

Dysregulation of monocyte differentiation in asthmatic subjects is reversed by IL-10

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Summary

Background IL-10 can modulate the differentiation of normal monocytes to macrophages, increasing the proportion of maturing cells with a phenotype consistent with T cell suppressive activity. Analysis of the immunopathology in endobronchial biopsies from asthmatic subjects has revealed significantly reduced proportions of suppressive macrophage populations associated with chronic T-cell mediated inflammation.

Objective This study investigates whether the altered homeostasis within the lung macrophage populations in asthma is reflected in aberrant differentiation of peripheral blood monocytes and whether this differentiation may be influenced by IL-10.

Methods Monocytes from 14 normal individuals and 14 atopic asthmatics were grown in culture for 7 days in the presence or absence of IL-10, added on day 5. Double immunofluorescence studies were performed on cytopins of the differentiated macrophages using the monoclonal antibodies RFD1 and RFD7 to distinguish inductive and suppressive macrophages by their respective phenotypes. HLADR expression was quantified using the monoclonal antibody RFDR1. Macrophage function was determined by quantifying allostimulation in a mixed leucocyte reaction and by measuring $\text{TNF}\alpha$ and $\text{TGF}\beta$ production.

Results With no cytokine addition the proportion of maturing macrophages with a suppressive phenotype ($\text{D1}^+ \text{D7}^+$) at day 7 was lower in the asthmatic samples (18%) compared with normals (25%). IL-10 increased the proportion of suppressive cells in cultures of both asthmatic and normal monocytes with the increase in the asthmatic subjects (94% increase) being significantly greater than that in normal subjects (32% increase) ($P < 0.01$). Asthmatic monocytes had a greater effect in stimulating MLR than normals ($P < 0.05$) but the addition of IL-10 reduced T cell proliferation in an MLR to a equivalent level in both groups.

Conclusions These results suggest that a fundamental problem may exist in the differentiation of monocytes in asthma which may be reversed by IL-10.

Keywords: asthma, monocyte differentiation, IL-10

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Introduction

The heterogeneity among normal lung macrophages is striking: there are subsets of cells with the capacity to

induce T-cell responses such as dendritic antigen presenting cells, subsets with the capacity to suppress T-cell responses (suppressive macrophages), and phagocytic effector cells [1–3]. These subsets may be phenotypically discriminated using the monoclonal antibodies RFD1 and RFD7 and can be functionally discriminated *in vitro* [3–7]. The RFD1 and RFD7 antigens are expressed on more than 80% of mature

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macrophages in culture but on less than 3% of peripheral blood monocyte [4]. The expression of these antigens increase as the cells differentiate reflecting diverging phenotype.

Analysis of the immunopathology of endobronchial biopsies from asthmatic subjects has revealed an imbalance within these functionally distinct macrophage populations in that reduced proportions of suppressive cells are associated with chronic peribronchial inflammation [8]. It has thus been suggested that there is a clear cause and effect relationship between the reduced capacity of local macrophage populations to suppress T cell activity and the chronic nature of the T-cell mediated inflammation [2]. A reduction in suppressive macrophages in the bronchial wall of asthmatics could be a consequence of either phenotypic and functional changes to the mature populations and/or aberrations in the differentiation and maturation of recruited monocytes.

Both changes to mature cells and monocyte differentiation are controlled at least in part by cytokines. More specifically IL-10 (cytokine synthesis inhibitory factor) produced by TH₂ type T helper cells (and macrophages) reduces cytokine production by activated macrophages [9,10] downregulates MHC class II expression [9] and inhibits antigen specific proliferation of human T cells [11,12]. Furthermore, it has been shown to regulate the emergence of functionally distinct macrophages from peripheral blood monocytes with an increase in the proportion of RFD1⁺ RFD7⁺ suppressive cells [7]. IL-10 therefore has the potential to redress the imbalance within the tissue macrophage subpopulations seen in bronchial asthma.

This study investigates whether the altered homeostasis within the lung macrophage populations results from aberrant differentiation of peripheral blood monocytes and whether this differentiation could be influenced by IL-10.

