas *et al*, 2000). Furthermore, dexamethasone at 1 μmol/l reduced eosinophil colony formation from T cell-depleted MNCs isolated from asthmatics, but not from healthy persons (Kuo *et al*, 2001). The differences observed in these results could be attributed to the origin of the stem cells, the type of the glucocorticoid used, and the level or type of cytokines present in the medium.

IL-3, IL-5 and granulocyte/macrophage colony-stimulating factor (GM-CSF) are cytokines that influence the proliferation, differentiation, survival and activation of eosinophils (Lopez *et al*, 1986; Rothenberg *et al*, 1988; Yamaguchi *et al*, 1988a). The effect of IL-5 is mainly restricted to eosinophils and basophils, while IL-3 and GM-CSF act on other haematopoietic lineages. IL-5 is essential for terminal eosinophil differentiation and is important for the function of mature eosinophils (Lopez *et al*, 1988; Yamaguchi *et al*, 1988b). The human IL-5 receptor (IL-5R) is composed of an IL5-specific α chain and a

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β chain, which is shared with the receptors for IL-3 and GM-CSF (Tavernier *et al*, 1991). The α subunit is also present at the surface of $CD34^+$ stem cells (Sehmi *et al*, 1997). IL-5R belongs to the cytokine receptor family, which lacks the intrinsic kinase activity and transduces signals through the Janus kinases (JAKs). Immediate targets of JAKs are the signal transducers and activators of transcription (STATs). Tyrosine-phosphorylated STAT molecules are able to form dimers (homo- or heterodimers) that translocate to the nucleus, and interact with promoter elements regulating cytokine-responsive genes. IL-5 stimulation resulted in tyrosine phosphorylation of JAK2 and JAK1, associated with α and β chains, STAT1, STAT3, and STAT5 (Caldenhoven *et al*, 1995; Pazdrak *et al*, 1995; Ogata *et al*, 1998). Four JAKs, STAT1, STAT3, and STAT5 are strongly expressed by $CD34^+$ cells (Biethahn *et al*, 1999). STAT5 is activated by IL-5 in the stem cells and is required for their differentiation in eosinophils (Buitenhuis *et al*, 2003). JAK2 and STAT5 are activated upon stimulation with IL-5 in human peripheral eosinophils (Ogata *et al*, 1997). In mammals, two isoforms of this protein, STAT5A and STAT5B, are encoded by distinct genes (Wakao *et al*, 1994). Du *et al* (2000) have shown that complexes obtained with IL-5-stimulated blood eosinophils contain STAT5A and STAT5B. Carboxy-terminally truncated STAT5 isoforms (STAT5β), as opposed to the full length form (STAT5α), lack the functional transactivation domains and exert their dominant negative effects by blocking the DNA-binding site in STAT5-responsive gene promoters (Moriggl *et al*, 1996). A carboxy-terminally truncated 80-kDa STAT5 isoform is activated by IL-5 in mature eosinophils and blocks transcription mediated by STAT3 (Caldenhoven *et al*, 1999). There is a functional interaction between STAT5 and the glucocorticoid receptor (GR) in transient cotransfected COS cells (Stocklin *et al*, 1996). In mammary epithelial cells, a specific association of a portion of the STAT5 and GR proteins might provide a mechanism for a synergistic action of prolactin and glucocorticoids in the induction of β-casein gene expression (Cella *et al*, 1998).

In this study, we have investigated the effects of dexamethasone on the proliferation, differentation and apoptosis during eosinophil differentiation from human cord blood $CD34^+$ cells, and on activation of STAT5 by IL-5. Our data show that dexamethasone increased proliferation and differentiation by acting on immature cells, and has variable effects on apoptosis, depending on cell maturation but not on proliferation or differentation. Long isoforms of STAT5 were always activated by IL-5, whereas short isoforms were activated only during early differentiation. In the presence of dexamethasone, short isoforms were less activated, suggesting a regulation of STAT5 by glucocorticoids.

MATERIALS AND METHODS

Antibodies. Rabbit anti-human STAT5A raised against the carboxy-terminal region of STAT5A, rabbit anti-human STAT5B raised against the carboxy-terminal region of STAT5B, chicken anti-human STAT5 raised against the N-terminal region of STAT5A and STAT5B, previously described (Gouilleux *et al*, 1995; Moriggl *et al*, 1996), anti-human STAT1 and anti-human STAT3 (BD Biosciences, Mississauga, Canada) were used for the supershift analysis. Mouse anti-human STAT5 raised against SH2-SH3 region (BD Biosciences) was used for Western blotting.

Isolation and culture of human cord blood $CD34^+$ cells. Human umbilical cord blood was collected using syringes containing citrate–phosphate–dextrose solution (Sigma, Saint Louis, MO, USA). The MNCs were isolated by the Ficoll procedure (Techgen International, Les Ulis, France). Cells were washed and resuspended in phosphate-buffered saline (PBS) containing 5 mmol/l EDTA and 0·5% human serum albumin (LFB, Les Ulis, France). The $CD34^+$ subpopulation was isolated from the MNCs using immunomagnetic beads according to the CD34 isolation kit instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) (Miltenyi *et al*, 1990). $CD34^+$ cell purity was determined by flow cytometry (Elite ESP™, Beckman Coulter, Brea, CA, USA) with a CD34 antibody (BD Biosciences). Cells were cultured in medium of defined components (MDC) (DAP, Vogelgrun, France) supplemented with 15% fetal calf serum (FCS; Biological Industries, Kibbutt Beit Haemek, Israel), 2 mmol/l glutamine (Biowhittaker, Walkersville, MD, USA), 3 U/ml IL-5, 3 ng/ml IL-3 and 3 ng/ml GM-CSF (Genzyme S.A.S., Cergy-Pontoise, France) at 37°C in 5% CO_2. SCF (Sigma) was added during the first week (10 ng/ml). Twenty-four hours after culturing, cells were divided and 10 nmol/l of dexamethasone (Sigma) were added to half of the culture. This concentration was chosen after a dose-response experiment on proliferation and differentiation using 0·1 nmol/l to 10 μmol/l dexamethasone. Medium and dexamethasone were changed weekly and viable cells were counted weekly in the presence of trypan blue. Cytospin slides were prepared weekly and differentiation of eosinophils was estimated using colorimetric assay of eosinophil peroxidase activity (EPO) in the presence of potassium cyanide (Ten *et al*, 1989).

