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TNF-α and IL-6 induce differentiation in the human basophilic leukaemia cell line KU812

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SUMMARY

The basophilic leukaemia cell line KU812 can be induced to differentiate into basophil-like cells *in vitro* when exposed to supernatant from the Mo T-cell line. KU812 cells express affinity receptors for IgE, produce histamine and tryptase and have the capacity for IgE-mediated histamine release. In this study we have examined the cytokines, produced by the Mo cell line, which are responsible for the observed differentiation-inducing effect in the KU812 cell line. It was shown that interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) induced differentiation in the KU812 cells and that these cytokines were responsible for the differentiation-inducing effect of the Mo supernatant. Other cytokines tested, IL-1β, IL-2, IL-4, IL-5, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF) and nerve growth factor (NGF) were without effect on the KU812 cells. KU812 was also shown to express receptors for both TNF-α and IL-6 after 3 days cultivation with conditioned media from the Mo T-cell line. Untreated cells showed no detectable levels of TNF-α or IL-6 receptors indicating induction of these receptors during differentiation. Spontaneous differentiation was shown to occur under serum-free conditions which may be the result of endogenous IL-6 production through an autocrine loop. The activity of TNF-α and IL-6 could be blocked by specific monoclonal antibodies (mAb) to the respective cytokine.

INTRODUCTION

Basophils and mast cells are the most important effector cells of immediate hypersensitivity reactions. Both cell types originate from the haematopoietic stem cells and share several biochemical and functional properties, e.g. the expression of the high-affinity receptor for IgE, and the capacity for release of histamine and other mediators upon activation. Mature basophils are typically found in the circulation, although they can be found in inflamed tissues. Mast cells, on the other hand, are exclusively found in the tissue. The cytokines involved in the control of growth and differentiation of human basophils and mast cells seem to be distinct. *In vitro* growth of basophils has been induced after exposure to interleukin-3 (IL-3),[1,2] but other factors, i.e. IL-5,[1] granulocyte–macrophage colony-stimulating factor (GM-CSF),[3,4] transforming growth factor-β (TGF-β)[5] and nerve growth factor (NGF),[4] have also been shown to regulate basophilic differentiation. In contrast, none of these cytokines have so far been shown to promote differentiation of human mast cells. The main growth factor for human mast cells was recently shown to be the stem cell factor,[6-8] the ligand to the Kit receptor encoded by the protooncogene *c-kit*.

Although methods for purification and *in vitro* differentiation of human basophils and mast cells have advanced in recent years, it has been difficult to establish continuous human basophil or mast cell lines. Today three cell lines are available for studies on differentiation of human basophils and mast cells: the human mast cell line, HMC-1, established from a patient with mast cell leukaemia;[9] the promyelocytic leukaemia cell line HL-60;[10] and KU812,[11] which was established from a patient with chronic myeloid leukaemia. Both KU812 and HL-60 have been shown to differentiate into basophil-like cells, e.g. they increased the synthesis of histamine, when cultured with a combination of sodium butyrate and conditioned media from the human T-cell line Mo.[3,12] The factor(s) in the Mo-conditioned media (CM) supernatant responsible for this effect has not yet been characterized. We have earlier shown that conditioned media from cultured peripheral blood mononuclear cells (PBMC) from atopic individuals induce differentiation in KU812 cells,[13] an effect which might be induced by factors identical to those found in the Mo-CM. It has also been shown that KU812 cells may undergo differentiation in the absence of exogenous factors,[14] i.e. under serum-free conditions.

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Abbreviations: CM, conditioned media; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; NGF, nerve growth factor; PBMC, peripheral blood mononuclear cells; r, recombinant; R, receptor; TNF, tumour necrosis factor.

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The present study aims at identifying the factor(s) present in the Mo-CM which induce basophilic differentiation of KU812 cells, assessed as increased histamine production. We show that tumour necrosis factor-α (TNF-α) and IL-6 induce differentiation of these cells while other cytokines tested had no or only minimal effects on the differentiation of KU812. We also demonstrate the presence of receptors for TNF-α and IL-6 on the KU812 cells, and an endogenous production of IL-6 but not TNF-α in the KU812 cell cultures.

