

Original article

Allergen-dependent CD14 modulation and apoptosis in monocytes from allergic patients

Background: CD14 is a most important monocyte surface molecule. Recently, it has been reported that there is an important relationship between CD14 and immunoglobulin E, and that regulation of CD14 expression is an effector mechanism mediating apoptosis of monocytes.

Objective: The present study was designed to determine whether specific allergens were able to modulate CD14 expression and apoptosis by monocytes from allergic patients or whether specific immunotherapy (IT) might affect these processes.

Methods: One group of adult allergic asthmatic patients had received IT for the previous 3 years. Another similar group was not treated with IT. We challenged peripheral blood monocytes from both groups of asthmatic patients *in vitro* with the specific allergen that produced clinical symptoms in asthmatic patients. The cells were also challenged with allergen to which the patients were not sensitive. Monocytes from normal subjects were also challenged with allergens. Expression of CD14 on the monocyte surface was analyzed by flow cytometry, and soluble CD14 (sCD14) in culture supernatant by enzyme-linked immunosorbent assay. The three groups of subjects were challenged with allergens, and apoptosis was analyzed by flow cytometry.

Results: When monocytes from non-IT-treated asthmatic patients were cultivated with the allergens to which the patients were sensitive, a significant up-regulation on the monocyte surface was observed compared with results from the healthy group ($P < 0.003$) and from the IT asthmatic group ($P < 0.003$). A significantly higher sCD14 level was observed in the culture supernatant of the monocytes from the IT asthmatic group were observed compared with those from the healthy group ($P < 0.001$) and those from the non-IT asthmatic group ($P < 0.001$). A significantly higher apoptosis level was observed in monocytes from the IT asthmatic group compared with those from the healthy group ($P < 0.001$) and those from the non-IT asthmatic group ($P < 0.001$).

Conclusions: We present evidence that the expression of CD14 on the surface of monocytes and the apoptosis of the same cells can be modulated by an allergen-dependent mechanism. These processes can be affected by IT.

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Monocytes circulate in the bloodstream for a short time (1–3 days), after which they die or emigrate into organs and tissues where differentiation into macrophages takes place. The presence of the three forms of immunoglobulin E (IgE) receptor the high-affinity receptor for IgE (FcεRI), the low-affinity receptor for IgE (FcεRII/CD23), and the Mac-2/IgE-binding protein (εBP), has been demonstrated on human monocytes (1–3). The observation that monocytes express IgE receptors suggests that allergen-dependent activation of monocytes plays an important role in the pathogenesis of allergic tissue injury. Alveolar macrophages and/or peripheral blood monocytes from

subjects with asthma have been reported to display an activated phenotype having up-regulated surface expression of human leukocyte antigen (HLA)-DR, type-1 complement receptor (CD35), and both high- and low-affinity IgE receptors (4–8). CD14, a glycoprotein attached to the cell surface by a glycosyl-phosphatidylinositol (GPI) anchor, is primarily expressed on myeloid cells. There is a second soluble form (sCD14) that lacks the GPI tail and is present in serum in micromolar concentrations (9, 10). CD14 is a most important monocyte surface molecule that induces activation in response to lipopolysaccharide (LPS) and obviously to other triggers present at the inflammatory

site. Recently, an important relationship between CD14 and IgE has been reported (11, 12). CD14 is involved in the regulation of monocyte apoptosis (13). The aim of our study is to investigate whether the CD14 molecule and apoptosis in monocytes can be modulated by an allergen mechanism. Here, we present evidence that specific allergens were able to modulate CD14 and apoptosis by monocytes from allergic patients, and that specific IT can modulate these processes.

Materials and methods

Materials

The allergens were commercially available antigen extracts, including D₁ (*Dermatophagoides pteronyssinus*), G₃ (*Dactylis glomerata*), T₉ (*Olea europaea*), and W₆ (*Artemisiavulgaris*). They were purchased from Bial-Aristegui (Bilbao, Spain). Other biochemicals were from Sigma (Madrid, Spain) or Merck (Barcelona, Spain). Anti-Mac-1, HLA-DR, CD14, CD4, CD16, CD32, CD3, and CD19 mAb were from Coulter-IZASA (Barcelona, Spain).