Isolation of human peripheral blood eosinophils. Peripheral blood eosinophils were separated using negative selection with an immunomagnetic method (Hansel *et al*, 1991). Blood was diluted with PBS–EDTA and overlaid onto ficoll solution. The erythrocyte fraction containing the granulocytes was collected and the erythrocytes were lysed using a 0·2% NaCl solution. The osmolarity was restored using a 1·6% NaCl solution. The polymorphonuclear cells were washed in PBS supplemented with 5 mmol/l EDTA, and incubated for 30 min at 4°C using CD16 immunomagnetic beads. A magnetic separation column (Miltenyi Biotec) retained the $CD16^+$ neutrophils and the column effluent containing $CD16^-$ eosinophils was collected with PBS–EDTA. The purity was determined using differential cell counts of cytospin preparation stained with May–Grünwald–Giemsa reagent.

Detection of apoptotic cells. The percentage of apoptotic cells was measured by fixation of Annexin-V-fluorescein isothiocyanate (FITC) to phosphatidylserine residues, exposed at the external surface of the cells during the

apoptotic process (Vermes *et al*, 1995); $0{\cdot}2 \times 10^6$ cells were washed with PBS, centrifuged at 200 ***g*** for 5 min. The cell pellet was resuspended in 100 µl of incubation buffer (10 mmol/l HEPES/NaOH pH 7·4, 140 mmol/l NaCl, 5 mmol/l $CaCl_2$) with 20 µl Annexin-V-FITC (Roche Diagnostics S.A, Meylan, France) and 1 µg/ml propidium iodide (Sigma). The cells were incubated for 15 min in the dark. The percentage of apoptotic cells was analysed with Elite ESP^{TM} flow cytometer using a 515 nm filter.

Whole cell extracts. Peripheral blood eosinophils or $CD34^+$-derived eosinophils were centrifuged for 5 min at 10 000 ***g*** and the pellets were resuspended in buffer containing 20 mmol/l Tris pH 7·9, 400 mmol/l NaCl, 1 mmol/l EDTA, 20% glycerol, 1 mmol/l dithiothreitol (DTT), 1 mmol/l phenylmethylsulphonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml aprotinin and 100 µmol/l vanadate. Lysates were obtained using a freezing/thawing technique followed by centrifugation for 10 min at 11 000 ***g***. Proteins were quantified using Coomassie blue reagent according to manufacturer's protocol (Pierce Biotechnology Inc., Rockford, IL, USA). For electrophoretic mobility shift assays (EMSA), cells were stimulated for 15 min with 50 U/ml of IL-5 prior to whole cell extraction.

Electrophoretic mobility shift assays. For EMSA analysis, 8 µg of whole cell extracts proteins were incubated with 25 fmol double-stranded oligonucleotide, corresponding to the STAT5 binding site of the bovine β-casein gene promoter (5′-AGATTTAGGAATTCAAATC-3′). Oligonucleotides were end-labelled with [γ-^{32}P]ATP (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA) using T4 polynucleotide kinase (Epicentre, Madison, WI, USA). Shift assays were performed in a total volume of 20 µl using the following buffer: 10 mmol/l Tris–HCl pH 7·5, 10 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l DTT, 0·5 mg/ml bovine serum albumin, 50 µg/ml polydeoxyinosinic deoxycytidylic acid (poly-dIdC; Amersham, Buckinghamshire, UK), 5% glycerol, 0·1% nonidet P40 (NP40). The reaction was carried out at room temperature for 30 min. Supershift analysis was performed by including 1 µg of anti-STAT1, anti-STAT3, or different anti-STAT5 antibodies for 15 min, followed by the addition of the labelled probe and further incubation for 30 min at room temperature. Specificity of complex formation was assessed using a 50-fold molar excess of unlabelled oligonucleotide competitor, added to the binding reaction 10 min prior to addition of the labelled probe. Complexes were then analysed using electrophoretic separation on a 6% non-denaturing polyacrylamide gel in TBE buffer (22 mmol/l Tris pH 8·3, 22 mmol/l borate, 0·6 mmol/l EDTA). The gels were dried and then exposed to X-ray films for autoradiographic analysis.

Western blot analysis. Whole cell proteins (40 µg) extracted from peripheral blood eosinophils or $CD34^+$-derived eosinophils were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (8%) and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked for 30 min in PBS containing 0·1% Tween-20 (PBS-T) and 5% non-fat dried milk and then incubated for 3 h with anti-STAT5 antibody 1:1000 in PBS-T. After washing, the membrane was incubated for 1 h with anti-mouse HRP-conjugated antibody (Amersham) 1:10 000 in PBS-T. Immunoreactive bands were visualized by an enhanced chemilumunescence detection system according to the manufacturer's instructions (Amersham).

Statistical analysis. Non-parametric Wilcoxon's test and Spearman's test were adopted for statistical evaluation, and *P*-values <0·05 were considered significant.

RESULTS

Effect of dexamethasone on the proliferation and differentiation of eosinophils from $CD34^+$ cells

To study the effect of dexamethasone on the proliferation and differentiation of cells of the eosinophil lineage, $CD34^+$ cells were isolated from human cord blood MNCs using an immunomagnetic method. The highly purified cells (>95% of $CD34^+$) were then cultured in MDC medium, supplemented with 15% FCS and the cytokines essential for inducing differentiation of the stem cells towards eosinophils (IL-3, IL-5, GM-CSF and SCF). Dexamethasone was added after 24 h at concentrations ranging from 0·1 nmol/l to 10 µmol/l. Proliferation was measured weekly by counting living cells, and the differentiation of eosinophils was evaluated by measuring the specific EPO. As shown in Fig 1A, the two lowest concentrations of dexamethasone (0·1 and 1 nmol/l) had no effect on proliferation during the course of the culture. In the presence of 10 nmol/l to 10 µmol/l dexamethasone, cell proliferation was strongly increased after 21 d. This effect was noticed after 14 d treatment with 10 nmol/l of dexamethasone. The percentage of EPO positive cells increased with 10 nmol/l to 1 µmol/l of dexamethasone at day 7, with all the concentrations tested at day 14, and in the presence of the three highest concentrations (0·1–10 µmol/l) at day 21 (Fig 1B). All further experiments were performed with 10 nmol/l of dexamethasone, the lowest concentration inducing a visible effect on proliferation and differentiation. Statistical analyses of nine cultures revealed that 10 nmol/l of dexamethasone significantly enhanced the proliferation of the cells after 14 d ($P < 0{\cdot}03$ Wilcoxon's test), 21 d ($P < 0{\cdot}02$) and 28 d ($P < 0{\cdot}01$) of culture (Fig 1C). Similarly, 10 nmol/l of dexamethasone enhanced differentiation of $CD34^+$ cells to eosinophils, and the percentage of EPO positive cells was higher in comparison with the control culture as early as day 7 ($P < 0{\cdot}02$) until day 28 (Fig 1D).