MATERIALS AND METHODS

Cell cultures and reagents

The KU812 cell line[11] was maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 μg/ml gentamycin) (Flow Laboratories, Irvine, U.K.). The cells were passaged every 3–4 days. To test for basophilic differentiation, KU812 cells were cultured at 3×10^5 cells/well in 96-well plates. Each well contained 250 μl of medium supplemented with 0·3 mM sodium butyrate (Sigma, St Louis, MO) as described previously.[12] After 3 days of culture the cells were resuspended and samples were harvested for analysis. Cell growth was determined by counting of viable cells with the use of a Bürker haemocytometer and trypan blue exclusion. Conditioned medium from the human T-cell line Mo[15] (Mo-CM) was prepared as previously detailed.[12,16] Human recombinant IL-1α (Genzyme, Cambridge, MA), rIL-2 (Amgen, Thousand Oaks, CA), rIL-3 (Genzyme), rIL-4 (Genzyme), rIL-5 (Genentech Inc., San Francisco, CA), rIL-6 (specific activity $1{\cdot}9 \times 10^5$ U/μg) (kindly provided by Dr T. Kishimoto, Osaka, Japan), rIL-8 (a kind gift from Dr K. Testrup-Pedersen, Århus, Denmark), rGM-CSF (Genzyme), rTNF-α (a generous gift from Dr G. R. Adolf, Ernst-Boehringer Inst., Vienna, Austria) and rNGF (kindly provided by Dr T. Ebendal, Uppsala, Sweden) were tested for their effect on the differentiation of KU812 cells. Antibodies (Ab) against IL-6 (goat anti-rIL-6) were kindly provided by Dr T. Kishimoto and Ab against TNF-α (rabbit anti-rTNF-α) were purchased from Genzyme. The Ab were preincubated for 2 hr at 37° with the cytokines or Mo-CM before the cells were added to the cultures.

Histamine assay

Histamine in the cell suspension was analysed by using the Pharmacia Histamine radioimmunoassay (RIA) kit (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden). The results were expressed as the ratio between histamine formation (ng histamine/10^6 cells) in treated cultures and in untreated control cultures.

Assay of IL-6 and TNF-α

The levels of IL-6 and TNF-α were measured in supernatants from Mo and KU812 by IL-6-Quantikine™ (R & D Systems, Minneapolis, MN) and TNF-α-IRMA (Medgenix Diagnostics, Brussels, Belgium), respectively.

TNF-α and IL-6 binding assay

The binding assays for TNF-α and IL-6 were performed as previously described.[17,18] Briefly, KU812 cells were washed in 10 mM EDTA-containing phosphate-buffered saline (PBS) for 15 min at 37° and then washed again with culture medium. The binding assay was performed with ^{125}I-labelled TNF-α (kindly provided by Dr I. Olsson, Lund, Sweden) (specific activity of 15·5 μCi/μg protein) or ^{125}I-labelled IL-6 (Phage, La Jolla, CA) (specific activity of 63 μCi/μg protein). Unlabelled cytokines were added at concentrations as indicated. The cell suspensions were incubated on a rocking platform at +4° for 4 hr and unbound cytokine was removed by three washes with ice-cold medium before the activity was determined by counting in a gamma-counter (LKB-Wallac, Bromma, Sweden). The data for Scatchard plots were calculated by the LIGAND program[19] on an IBM PC.

RESULTS

Effect of Mo-CM on basophilic differentiation of KU812 cells

Induction of basophilic differentiation was analysed on the basis of increased histamine production in the KU812 cell line. We have previously shown that Mo-CM strongly induced histamine production reaching a plateau after approximately 4 days, and granulation with slower kinetics reaching a maximum of approximately 4% after 7 days.[12] Figure 1a shows that histamine production in KU812 was induced in a dose-dependent pattern by the addition of 1–20% of Mo-CM to the culture. The cell growth was slightly reduced in the presence of Mo-CM (Fig. 1b), without affecting the viability compared to control cultures (viability 80–95%).