Patients and controls

The studied groups included adult atopic patients with bronchial asthma, and healthy adult nonatopic volunteer controls. One group of asthmatic patients had positive skin prick test (Bial-Aristegui) and specific IgE (CAP, Pharmacia Ibérica, Barcelona, Spain) to *Dermatophagoides pteronyssinus*. The subjects received no specific hyposensitization (non-IT asthmatic group). Another group of asthmatic patients had been immunotherapy (IT)-treated with *Dermatophagoides pteronyssinus* extract (Bial-Aristegui) for the previous 3 years, and continued to receive a maintenance dose within the highest potency of the extract (IT asthmatic group). The patients suffering an intermittent asthma (14) were not allowed to take any bronchodilators within the 8 h before challenge of monocytes *in vitro*. Oral bronchodilators were withheld for 24 h, and none of the subjects had taken corticosteroids, disodium cromoglycate, or nedocromil sodium in the previous week. The healthy controls had no history of allergy or bronchial symptoms, and had negative skin prick test (Bial-Aristegui) and specific IgE (CAP) to a battery of inhalant allergens (house-dust mites, pollens, molds, and animal danders). The study was approved by the Hospital Research Committee of the Department of Medicine, and each subject gave informed consent.

Isolation of monocytes

Human leukocytes were isolated from freshly drawn heparinized blood. Peripheral blood mononuclear cells (PBMC) were obtained as described previously (15). Total cells were overlaid on an equal volume of Histopaque (Sigma) and centrifuged at 400 *g* for 30 min at room temperature. Buffy interface cells (PBMC) were collected and washed in isotonic phosphate buffered saline (PBS). The PBMC population was further enriched by 46% (v/v) Percoll gradient (Pharmacia Biotech, Barcelona, Spain) separation. Cells were washed in PBS and resuspended in RPMI 1640 medium [10% fetal bovine serum (FBS); Bio Whittaker, Rockville, MD], and viable cells were enumerated by vital staining (trypan blue exclusion). The resultant cell population was assessed for purity of isolated cells by morphology and fluorescent activated cell sorter analysis, and typically comprised >98% monocytes (Mac-1⁺, HLA-DR⁺,

CD14⁺, CD4⁺, CD16⁺, CD32⁺) (15). To obtain enriched monocytes (lymphocyte-depleted), cells were incubated on ice for 30 min with an Ab mixture consisting of mouse anti-human CD3 and CD19 (Coulter-IZASA), at 5 µg of each mAb per 10 × 10⁶ cells in 100 µl PBS containing 10% FBS (PBS/FBS). The cells were washed three times in PBS/FBS, the cell pellet was resuspended in PBS/FBS in the presence of Dynabeads M280 sheep anti-mouse IgG (Dyna, Oslo, Norway) (10 beads/cell), and the mixture was incubated on ice for 30 min. The cell-bead mixture was exposed to a magnet through three cycles of magnetic separation and washing of the unattached cells (16).

Monocyte stimulation with allergens

Cells, resuspended at 1 × 10⁶ in RPMI, were stimulated with different doses of allergens (17) at 37°C, for 24 h for the analysis of membrane CD14 (mCD14) and 48 h for the analysis of sCD14 in the culture supernatant (18). For apoptosis experiments, monocytes, resuspended at 1 × 10⁶ in apoptosis culture medium, were stimulated with 10 µg/ml of allergens (17) at 37°C for 16 h (13). In all cases, two independent experiments were performed in each subject. All the subjects of each group were tested in the designed experiment. All cultured reagents had endotoxin levels of ≤0.01 ng/ml, as tested by the *Limulus* lysate assay (Coatest, Chromogenix, Mölndal, Sweden).