Effect of dexamethasone on apoptosis during eosinophil differentiation from $CD34^+$ cells

To determine whether dexamethasone acts via modulation of cell death to increase the proliferation of eosinophils, cells were cultured in the presence of 10 nmol/l of dexamethasone and apoptosis was evaluated weekly using the Annexin-V-FITC staining method. At day 7 of culture, dexamethasone significantly reduced the percentage of apoptotic cells in comparison with the control culture ($P < 0{\cdot}02$) (Fig 2A). In contrast, the percentage of apoptotic cells was increased in the presence of dexamethasone after

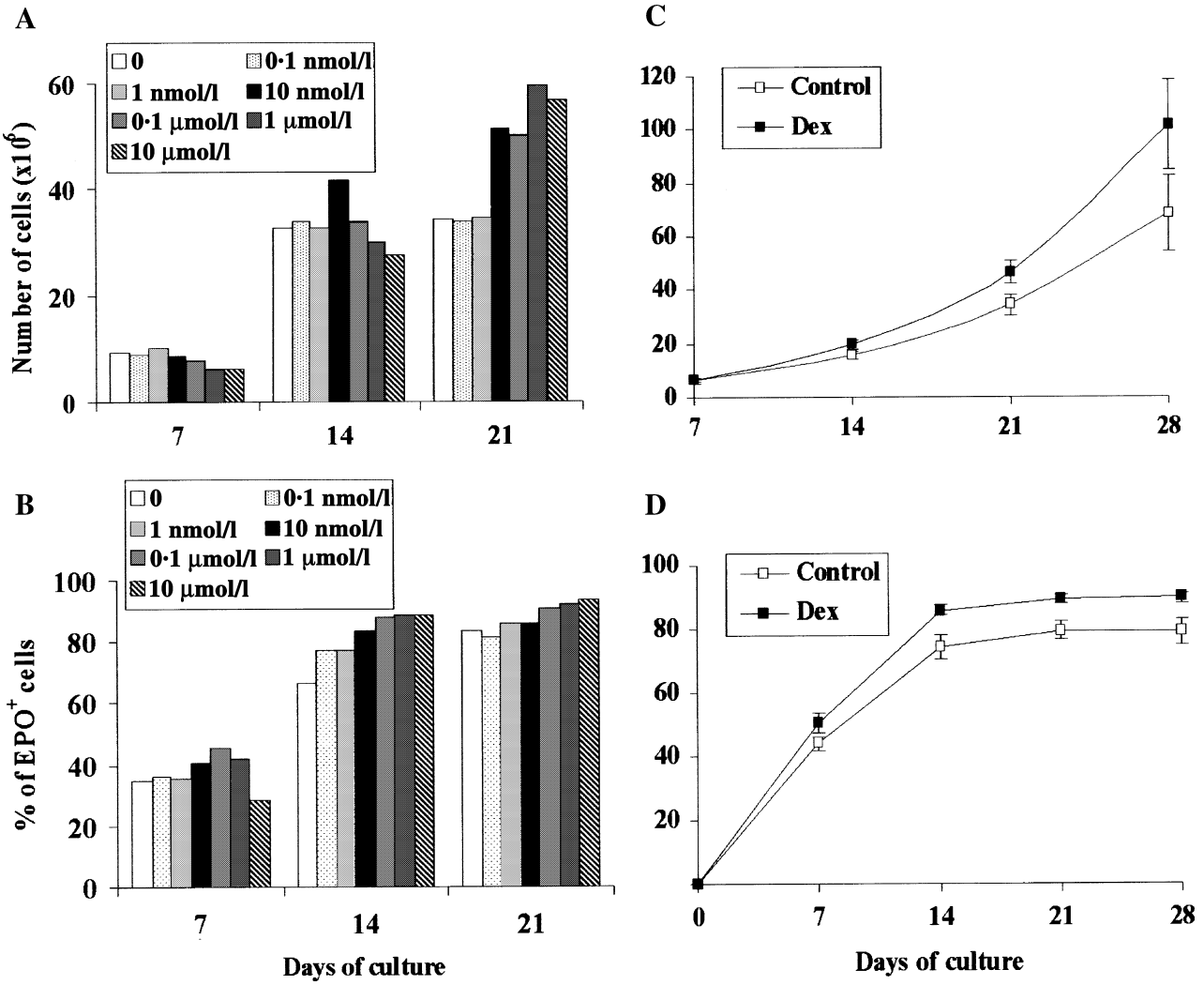

Fig 1. Dose-response experiment on proliferation (A) and eosinophil differentiation (B) in the presence of 0·1 nmol/l to 10 μmol/l dexamethasone. Effect of dexamethasone on proliferation (C) and differentiation (D) of nine cultures of $CD34^+$ cells. Cells were cultured with or without 10 nmol/l dexamethasone (Dex) (mean ± SEM).

21 and 28 d culture ($P < 0·02$). No significant difference was observed between both conditions after 14 d culture. Growth rate and apoptosis were positively correlated in the control culture (Spearman correlation coefficient: $r = 0·52$, $P = 0·006$), but not in culture treated with dexamethasone ($r = 0·17$, $P = 0·37$) (Fig 2B). Apoptosis was negatively correlated with differentiation rate in the control culture ($r = -0·49$, $P = 0·009$), but not in the presence of dexamethasone ($r = -0·17$, $P = 0·37$) (Fig 2C). These results suggest that cell death induced by dexamethasone is independent of the increase of proliferation and differentiation.