Materials and methods

Subjects

Venous blood (20 mL) was taken from 14 normal subjects and 14 atopic asthmatics (age 18–50 years). The asthmatic subjects fulfilled the American Thoracic Society (ATS) criteria for the diagnosis of asthma and all showed a positive skin-prick response to one or more of a battery of eight common allergens including house dust mite and grass pollen. All patients were on a stable dose of inhaled corticosteroids. The control group consisted of both atopic and non-atopic individuals.

Monocyte harvest

Mononuclear cells were separated by density centrifugation

(Nycomed Pharma As, Oslo, Norway) at 650 *g* for 15 min. Mononuclear cells were washed with phosphate buffered saline (PBS) three times and suspended at a density of 1×10^6 cells/mL in RPMI 1640 culture medium (Sigma-Aldrich Co. Ltd, Dorset, UK) supplemented with 10% heat inactivated Fetal Bovine Serum, 1.25% penicillin/streptomycin and 1.25% 200 mM glutamine aliquots (2 mL) were then transferred to each well of 24 well culture plates. The cultures were incubated at 37°C in 5%CO₂ to separate monocytes by adherence. After 2 h the non-adherent cells were removed by aspiration and each well was washed three times in PBS preheated to 37°C. Fresh supplemented RPMI medium (2 mL) was then added to each well. For each culture experiment triplicate wells were harvested at this time (= T₀). The method of harvest is described below. The cell populations at T₀ contained consistently greater than 90% monocytes as determined by morphology; and viability (determined by trypan blue exclusion) was consistently greater than 95%. The sensitivity of this separation technique was verified by immunophenotyping with CD14 and CD68 as described in a previous study [4]. The remaining 10% of cells were predominantly B lymphocytes.

Cell culture

Adherent monocytes were cultured in 24 well plates in supplemented RPMI (see above) for 7 days either with no addition or with the addition of 10 ng/mL IL-10 added on day 5. Time course and dose-response effect for IL-10 addition were determined in a previous study by this laboratory [4]. Recombinant human IL-10 obtained from R & D Systems (Abingdon, UK) was added in 20 µL aliquots with control cultures receiving 20 µL of sterile PBS. All solutions added were warmed to 37°C before addition. Cultures were all harvested after 7 days. At time of harvest plates were placed at 4°C for 30 min and then vigorously aspirated with fresh cold PBS. All cells from the wells were collected including any cells no longer adhering to the plastic substrate. Viability was reassessed and only cultures with a viability greater than 90% were used for analysis.

Cytospin preparation

After harvest at day 7 the cells were washed with PBS and centrifuged at 650 *g* for 5 min. The cell density was adjusted to $3\text{--}5 \times 10^5$ cells per mL and cytopspins prepared by spinning 50 mL aliquots at 80 *g* for 2 min in a Shandon cytocentrifuge (Shandon Southern Products Ltd, Runcorn, UK). Cytopspins were air dried for 1 h and fixed in a 1:1 mixture of chloroform acetone for 10 min. These were then wrapped in cling film and stored at –20°C until analysed.

Immunocytological analysis

The proportions of mature macrophage subsets within the harvested cell populations were determined by double immunofluorescence methods in which monoclonal antibodies RFD1 (mouse IgM) and RFD7 (mouse IgG1 (Royal Free Hospital School of Medicine, London, UK) were used in combination [13]. These reagents have been extensively used in this laboratory and by many independent workers [14–17] to discriminate phenotypically distinct macrophage subsets. (They are commercially available from Serotec UK Ltd). By using two immunoglobulin class specific second layer reagents conjugated, respectively, to fluorescein isothiocyanate (FITC) and tetraethyl rhodamine isothiocyanate (TRITC) (Europath Ltd, Cornwall, UK), the relative proportions of D1⁺ stimulating cells fluorescing red, D7⁺ phagocytes fluorescing green, and D1D7⁺ suppressive cells fluorescing both red and green could be determined [6]. These proportions were quantified by counting multiple high powered fields using a Zeiss fluorescence microscope with epi-illumination and appropriate barrier filters for FITC and TRITC. Approximately two hundred cells were counted in each experiment always by the same observer.