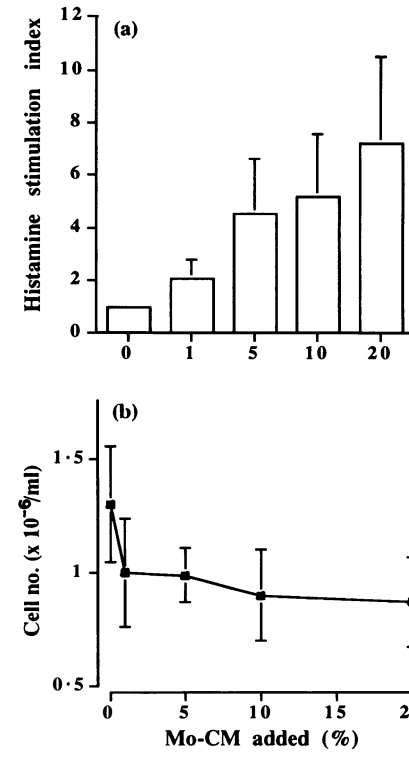

Figure 1. Histamine production by KU812 (a) and cell growth (b) after 3 days of culture in the presence of Mo-CM, 1–20% and 0·3 mM sodium butyrate. Histamine syntheses are related to the value in untreated control cultures and given as histamine stimulatory index. Results are given as the mean ± SD ($n = 5$–9).

Effect of recombinant cytokines on histamine synthesis and cell growth in KU812

Among the cytokines tested only IL-6 and TNF-α were found to increase the histamine production in KU812 cells consistently. TNF-α induced histamine synthesis in a dose-dependent manner with a maximum stimulation at 40 ng/ml (Fig. 2a). The maximum level varied somewhat and in some experiments a ninefold increase in the histamine levels was found. IL-6 at 10 U/ml increased the histamine level about twofold. Almost the same stimulatory effects were observed after addition of higher concentrations of IL-6, up to 1000 U/ml (Fig. 2b). The effect of TNF-α on the cell growth was similar to the effect observed with Mo-CM. The cell growth was inversely related to the histamine production. IL-6, however, had only a minor effect on the cell growth.

Several other recombinant cytokines were tested for their ability to induce basophilic differentiation in KU812 cells. IL-1α (1, 10, 100 U/ml), IL-2 (10, 100, 1000 U/ml), IL-4 (10, 100, 1000 U/ml), IL-5 (5, 50, 500 CFU/ml), IL-8 (0·1, 1, 10 ng/ml), GM-CSF (5, 50, 500 U/ml) or NGF (28, 280, 560 ng/ml) did not increase histamine production. IL-3 (14 and 144 U/ml) induced a minor, maximally one- to twofold increase in histamine synthesis. IL-8 was observed to have a small but reproducible inhibitory effect on the histamine production by KU812. We also tested whether IL-2, IL-3, IL-4, GM-CSF or NGF had any synergistic effects with Mo-CM. These cytokines were not found to have any significant effect.

Analysis of IL-6 and TNF-α in supernatants

To determine the factor(s) in the Mo-CM which induce basophilic differentiation, we measured the presence of IL-6 and TNF-α in the Mo-CM by using an enzyme-linked immunosorbent assay (ELISA) for IL-6 and a RIA for the detection of TNF-α. The concentration of IL-6 in the Mo-CM was calculated to be approximately 50 ng/ml and for TNF-α approximately 2 ng/ml. These data are in accordance with our earlier report on high expression of mRNA for IL-6 and TNF-α in Mo.[16] We also measured supernatants from KU812 to investigate if KU812 cells produced any of these cytokines. We did not find any TNF-α in the CM from KU812 but significant levels of IL-6 (140 pg/ml) could be detected.

Blocking of IL-6 and TNF-α activity

The specificity of the effects of IL-6 and TNF-α on histamine production in KU812 were evaluated by using polyclonal antibodies against IL-6 and TNF-α, respectively. Antibodies against TNF-α (diluted 1/250) inhibited completely the effect of TNF-α (Fig. 3). Although anti-TNF-α reduced the effect of Mo-CM (5%) an effect of Mo-CM on the histamine production could still be obtained. A similar effect was noted when antibodies against IL-6 were used. Anti-IL-6 Ab (1/500) completely inhibited the effect of rIL-6, but did not completely block the effect of Mo-CM. Furthermore, the combination of anti-IL-6 and anti-TNF-α antibodies did not completely inhibit the effect of Mo-CM on histamine production in KU812. The antibodies, by themselves, did not affect histamine production in KU812. Thus anti-IL-6 did not affect the activity of TNF-α and anti-TNF-α did not change the effect of IL-6.