STAT5 DNA-binding activity in response to IL-5 during eosinophil differentiation and in blood eosinophils

The STAT5 is required for eosinophil differentiation and interacts with the GR, suggesting that STAT5 is involved in the effects of dexamethasone. To investigate the activation of STAT5 during eosinophil differentiation, proteins were extracted from the cells after 15 min of stimulation by IL-5, and EMSA were performed using a specific STAT5 binding oligonucleotide derived from the β-casein gene promoter. Figure 3A shows that the amount of complexes was very high at days 7 and 14 (lanes 1 and 2), and then decreased after 21 and 28 d of culture (lanes 3 and 4). Comparison with eosinophils isolated from human peripheral blood (lane 5) revealed that the band had the same intensity as the band obtained at day 28. The addition of 10 nmol/l of dexamethasone during eosinophil differentiation, produced an identical profile in response to IL-5 stimulation (Fig 3B). A slight decrease in complex intensity was observed only at day 14 (lane 2).

To identify the proteins that form the complexes, supershift analysis was performed using specific antibodies raised against the carboxy-terminal region of STAT5A and STAT5B, which recognize the long isoforms of STAT5,

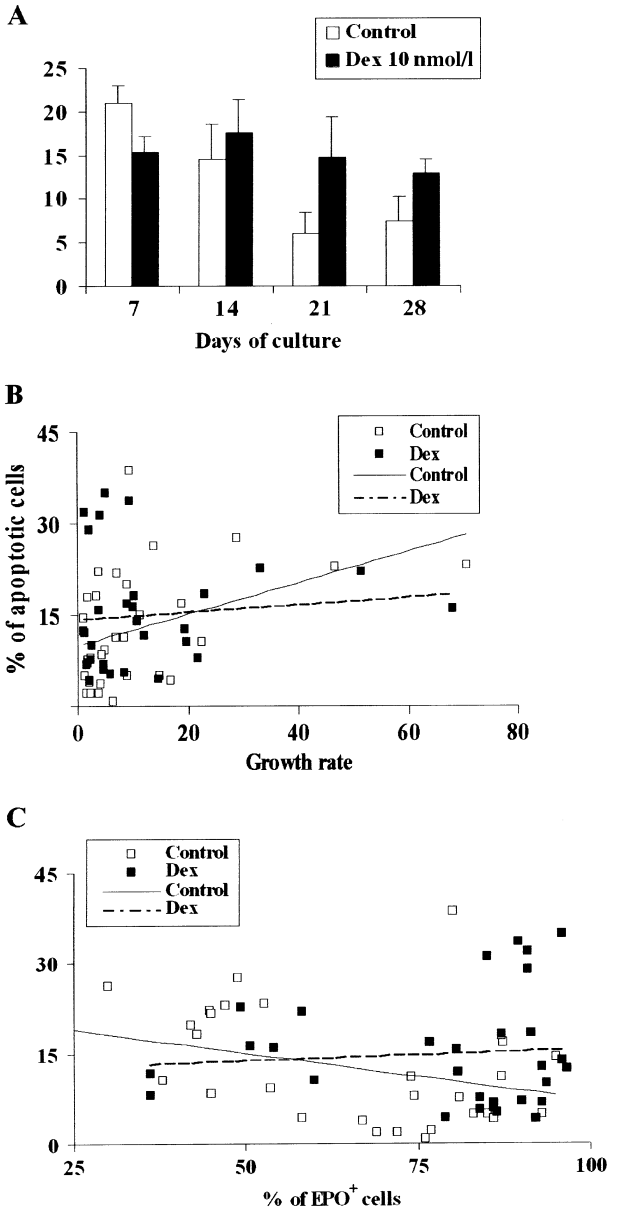

Fig 2. (A) Analysis of apoptosis using the Annexin-V-FITC staining method during eosinophil differentiation in the presence or absence of 10 nmol/l dexamethasone (Dex) (mean ± SEM, $n = 7$). (B) Correlation (Spearman's test) between growth rates and apoptosis with or without 10 nmol/l dexamethasone (Dex). (C) Correlation (Spearman's test) between eosinophil differentiation and apoptosis with or without 10 nmol/l dexamethasone (Dex).

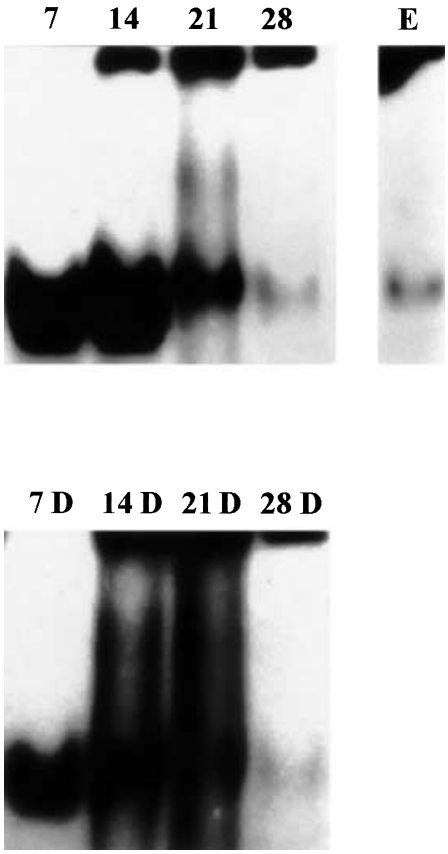

Fig 3. Analysis of STAT5 activation by EMSA using a ^{32}P-β-casein oligonucleotide after stimulation for 15 min with 50 U/ml IL-5. Whole cell extracts were prepared from cells differentiated towards eosinophils after 7, 14, 21 and 28 d with (7D, 14D, 21D, 28D) or without (7, 14, 21, 28) 10 nmol/l dexamethasone, or from human peripheral blood eosinophils (E).