For each subset the proportion of the cells was calculated by the formula

$$\frac{(\text{specific subset})}{(\text{D1}^+) + (\text{D7}^+) + (\text{D1D7}^+)}$$

To determine the level of expression of HLADR on the cultured cells the indirect immunoperoxidase method was applied using MoAb. RFDR1 (Royal Free Hospital School of Medicine) which identifies a framework epitope on the HLADR molecule [18]. The level of HLADR expression was quantified by measuring the optical density of the peroxidase reaction product using a computerised image analyser (Seescan Imaging Ltd, Cambridge, UK) [19]. Human palatine tonsil was used as a positive control. For negative controls using the cell cytospin PBS was substituted for the RFDR1 first layer antibody.

Mixed lymphocyte reactions (MLR)

In some experiments (four normal individuals and three asthmatics) the peripheral blood monocytes cultured for 7 days in medium alone or with IL-10 added on day 5, were harvested and treated with mitomycin C [20]. The addition of mitomycin C inhibits lymphocyte proliferation thus preventing contaminating lymphocytes from being stimulated in a two-way MLR. Aliquots of 2×10^5 cells were introduced into 96 well microtitre plates and cocultured with autologous or allogeneic T cells added to each well at a final concentration of 1×10^6 cells per well. Each experiment was performed in triplicate. This coculture was

incubated for 6 days, 18 h before termination of cultures $1 \mu\text{Ci} [^3\text{H}]$ thymidine was added to each well. Cells were harvested using an automatic cell harvester (Tomtec, Connecticut, USA) and counted in a scintillation counter. The result are expressed as counts per minute.

Cytokine ELISA

The production of $\text{TNF}\alpha$ and $\text{TGF}\beta$ by differentiating monocytes were determined by cytokine specific ELISA. Supernatant from the 7 day cultures was stored at -20°C . Cytokine assays were performed on culture supernatant with ELISA systems according to manufacturers instructions. Both $\text{TNF}\alpha$ and $\text{TGF}\beta$ ELISA kits were from R & D systems (Abingdon, UK) and had sensitivities down to 15.6 pg/mL . The plates were read at 490 nm using an automated ELISA reader. No specific background presence of $\text{TNF}\alpha$ was detected in the culture media. The background level of activated $\text{TGF}\beta$ in serum based culture media was subtracted from the total $\text{TGF}\beta$ assayed in the cell culture supernatant to determine monocyte cytokine production.

Statistical analysis

The effect of IL-10 on monocyte maturation was analysed using a paired non parametric Mann–Whitney test. The comparison between normal and asthma was analysed using the non paired Wilcoxon test.

Results

Macrophage phenotype

In establishing the control group, the effect of IL-10 on the phenotype of differentiating monocytes in culture was first compared between nine normal subjects and nine atopic non-asthmatic subjects. As there was no significant difference in either monocyte differentiation or response to IL-10 between atopic and non-atopic individuals (Fig. 1) both were included in the control group.

With no cytokine addition similar proportions of monocytes derived from normal or atopic asthmatic subjects differentiated into macrophages expressing an antigen presenting cell phenotype (D1⁺D7⁺), 52% and 53%, respectively (Fig. 2). The proportions of maturing monocytes expressing a suppressive (D1⁺D7⁺) phenotype at day 7 was however, lower in the asthmatic subjects (18%) compared with normal (25%) ($P < 0.02$). Consequently the proportion of cells expressing the (D1D7⁺) phenotype of mature phagocytes was greater in the asthmatic samples. The addition of IL-10 resulted in an increase in the proportion of D1⁺D7⁺ suppressive macrophages in both groups (Fig. 3). This change was significantly greater in the asthmatic