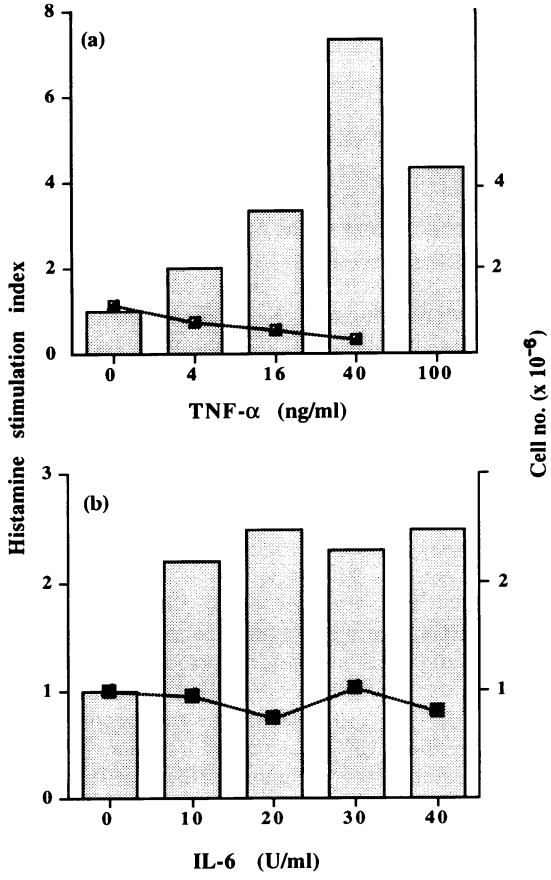

Figure 2. Induction of histamine production and the cell number in KU812 after treatment with human recombinant TNF-α (4–100 ng/ml) (a) and IL-6 (10–40 U/ml) (b). Histamine syntheses are related to the value in untreated control cultures and are given as histamine stimulatory index. The dotted line represents the cell number. Results from one representative experiment out of four.

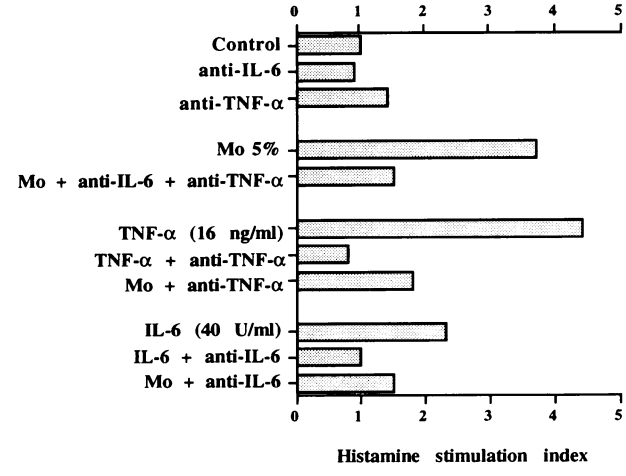

Figure 3. Induction of histamine production by Mo-CM (5%), IL-6 (40 U/ml) and TNF-α (16 ng/ml); and blocking with mAb against IL-6 (diluted 1/500) or TNF-α (1/250). Results shown from one of two experiments.