and an antibody raised against the N-terminal region of STAT5 that recognizes the long and the short isoforms of STAT5. A mix of anti-STAT5A and anti-STAT5B antibodies (5A/5B) induced a supershift of some of the complexes at days 7 and 14 (Fig 4A, lanes 2 and 5), indicating that the long isoforms of STAT5 were involved in the formation of these complexes in association with other proteins. The complexes obtained at day 21 (lane 8), day 28 (lane 11) and in peripheral blood eosinophils (lane 13) were completely supershifted by the mix of anti-STAT5A and anti-STAT5B antibodies. Therefore, the long isoforms of STAT5 were only activated by IL-5 in these cells. The anti-STAT5 antibody raised against the N-terminal region (5N) supershifted all the bands at day 14 (Fig 4A, lane 6), indicating that complexes not supershifted by the anti-STAT5A and anti-STAT5B antibodies constituted the short isoforms of STAT5. In contrast, at day 7 the anti-N-terminal antibody was unable to supershift all the bands (lane 3). STAT1 and STAT3 are known to be activated by IL-5 and can bind the β-casein oligonucleotide with a lower affinity than STAT5. Specific antibodies were tested at day 7 but neither anti-STAT1 antibody (Fig 4B, lane 3) nor the anti-STAT3 antibody (Fig 4B, lane 4) induced a supershift of the bands. In the same way, the use of an oligonucleotide specific for the binding of STAT1 and STAT3 (probe SIE, serum inducible element) did not result in the formation of any complex (data not shown). The specificity of the complexes was demonstrated after the addition of unlabelled β-casein oligonucleotide probe as competitor (Fig 4B, lane 2).

When the cells were treated with 10 nmol/l of dexamethasone, long and short isoforms of STAT5 were

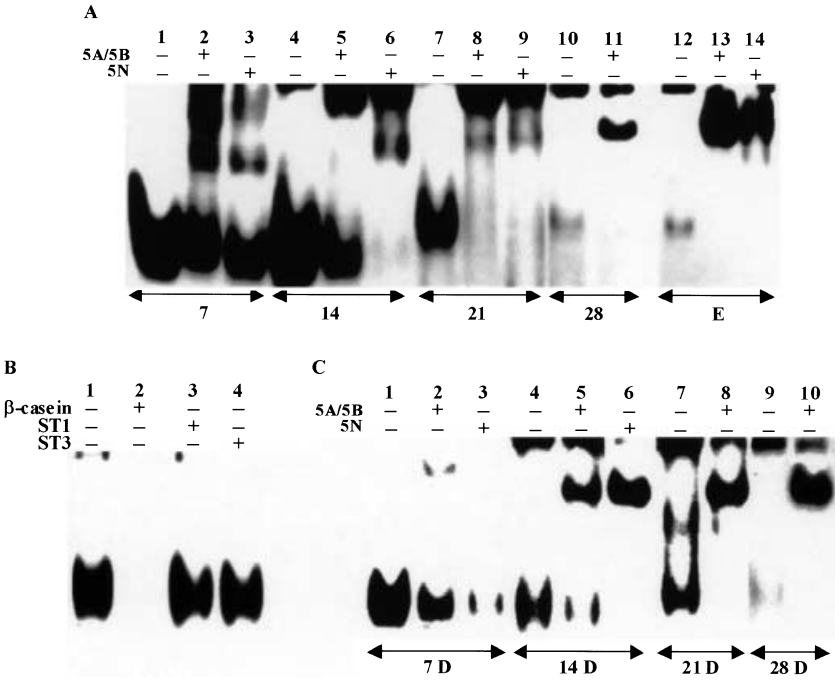

Fig 4. Supershift analysis was performed with a mixture of antibodies raised against the carboxy-terminal end of STAT5A as well as the carboxy-terminal end of STAT5B (5A/5B) or with an antibody raised against the N-terminal region of STAT5 (5N). (A) Supershift assays of proteins extracted from cells cultured for 7, 14, 21 and 28 d without dexamethasone, or from peripheral blood eosinophils (E). (B) Supershift assays performed at day 7 with antibodies raised against STAT1 (ST1) or STAT3 (ST3). Specificity of complex formation was assessed with 50-fold molar excess of unlabelled β-casein probe. (C) Supershift assays of proteins extracted from cells cultured for 7, 14, 21 and 28 d with 10 nmol/l dexamethasone.

activated in response to IL-5 at days 7 and 14, as shown in Fig 4C. However, the mix of anti-STAT5A and anti-STAT5B delayed a higher amount of complexes than in the control, indicating that short isoforms were less activated in the cells exposed to the glucocorticoid. At days 21 and 28, complexes were only formed with long isoforms completely supershifted by the mix of anti-STAT5A and anti-STAT5B (Fig 4C, lanes 8 and 10), as in the control.

Expression of STAT5 during eosinophil differentiation and in blood eosinophils

The short isoforms of STAT5 were activated in immature but not in mature cells. In order to determine if this inactivation was due to the absence of short isoforms of STAT5, Western blot analysis was performed using an anti-STAT5 antibody raised against the N-terminal domain. Figure 5 shows that the long (STAT5α) and the short isoforms (STAT5β) of STAT5 were expressed during the 4 weeks of culture and in peripheral blood eosinophils (Fig 5). The absence of activation of the short isoforms of STAT5 was not due to a lack of expression of these proteins in mature cells.

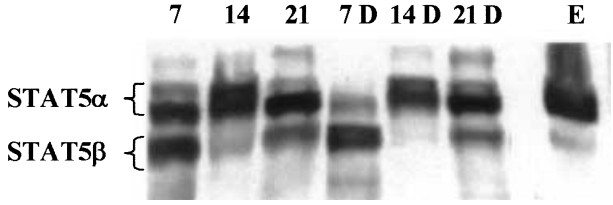

Fig 5. STAT5 expression during eosinophil differentiation with or without dexamethasone. Whole cell extracts were prepared from cells differentiated towards eosinophils for 7, 14 and 21 d with (7D, 14D, 21D) or without (7, 14, 21) 10 nmol/l dexamethasone, or from human peripheral blood eosinophils (E). Western blot analysis was performed using an antibody raised against the N-terminal region of STAT5. STAT5α: long isoforms of STAT5; STAT5β: short isoforms of STAT5.