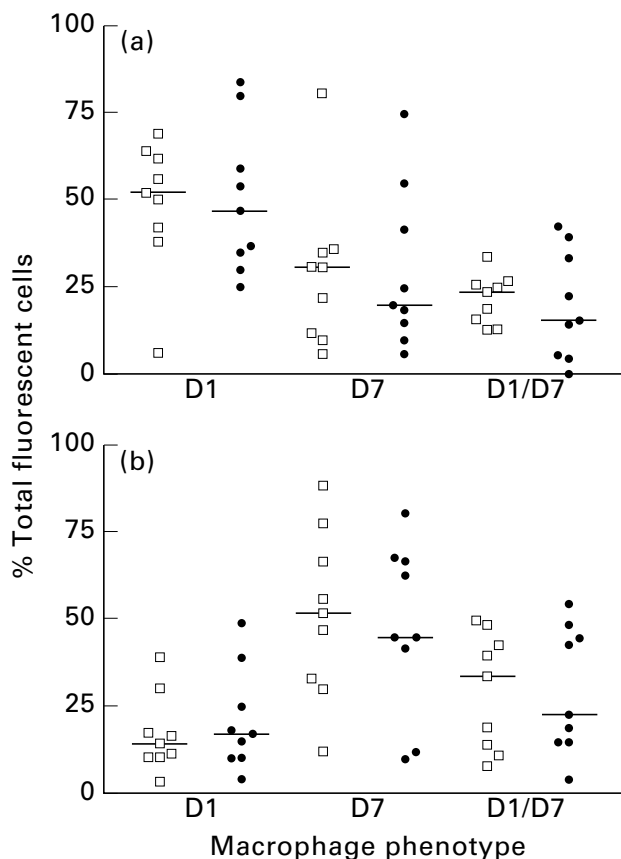


Fig. 1. The proportions of monocytes from normal [□] and atopic nonasthmatic individuals [●] expressing the stimulatory phenotype D1⁺; phagocytic phenotype D7⁺; and suppressive phenotype D1⁺D7⁺ after 7 days culture without the addition (a) and following the addition (b) of IL-10 on day 5. Horizontal bars indicate median values.

(94% increase) compared to normal subjects (32% increase) ($P < 0.01$) (Fig. 4), thus eliminating the initial disparity between these groups after 7 days culture (Fig. 1). IL-10 significantly decreased the proportion of inductive macrophages in both normal and asthmatic subjects ($P < 0.01$); the magnitude of this reduction was similar in both groups (Fig. 5). The level of HLA-DR expression was reduced by IL-10 to an equivalent extent in both normal and asthmatic subjects; the difference between the two groups not reaching statistical significance (data not shown).

Cytokine production

Seven day supernatant from all cultures were tested for levels of cytokines TNF α and TGF β . IL-10 addition did not significantly influence TNF α production in cultures of either normal or asthmatic monocytes. Levels of TGF β in culture supernatants were increased in the majority of

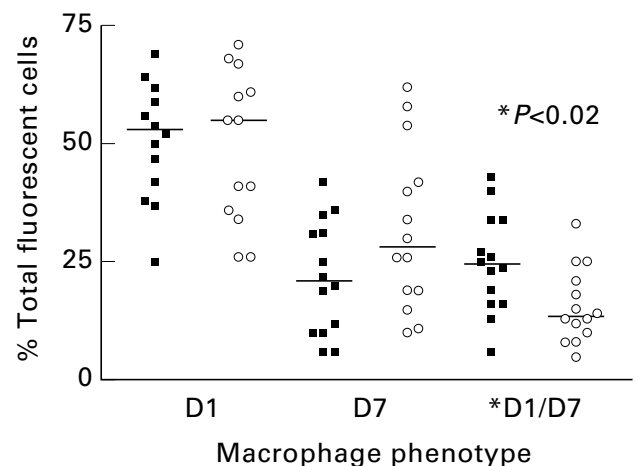


Fig. 2. The proportions of monocytes from normal [■] and asthmatic individuals [○] expressing the stimulatory phenotype D1⁺; phagocytic phenotype D7⁺; and suppressive phenotype D1⁺D7⁺ after 7 days culture. Horizontal bars indicate median values.

cultures from both normal and asthmatic subjects but group analysis revealed no significant difference over IL-10 free controls. There was no correlation between cytokine levels and proportions of macrophage subsets.

Mixed lymphocyte reaction

After 7 days in culture monocytes from asthmatic subjects have a greater capacity to stimulate T cell proliferation in an allogenic MLR than monocytes from normal individuals ($P < 0.05$) (Fig. 6). The addition of IL-10 to the monocyte stimulator population significantly inhibited T cell

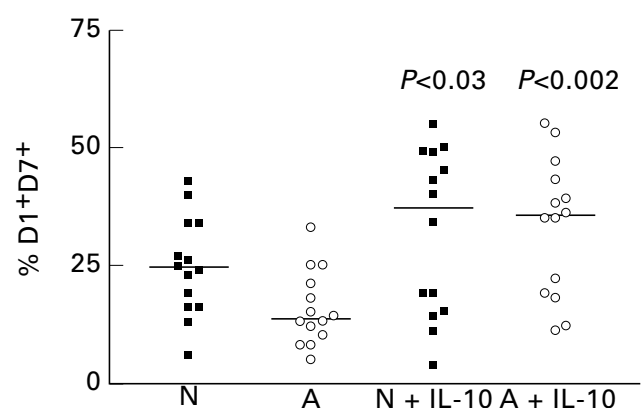


Fig. 3. The effect of IL-10 on the proportion of D1⁺D7⁺ suppressive cells from normal [■] and asthmatic individuals [○] which have matured in culture. Horizontal bars indicate median values.