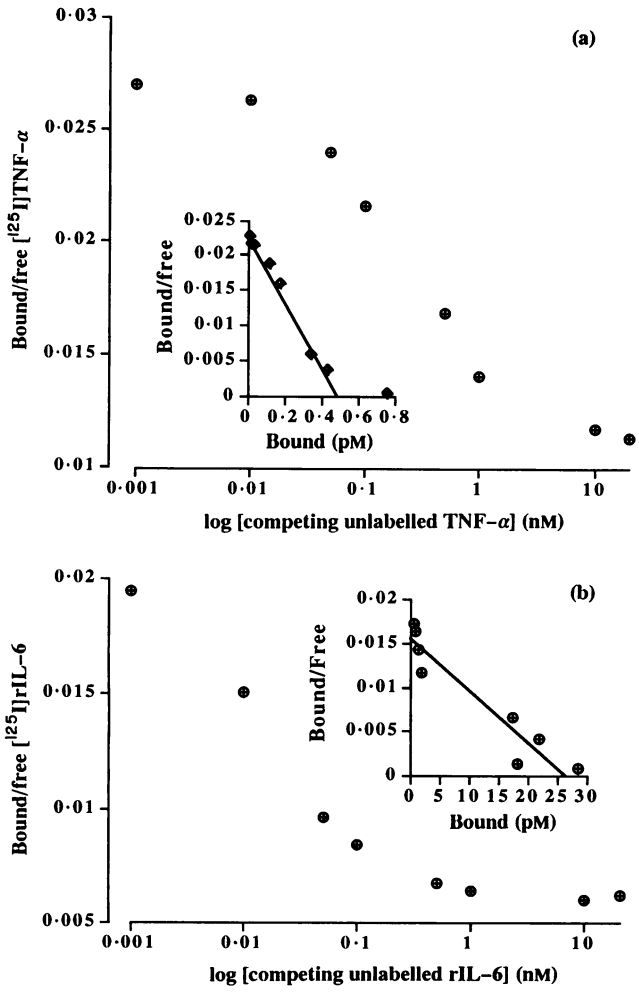

Figure 4. TNF-αR and IL-6R expression on KU812 cells after treatment with Mo-CM (5%) for 3 days. Cells were incubated with increasing concentrations of radiolabelled rTNF-α or rIL-6 at +4°. The figures show specific binding of ^{125}I-labelled TNF-α (a) and IL-6 (b) and a Scatchard transformation of the binding data (one of two experiments).

IL-6 and TNF-α receptor expression

As KU812 cells responded to IL-6 and TNF-α, we investigated the presence of cell surface TNF-αR and IL-6R on the cells by a binding assay using ^{125}I-labelled TNF-α and IL-6. No receptors were detected on untreated cells, although a specific binding of [^{125}I]TNF-α and [^{125}I]IL-6 was observed after treatment with Mo-CM (10%) (Fig. 4). A mean of 1324 TNF-α and six IL-6 receptors/cell and a dissociation constant of $1{\cdot}6 \times 10^{-9}$ M and $5{\cdot}9 \times 10^{-12}$ M, respectively, were calculated from Scatchard plots. The number of receptors/cell was calculated based on the total number of cells in the cell suspension and no adjustments for the number of granulated cells were made.

DISCUSSION

Factors derived from the human T-cell line Mo have been shown to induce basophilic differentiation in the pre-basophilic cell line KU812,[12] the promyelocytic cell line HL-60[3] and from PBMC.[20] IL-3 has been described as being an important cytokine for basophilic differentiation both in primary cell cultures and in differentiation of cell lines such as KU812 and HL-60.[1,20,21] However, IL-3 has not been found in supernatants from Mo cells and this cytokine showed only a minor and not reproducible stimulatory effect on the KU812 cell line used in our experiments. Factors produced by the Mo cells are GM-CSF, IL-6, IL-8, TNF-α, IFN-γ and TGF-β. However, IFN-γ and TGF-β in the CM from Mo are inactivated due to the treatment of the Mo supernatant. In addition, it has been shown that Mo does not produce IL-1β, IL-2, IL-5, M-CSF, IFN-α or β.[15,16] Basophilic differentiation has been assessed by the potential to produce histamine, by increased granulation, and by expression of high-affinity receptors for IgE. The KU812 cell line has been shown to possess the potential to increase granulation and produce histamine,[12] and it has been shown to express both mRNA[22] and protein levels of tryptase (G. Nilsson, manuscript in preparation), a protease commonly found in mast cells but also found in small amounts in human peripheral blood basophils.[23] Furthermore, KU812 cells responded by histamine release to cross-linking IgE added to the cells by either anti-IgE or specific allergen (unpublished results). It is therefore a strong indication that KU812 cells can be used as a model in studies of basophilic differentiation. In the experiments presented herein we have tested different cytokines independently and in combination with Mo-CM containing a variety of different cytokines. The cytokines IL-1β, IL-2, IL-4, IL-5, IL-8, GM-CSF, or NGF did not have any stimulatory effect on histamine production in KU812, either by themselves or in synergy with Mo-CM. A slight increase by IL-3, a strong response with IL-6, and a very strong response to TNF-α were obtained. The responses to recombinant TNF-α and IL-6 could be specifically and completely blocked by specific Ab against the respective cytokines. As IL-3 was not present in the Mo-CM we conclude that the main response in this model system was due to TNF-α and IL-6 stimulation. Valent *et al.* have described that IL-3 promotes basophilic differentiation of KU812F through high-affinity binding sites.[21] The effect of IL-3 found in this study could be due to the time for histamine analysis, day 3, compared to Valent *et al.* who reported that increased histamine levels could be seen after 7 days of culture.