DISCUSSION

A decrease in the number of circulating eosinophils is usually observed after *in vivo* administration of glucocorticoids. Different mechanisms might explain this phenomenon, such as induction of cell death or inhibition of growth factor production. In this study, we have investigated the effect of dexamethasone on eosinophil differentiation from $CD34^+$ stem cells. The opposite effects of glucocorticoids on eosinophil differentiation reported in the literature (Bjornson *et al*, 1985; Butterfield *et al*, 1986; Gaspar Elsas *et al*, 2000; Kuo *et al*, 2001) were perhaps due to the presence of $CD34^-$ MNCs. To rule out the participation of T cells that produce cytokines, we isolated $CD34^+$ cells from human cord blood MNCs and cultured them in the presence of IL-3, IL-5 and GM-CSF to obtain eosinophils. Dexamethasone at 10 nmol/l increased proliferation and differentiation of eosinophils. A 10-fold higher concentration was required to obtain the same effect on total cord blood MNCs (data not shown). This is certainly due to the expression of GR by $CD34^-$ MNCs. Shalit *et al* (1995) tested the effect of dexamethasone on $CD34^+$ cells purified from peripheral blood MNCs. In contrast to our results, doses ranging from 0·1 to 10 μmol/l were found to have no effect on eosinophil colony growth (Shalit *et al*, 1995). It is possible that $CD34^+$ cells isolated from different origins do not have the same sensitivity against glucocorticoids.

Dexamethasone induces apoptosis of peripheral blood eosinophils (Meagher *et al*, 1996), through permeabilization

of the mitochondrial membrane and activation of caspases (Letuve *et al*, 2002). The development of apoptosis during eosinophil differentiation in the presence of dexamethasone was unclear to date. Our investigations showed that dexamethasone not only reduced the apoptosis of immature cells, but also increased apoptosis after 3 weeks of culture. Dexamethasone has been shown to exert different effects on apoptosis of thymocytes with regard to their development. Indeed, dexamethasone enhanced the apoptosis of mature thymocytes, while immature thymocytes were resistant to apoptosis (Chow *et al*, 1997). A previous study showed that dexamethasone inhibited apoptosis of blood eosinophils isolated from some hypereosinophilic patients (Debierre-Grockiego *et al*, 2001) and, therefore, we investigated whether they express markers of immature cells.

The STATs are transcription factors involved in the proliferation, differentiation and survival of several haematopoietic cells in response to cytokines. We have investigated the activation of STAT5 by IL-5 during eosinophil differentiation from $CD34^+$ stem cells. During the first 2 weeks, IL-5 stimulation induced activation of short and long isoforms of STAT5. Antibody raised against the N-terminal region of STAT5, which recognizes all isoforms, completely supershifted the complexes at day 14 but not, surprisingly, at day 7. A possible explanation is that the recognized epitope was inaccessible because of the nature of the complexes formed at day 7. A study performed on STAT4 has shown that N-terminal domains participate in the tetramerization of STAT4. Antibodies raised against the N-terminal region did supershift the STAT4 dimers but not the STAT4 tetramers, confirming that the epitope was not accessible (Xu *et al*, 1996). Furthermore, a lysine residue in the STAT5 N-terminal domain is involved in stable tetramer formation (John *et al*, 1999). This suggests that tetramers of STAT5 are present in the complexes obtained after 7 d of culture.

During the last 2 weeks of culture, IL-5 only activated the long isoforms of STAT5 despite the expression of the short isoforms. IL-5 stimulation of mature eosinophils isolated from peripheral blood had same pattern of STAT5 activation observed at day 28. Activation of the different isoforms of STAT5 seems to be dependent on the stage of maturation of the cells. Similarly, short isoforms of STAT5 are activated by IL-3 in immature myeloid cells, whereas long isoforms are activated in mature myeloid cells (Azam *et al*, 1995). The truncated isoforms of STAT5 are also preferentially activated in murine early progenitor cells (factor-dependent cell progenitor 1; FDC-P1) stimulated with IL-3. In contrast, predominantly full-length isoforms are activated by IL-3 in FDC-P1M cells lacking the markers of early progenitors (Meyer *et al*, 1998). Our data suggest that the distinct isoforms of STAT5 might be involved in regulating different biological functions during eosinophil differentiation. A recent study investigated the role of STAT5 in haematopoietic cell survival. Ba/F3 cells expressing STAT5Δ749, a truncated dominant negative of STAT5, were more sensitive to apoptosis induced by IL-3 withdrawal than control Ba/F3 cells (Dumon *et al*, 1999). The expression of Bcl-x, a protein that belongs to Bcl-2 family and inhibits apoptosis, was impaired in different cells that express a dominant negative STAT5 mutant (Dumon *et al*, 1999; Lord *et al*, 2000). Expression of Bcl-2 is high in $CD34^+$ cells and gradually decreases during eosinophil differentiation, becoming barely detectable in mature blood eosinophils (Delia *et al*, 1992; Druilhe *et al*, 1998; Buitenhuis *et al*, 2003). Overexpression of STAT5A in $CD34^+$ cells results in enhanced expression of Bcl-2, while expression of a truncated mutant STAT5Δ750 in $CD34^+$ cells decreases the expression of Bcl-2 (Buitenhuis *et al*, 2003). We have shown that the short isoforms of STAT5 were less activated in the presence of dexamethasone. This could explain the inhibition of apoptosis by dexamethasone that was observed at day 7. The drug inhibits apoptosis of hepatocytes by increasing the expression of Bcl-2 and Bcl-x, and by decreasing expression of the pro-apoptotic protein Bax (Bailly-Maitre *et al*, 2001). This suggests that dexamethasone could enhance the expression of anti-apoptotic proteins by modulating activation of STAT5 through interaction with the GR.

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REFERENCES

Azam, M., Erdjument-Bromage, H., Kreider, B.L., Xia, M., Quelle, F., Basu, R., Saris, C., Tempst, P., Ihle, J.N. & Schindler, C. (1995) Interleukin-3 signals through multiple isoforms of Stat5. *EMBO Journal*, **14**, 1402–1411.

Bailly-Maitre, B., de Sousa, G., Boulukos, K., Gugenheim, J. & Rahmani, R. (2001) Dexamethasone inhibits spontaneous apoptosis in primary cultures of human and rat hepatocytes via Bcl-2 and Bcl-xL induction. *Cell Death and Differentiation*, **8**, 279–288.