Flow cytometry analysis

After each experiment, cells were rapidly cooled on ice, and 1 ml of cold wash buffer (PBS) was added. Cells were then centrifuged at 500 × *g* for 10 min, supernatants were removed, and cell pellets were washed three times with cold wash buffer before staining with 20 µl of PE-labeled CD14 along with control cells stained with PE-labeled IgG2b-nonspecific control antibody (Coulter-IZASA). Flow cytometry was performed on an Epics Elite flow cytometer (Coulter-IZASA), calibrated by standard techniques. For each stained sample, 2 × 10⁴ cells were analyzed for fluorescence intensity. The fluorescence distribution for both control and test cells was analyzed with a Coulter Elite software workstation. Results are expressed as mean fluorescence intensity (MFI) (17). For apoptosis experiments, after treatment with the allergen, monocytes were harvested from the culture dishes with ice-cold PBS. Blocking of nonspecific binding sites was performed by incubation in PBS containing 2% bovine serum albumin (BSA), 0.01% NaN₃, and 10% casein for 20 min on ice. Subsequently, the cells were washed with staining buffer (SB, containing 1% BSA in 50 mM HEPES buffer, pH 7.4) and fixed with 4% paraformaldehyde in SB for 10 min. Fixed cells were first labeled with annexin-V-FITC (Coulter-IZASA) (2.5 µg/ml in HEPES buffer containing 150 mM/l NaCl, 5 mM/l KCl, 1 mM/l MgCl₂, and 1.8 mM/l CaCl₂, pH 7.4) for 15 min on ice to determine phosphatidylserine (PS) exposure to the outer face of the cell membrane. After repeated washing with SB, the cells were stained with propidium iodide (PI) (Coulter-IZASA) (5 µg/ml) for 15 min, and washed again with SB. Results from flow cytometry analysis were expressed as percentages of positive cells (13).

Analysis of soluble CD14 (sCD14) by ELISA

The sCD14 levels in culture supernatants were determined in replicate wells using a CD14 capture enzyme-linked immunosorbent assay (ELISA) (IBL, Hamburg, Germany). The lower detection limit was <1 ng/ml.

Statistics

Data are expressed as mean $\pm$ SEM. Comparisons between groups were made with one-way ANOVA. A P -value of <0.05 was considered significant.

Results

Figure 1 shows that when monocytes from non-IT-treated asthmatic patients ($n = 15$) were cultivated with the allergens to which the patients were sensitive, a significant up-regulation on the monocyte surface CD14 was observed compared with that on monocytes cultivated with allergens to which the patients were not sensitive to ($P < 0.003$), on monocytes from the healthy group ($P < 0.003$), and on monocytes from the IT asthmatic group ($P < 0.003$). No significant differences in monocyte surface CD14 were observed between monocytes from the healthy group ($n = 10$), the IT-asthmatic group

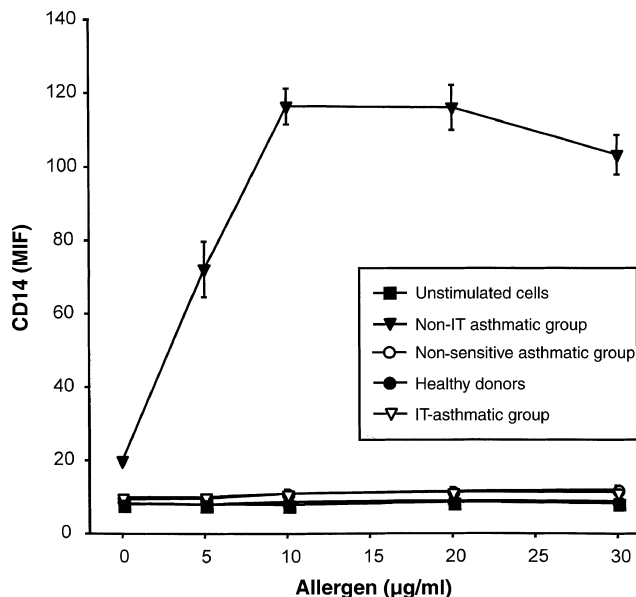


Figure 1. When monocytes from non-IT-treated asthmatic patients (∇) were cultivated with the allergens to which the patients were sensitive, a significant up-regulation on the monocyte surface CD14 was observed compared with that on monocytes cultivated with allergens to which the patients were not sensitive ($\circ$) ($P < 0.003$), on monocytes from the healthy group ($\bullet$) ($P < 0.003$), and on monocytes from the IT asthmatic group (∇) ($P < 0.003$). No significant differences in monocyte surface CD14 were observed between the healthy group, the IT asthmatic group, and the non-IT asthmatic group when challenged with allergens to which the patients were not sensitive. Present data also show that the activation of cells was dependent on dosage of allergens. Data are given as mean $\pm$ SEM of 30 experiments (∇), 20 experiments ($\circ$), 20 experiments ($\bullet$), and 30 experiments (∇).