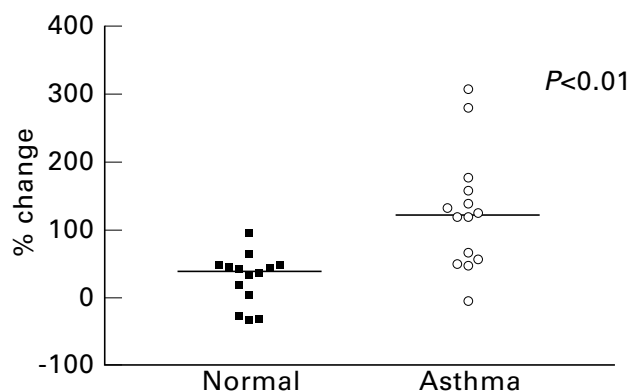


Fig. 4. The increase in D1⁺D7⁺ suppressive cells following the addition of IL-10 expressed as the percentage change in the proportion of suppressive cells cultured in the absence of IL-10. Horizontal bars indicate median values.

proliferation in both normal and asthmatic samples. The inhibition by IL-10 was significantly greater in the asthmatic compared with normal subjects thus reducing the initial disparity between the two groups.

Discussion

Impaired regulation of T-cell reactivity by macrophages may be responsible for the persistence of a T cell dominated peribronchial inflammation in asthma [8]. More specifically, macrophage subsets have been implicated in both the promotion and loss of suppression of T cell proliferation [1–3,7,8]. It is possible that the imbalance within lung macrophage subsets of the asthmatic individual may be secondary to either changes to the mature macrophage population within the lung microenvironment or an alteration in the maturation of recruited peripheral blood monocytes. It has previously

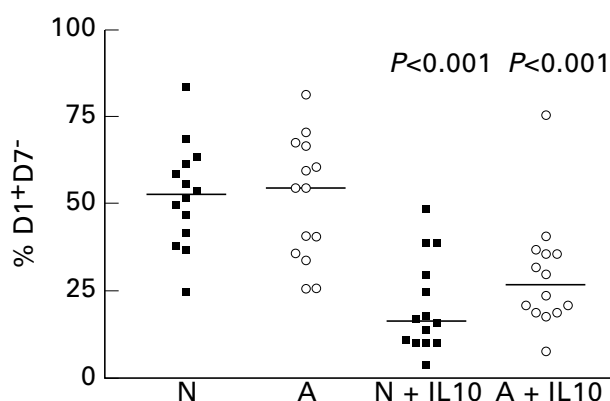


Fig. 5. The effect of IL-10 on the proportion of D1⁺D7⁻ antigen presenting cells from normal [■] and asthmatic individuals [○] which have matured in culture. Horizontal bars indicate median values.

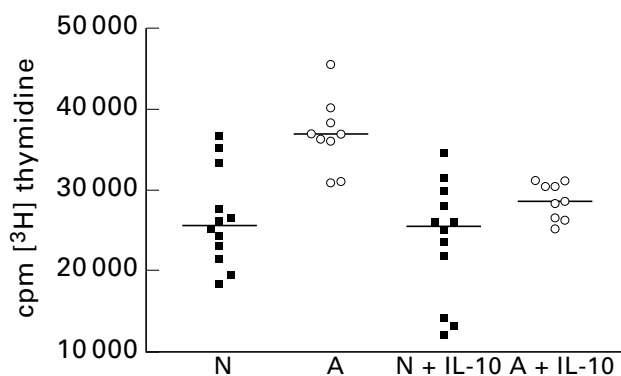


Fig. 6. The effect of IL-10 in inhibiting T cell proliferation in an allogenic MLR in normal and asthmatic individuals. The results are expressed as counts per minute of [³H] thymidine. Horizontal bars indicate median values.

been shown that T cell cytokines regulate the differentiation of peripheral blood monocytes and more specifically that IL-10 has the capacity to increase the proportion of lung macrophages with a suppressive phenotype [4]. The present study reveals that the aberrant maturation of monocytes occurring in asthmatic individuals and the resulting imbalance between macrophage subsets may be corrected by exogenous IL-10. We did not investigate the effect of other cytokines including IL-4, IFN γ , IL-1 and GM-CSF on asthmatic monocyte maturation, although it is likely that they also have a significant effect on macrophage phenotype.