Biethahn, S., Alves, F., Wilde, S., Hiddemann, W. & Spiekermann, K. (1999) Expression of granulocyte colony-stimulating factor- and granulocyte-macrophage colony-stimulating factor-associated signal transduction proteins of the JAK/STAT pathway in normal granulopoiesis and in blast cells of acute myelogenous leukemia. *Experimental Hematology*, **27**, 885–894.

Bjornson, B.H., Harvey, J.M. & Rose, L. (1985) Differential effect of hydrocortisone on eosinophil and neutrophil proliferation. *Journal of Clinical Investigation*, **76**, 924–929.

Buitenhuis, M., Baltus, B., Lammers, J.W., Coffer, P.J. & Koenderman, L. (2003) Signal transducer and activator of transcription 5a (STAT5a) is required for eosinophil differentiation of human cord blood-derived CD34+ cells. *Blood*, **101**, 134–142.

Butterfield, J.H., Ackerman, S.J., Weiler, D., Eisenbrey, A.B. & Gleich, G.J. (1986) Effects of glucocorticoids on eosinophil colony growth. *Journal of Allergy and Clinical Immunology*, **78**, 450–457.

Caldenhoven, E., van Dijk, T., Raaijmakers, J.A., Lammers, J.W., Koenderman, L. & De Groot, R.P. (1995) Activation of the STAT3/acute phase response factor transcription factor by interleukin-5. *Journal of Biological Chemistry*, **270**, 25778–25784.

Caldenhoven, E., van Dijk, T.B., Raaijmakers, J.A., Lammers, J.W., Koenderman, L. & de Groot, R.P. (1999) Activation of a functionally distinct 80-kDa STAT5 isoform by IL-5 and GM-CSF in

human eosinophils and neutrophils. *Molecular Cell Biology Research Communications*, **1**, 95–101.

Cella, N., Groner, B. & Hynes, N.E. (1998) Characterization of Stat5a and Stat5b homodimers and heterodimers and their association with the glucocortiocoid receptor in mammary cells. *Molecular and Cellular Biology*, **18**, 1783–1792.

Chow, S.C., Snowden, R., Orrenius, S. & Cohen, G.M. (1997) Susceptibility of different subsets of immature thymocytes to apoptosis. *FEBS Letters*, **408**, 141–146.

Debierre-Grockiego, F., Leduc, I., Prin, L. & Gouilleux-Gruart, V. (2001) Dexamethasone inhibits apoptosis of eosinophils isolated from hypereosinophilic patients. *Immunobiology*, **204**, 517–523.

Delia, D., Aiello, A., Soligo, D., Fontanella, E., Melani, C., Pezzella, F., Pierotti, M.A. & Della Porta, G. (1992) bcl-2 proto-oncogene expression in normal and neoplastic human myeloid cells. *Blood*, **79**, 1291–1298.

Druilhe, A., Arock, M., Le Goff, L. & Pretolani, M. (1998) Human eosinophils express bcl-2 family proteins: modulation of Mcl-1 expression by IFN-gamma. *American Journal of Respiratory Cell and Molecular Biology*, 18, 315–322.

Du, J., Alsayed, Y.M., Xin, F., Ackerman, S.J. & Platanias, L.C. (2000) Engagement of the CrkL adapter in interleukin-5 signaling in eosinophils. *Journal of Biological Chemistry*, **275**, 33167–33175.

Dumon, S., Santos, S.C., Debierre-Grockiego, F., Gouilleux-Gruart, V., Cocault, L., Boucheron, C., Mollat, P., Gisselbrecht, S. & Gouilleux, F. (1999) IL-3 dependent regulation of Bcl-xL gene expression by STAT5 in a bone marrow derived cell line. *Oncogene*, **18**, 4191–4199.

Finotto, S., Mekori, Y.A. & Metcalfe, D.D. (1997) Glucocorticoids decrease tissue mast cell number by reducing the production of the c-kit ligand, stem cell factor, by resident cells: in vitro and in vivo evidence in murine systems. *Journal of Clinical Investigation*, **99**, 1721–1728.

Gaspar Elsas, M.I., Joseph, D., Lintomen, L., Maximiano, E.S., Bodstein, M., Xavier Elsas, P. & Vargaftig, B.B. (2000) Murine myeloid progenitor responses to GM-CSF and eosinophil precursor responses to IL-5 represent distinct targets for downmodulation by prostaglandin E(2). *British Journal of Pharmacology*, **130**, 1362–1368.

Gouilleux, F., Pallard, C., Dusanter-Fourt, I., Wakao, H., Haldosen, L.A., Norstedt, G., Levy, D. & Groner, B. (1995) Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity. *EMBO Journal*, **14**, 2005–2013.

Hansel, T.T., De Vries, I.J., Iff, T., Rihs, S., Wandzilak, M., Betz, S., Blaser, K. & Walker, C. (1991) An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *Journal of Immunological Methods*, **145**, 105–110.

Her, E., Frazer, J., Austen, K.F. & Owen, Jr, W.F. (1991) Eosinophil hematopoietins antagonize the programmed cell death of eosinophils. Cytokine and glucocorticoid effects on eosinophils maintained by endothelial cell-conditioned medium. *Journal of Clinical Investigation*, **88**, 1982–1987.

John, S., Vinkemeier, U., Soldaini, E., Darnell, Jr, J.E. & Leonard, W.J. (1999) The significance of tetramerization in promoter recruitment by Stat5. *Molecular and Cellular Biology*, **19**, 1910–1918.

Kibe, A., Inoue, H., Fukuyama, S., Machida, K., Matsumoto, K., Koto, H., Ikegami, T., Aizawa, H. & Hara, N. (2003) Differential regulation by glucocorticoid of interleukin-13-induced eosinophilia, hyperresponsiveness, and goblet cell hyperplasia in mouse airways. *American Journal of Respiratory and Critical Care Medicine*, **167**, 50–56.

Kuo, H.P., Wang, C.H., Lin, H.C., Hwang, K.S., Liu, S.L. & Chung, K.F. (2001) Interleukin-5 in growth and differentiation of blood eosinophil progenitors in asthma: effect of glucocorticoids. *British Journal of Pharmacology*, **134**, 1539–1547.