Blocking experiments with anti-TNF-α and IL-6 could not completely abolish the effect of differentiating factors in Mo-CM, indicating that there may be other factors or a combination of factors involved in the differentiation of KU812 cells. Apart from the increase in histamine production in KU812, the combination of TNF-α and IL-6 also induced increased expression of mRNA for the growth factor PDGF-A[24] indicating that TNF-α and IL-6 are not restricted in their effects on KU812.

Both TNF-α and IL-6 are cytokines with pleiotropic effects on cells within the haematopoietic and immune system, and in inflammatory responses.[25,26] Neither TNF-α nor IL-6 have been shown to have any effect on human basophils in respect of activation of cells.[27,28] The effect of TNF-α or IL-6 on the differentiation of basophils has, to our knowledge, not been studied, nor has the expression of receptors for TNF-α or IL-6. However, the expression of IL-6R on a subline of KU812 (KU812 F) has been described earlier.[29] These cells expressed a low number of receptors (104 receptors/cell) with a K_d of $2{\cdot}5 \times 10^{-10}$ M. We investigated the amount of receptors for both TNF-α and IL-6 on the KU812 cell line. In uninduced untreated

cells, we found no receptor expression, whereas in stimulated cultures either by TNF-α, IL-6 or Mo supernatant, we found significantly increased numbers of both TNF-α and IL-6 receptors. This was clearly shown by displacement curves and Scatchard plots. We found that KU812 cells expressed low numbers of IL-6 receptors with an affinity constant of $6{\cdot}2 \times 10^{-12}$ M indicating the presence of a high-affinity receptor. The number of receptors was very low but the calculations of receptors/cell were based on the total number of cells in the cell suspension and not adjusted for the relative number of differentiated cells. As the KU812 is a heterogeneous cell line, defined by its morphology, and as induction of differentiation is likely to occur in less than 10% of the population after 7 days of exposure to Mo supernatant,[12] it is likely that the differentiated cells would express a higher number of IL-6 receptors. It has previously been reported that two classes of IL-6 receptors exist; one high-affinity binding site with a K_d around 10^{-11} M and a low affinity receptor with a K_d around 10^{-9} M.[30] Two distinct receptors for TNF with molecular weights of 55,000 and 75,000 have been described.[31] Both receptors have a high affinity for TNF-α as well as for TNF-β. Several cell lines including HL-60 have been found to express both type of receptors.[31] The number of TNF-αR expressed on KU812 was similar to the number reported for HL-60 (~1500 receptors/cell),[32] although the affinity of the receptors on KU812 was approximately 10 times lower.

TNF-α and IL-6 are both found in elevated levels in different inflammatory responses; e.g. allergy and asthma.[33,34] Under such conditions these cytokines could have important implications on the differentiation of basophils and the production of inflammatory mediators, e.g. histamine. We have previously shown that CM from atopic individuals stimulates the histamine synthesis in KU812 to a higher level compared to control individuals.[13] Other findings supporting these results are that Mo-CM stimulates differentiation of basophils from peripheral blood and that the effect of Mo-CM on HL-60 cells could not be blocked by mAb against GM-CSF, IL-3 or IL-5.[20] These results together provide greater understanding of histamine synthesis and differentiation of basophils, which could be of importance in allergic and asthmatic states.

In previous experiments we and others have shown that culturing KU812 cells under serum-free conditions also induces basophilic differentiation.[14,22] We therefore evaluated if supernatants from KU812 also contained increased levels of TNF-α or IL-6. We found significant amounts of IL-6 present in the KU812 supernatant, indicating an endogenous IL-6 production. Hence, IL-6 could in this case act in an autocrine fashion which might be the cause for spontaneous differentiation of KU812 under certain culturing conditions. Antibodies against rIL-6 did not block the effect of the endogenous IL-6, but this does not exclude the involvement of an autocrine loop in the spontaneous differentiation seen under serum-free conditions.[18]

In summary, we have shown that TNF-α and IL-6 strongly induce differentiation of KU812 cells towards a basophil-like cell as measured by increased histamine production. This effect was mediated through high affinity binding sites. We also show that KU812 cells can spontaneously differentiate into basophilic-like cells, probably due to endogenous IL-6 production. Although IL-3 has previously been shown to be an important cytokine in basophilic differentiation, IL-3 was not essential in this model system.

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