It has been shown previously that lung macrophages from asthmatics are better stimulators of allogenic T cells than those from normal subjects. The present study reveals that monocytes from asthmatic individuals which have matured in culture also have a greater capacity to stimulate T cell proliferation than monocytes from normal individuals. However, monocytes from both asthma and normal individuals have an equivalent functional response to IL-10 in terms of the relative inhibition of an MLR. Therefore in asthma, the reduction in the proportion of cells maturing into D1⁺D7⁺ suppressive macrophages may be seen as consistent with a greater capacity for T cell proliferation.

The control group consisted of both atopic and non atopic individuals. There was no significant difference in either monocyte differentiation or response to IL-10 between atopic and nonatopic normal individuals. Therefore, since atopy did not alter the balance within the macrophage populations derived from blood monocytes, we did not investigate intrinsic asthmatics as a specific subgroup.

One limitation of this study is that the asthmatics were taking maintenance inhaled corticosteroids which have been shown to alter macrophage phenotype within the bronchial wall in asthma [21]. More recently, John *et al.* demonstrated

that inhaled corticosteroids increased IL-10 expression in alveolar macrophages but not peripheral blood monocytes [22]. However, it is unlikely that this would alter the results of the present study. Indeed, if anything it would tend to decrease the probability of detecting a significant difference in both macrophage phenotype and sensitivity to IL-10 between asthma and normal individuals.

Thus in asthma, there is a reduction in the proportion of cultured macrophages expressing a $D1^+D7^+$ suppressive phenotype associated with an increased capacity to stimulate allogenic T cells. This relative deficiency in the differentiation of monocytes (not yet exposed to the lung microenvironment) into mature suppressive cells in asthma may offer a novel insight into pathogenesis. Since asthma is not a generalized systemic disorder, one may question why the altered maturation of monocytes reported here [4] is not reflected in concomittant extrapulmonary pathology. The local factors that influence macrophage development in the airway mucosa in asthma remain to be determined. Most evidence favours a TH2 cytokine profile in asthma [23–25], and this might be expected to include increased expression of IL-10 [10,27]. However, other data suggest that there may be deficient IL-10 expression by airway cells in asthma [26]. Our data would suggest that if IL-10 production is increased in the airway in asthma this would prevent accumulation of $D1^+D7^-$ phenotype macrophages, whereas such accumulation is reported. It is possible that the timing of IL-10 production in relation to allergen exposure [27], or interaction with other cytokines influencing macrophage development determine the airway macrophage phenotype in asthma.

The addition of IL-10 increased the proportion of $D1^+D7^+$ cells in both normal individuals and asthmatics to a similar level. However, given the initial deficiency of suppressive macrophages in asthmatics the proportionate increase in these subjects was significantly greater than in normal individuals. Given the nature of the cytokine control of monocyte differentiation, these results refute the possibility that asthmatic monocytes/macrophages are less sensitive to IL-10. Although it is recognized that other factors may impact on IL-10 responses *in vivo* the data support the alternate suggestion that there is deficient IL-10 production within mononuclear cells regulating monocyte differentiation in asthmatic individuals. This is supported by a recent study documenting decreased spontaneous and stimulated IL-10 production in asthmatic peripheral blood mononuclear cells [26]. Similarly, the appearance of IL-10 production may contribute to the resolution of the late asthmatic response after allergen challenge [27]. More recently, a comprehensive review summarized the evidence supporting the concept that IL-10 could be useful in controlling TH₂ mediated inflammatory processes by preventing the accumulation of activated eosinophils in target

tissues [28]. Thus, since IL-10 alters monocyte differentiation and the phenotype and function of the resulting macrophage pool in both normal and asthmatic subjects the intriguing possibility emerges that manipulation of IL-10 may be associated with therapeutic benefit in asthma [22].

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