Lantero, S., Sacco, O., Scala, C., Morelli, M.C. & Rossi, G.A. (1996) Eosinophil locomotion and the release of IL-3 and IL-5 by allergen-stimulated mononuclear cells are effectively downregulated in vitro by budesonide. *Clinical and Experimental Allergy*, **26**, 656–664.

Letuve, S., Druilhe, A., Grandsaigne, M., Aubier, M. & Pretolani, M. (2002) Critical role of mitochondria, but not caspases, during glucocorticosteroid-induced human eosinophil apoptosis. *American Journal of Respiratory Cell and Molecular Biology*, **26**, 565–571.

Lilly, C.M., Nakamura, H., Kesselman, H., Nagler-Anderson, C., Asano, K., Garcia-Zepeda, E.A., Rothenberg, M.E., Drazen, J.M. & Luster, A.D. (1997) Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *Journal of Clinical Investigation*, **99**, 1767–1773.

Lopez, A.F., Williamson, D.J., Gamble, J.R., Begley, C.G., Harlan, J.M., Klebanoff, S.J., Waltersdorph, A., Wong, G., Clark, S.C. & Vadas, M.A. (1986) Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. *Journal of Clinical Investigation*, **78**, 1220–1228.

Lopez, A.F., Sanderson, C.J., Gamble, J.R., Campbell, H.D., Young, I.G. & Vadas, M.A. (1988) Recombinant human interleukin 5 is a selective activator of human eosinophil function. *Journal of Experimental Medicine*, **167**, 219–224.

Lord, J.D., McIntosh, B.C., Greenberg, P.D. & Nelson, B.H. (2000) The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. *Journal of Immunology*, **164**, 2533–2541.

Meagher, L.C., Cousin, J.M., Seckl, J.R. & Haslett, C. (1996) Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *Journal of Immunology*, **156**, 4422–4428.

Meyer, J., Jucker, M., Ostertag, W. & Stocking, C. (1998) Carboxyl-truncated STAT5beta is generated by a nucleus-associated serine protease in early hematopoietic progenitors. *Blood*, **91**, 1901–1908.

Miltenyi, S., Muller, W., Weichel, W. & Radbruch, A. (1990) High gradient magnetic cell separation with MACS. *Cytometry*, **11**, 231–238.

Moriggl, R., Gouilleux-Gruart, V., Jahne, R., Berchtold, S., Gartmann, C., Liu, X., Hennighausen, L., Sotiropoulos, A., Groner, B. & Gouilleux, F. (1996) Deletion of the carboxyl-terminal transactivation domain of MGF-Stat5 results in sustained DNA binding and a dominant negative phenotype. *Molecular and Cellular Biology*, **16**, 5691–5700.

Ogata, N., Kikuchi, Y., Kouro, T., Tomonaga, M. & Takatsu, K. (1997) The activation of the JAK2/STAT5 pathway is commonly involved in signaling through the human IL-5 receptor. *International Archives of Allergy and Immunology*, **114**(Suppl. 1), 24–27.

Ogata, N., Kouro, T., Yamada, A., Koike, M., Hanai, N., Ishikawa, T. & Takatsu, K. (1998) JAK2 and JAK1 constitutively associate with an interleukin-5 (IL-5) receptor alpha and betac subunit, respectively, and are activated upon IL-5 stimulation. *Blood*, **91**, 2264–2271.

Pazdrak, K., Stafford, S. & Alam, R. (1995) The activation of the Jak-STAT 1 signaling pathway by IL-5 in eosinophils. *Journal of Immunology*, **155**, 397–402.

Rothenberg, M.E., Owen, Jr, W.F., Silberstein, D.S., Woods, J., Soberman, R.J., Austen, K.F. & Stevens, R.L. (1988) Human eosinophils have prolonged survival, enhanced functional properties, and become hypodense when exposed to human interleukin 3. *The Journal of Clinical Investigation*, **81**, 1986–1992.

Sehmi, R., Wood, L.J., Watson, R., Foley, R., Hamid, Q., O'Byrne, P.M. & Denburg, J.A. (1997) Allergen-induced increases in IL-5 receptor alpha-subunit expression on bone marrow-derived CD34+ cells from asthmatic subjects. A novel marker of progenitor cell commitment towards eosinophilic differentiation. *Journal of Clinical Investigation*, **100**, 2466–2475.

Shalit, M., Sekhsaria, S. & Malech, H.L. (1995) Modulation of growth and differentiation of eosinophils from human peripheral blood CD34+ cells by IL5 and other growth factors. *Cellular Immunology*, **160**, 50–57.

Stocklin, E., Wissler, M., Gouilleux, F. & Groner, B. (1996) Functional interactions between Stat5 and the glucocorticoid receptor. *Nature*, **383**, 726–728.

Tavernier, J., Devos, R., Cornelis, S., Tuypens, T., Van der Heyden, J., Fiers, W. & Plaetinck, G. (1991) A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific alpha chain and a beta chain shared with the receptor for GM-CSF. *Cell*, **66**, 1175–1184.

Ten, R.M., Pease, L.R., McKean, D.J., Bell, M.P. & Gleich, G.J. (1989) Molecular cloning of the human eosinophil peroxidase. Evidence for the existence of a peroxidase multigene family. *Journal of Experimental Medicine*, **169**, 1757–1769.

Vermes, I., Haanen, C., Steffens-Nakken, H. & Reutelingsperger, C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of Immunological Methods*, **184**, 39–51.

Wakao, H., Gouilleux, F. & Groner, B. (1994) Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO Journal*, **13**, 2182–2191.

Xu, X., Sun, Y.L. & Hoey, T. (1996) Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science*, **273**, 794–797.

Yamaguchi, Y., Hayashi, Y., Sugama, Y., Miura, Y., Kasahara, T., Kitamura, S., Torisu, M., Mita, S., Tominaga, A. & Takatsu, K. (1988a) Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor. *Journal of Experimental Medicine*, **167**, 1737–1742.

Yamaguchi, Y., Suda, T., Suda, J., Eguchi, M., Miura, Y., Harada, N., Tominaga, A. & Takatsu, K. (1988b) Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *Journal of Experimental Medicine*, **167**, 43–56.