($n = 15$), and the nonsensitive asthmatic group ($n = 10$) when challenged with allergens to which the patients were not sensitive. Present data also show that the activation of cells was dependent on dosage of allergens. In the same groups of patients and controls in which we had studied the monocyte CD14 surface, when cells were cultivated with the allergens to which the patients were sensitive, a significantly higher sCD14 level was observed in the culture supernatant of the monocytes from the IT asthmatic group compared with those of monocytes from the healthy group ($P < 0.001$), from the non-IT asthmatic group ($P < 0.001$), and from the non-IT asthmatic group when challenged with allergens to which the patients were not sensitive ($P < 0.001$) (Fig. 2). A significantly higher sCD14 level was observed in the culture supernatant of the monocytes from the non-IT asthmatic group than in that of monocytes from the

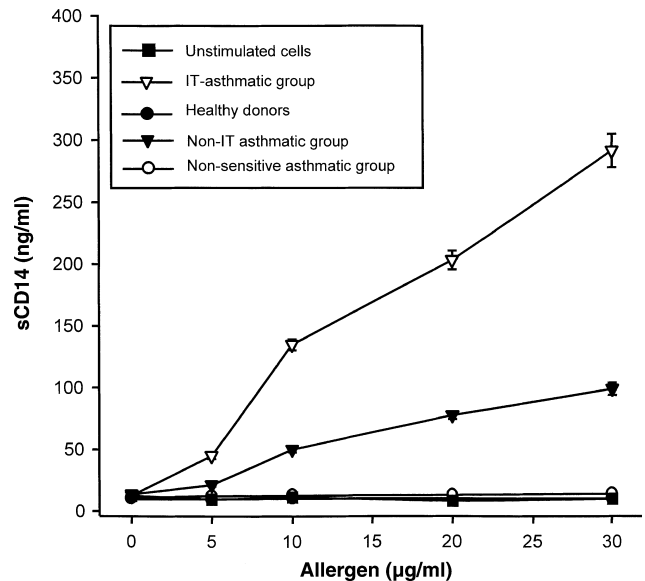


Figure 2. When cells were cultivated with the allergen to which the patients were sensitive, a significantly higher sCD14 level was observed in the culture supernatant of the monocytes from the IT asthmatic group (∇) compared with that of monocytes from the healthy group ($\bullet$) ($P < 0.001$), the non-IT asthmatic group (∇) ($P < 0.001$) and the non-IT asthmatic group when challenged with allergens to which the patients were not sensitive ($\circ$) ($P < 0.001$). A significantly higher sCD14 level was observed in the culture supernatant of the monocytes from the non-IT asthmatic group than in that of monocytes from the healthy group ($P < 0.001$). No significant differences in the sCD14 level were observed in the culture supernatant of the monocytes from the healthy group compared that of monocytes from the non-IT asthmatic group when challenged with allergens to which the patients were not sensitive. The levels of sCD14 in the culture supernatant were dependent on dosage of allergens. Data are given as mean $\pm$ SEM of 30 experiments (∇), 20 experiments ($\bullet$), 30 experiments (∇), and 20 experiments ($\circ$).

healthy group ($P < 0.001$). No significant differences were observed in the sCD14 levels in the culture supernatant of the monocytes from the healthy group compared with that of monocytes from the non-IT asthmatic group when challenged with allergens to which the patients were not sensitive. The levels of sCD14 in the culture supernatant were dependent on dosage of allergens.

As CD14 is involved in the regulation of monocyte apoptosis, further experiments were performed to analyze whether allergens could induce monocyte apoptosis. Expression of the phospholipid PS on the cell surface is thought to be one of the early features of apoptosis (19). Annexin-V has a high affinity for PS, and is used in conjunction with PI to distinguish apoptotic cells from necrotic cells or cells that have already died following apoptosis. This is based on the fact that PI selectively penetrates the cytoplasmic membrane of necrotic cells, but not that of apoptotic cells (20). Monocytes cultured with 10 $\mu\text{g}/\text{ml}$ of allergens for 16 h were resuspended in PBS, and stained using FITC-labeled annexin-V and PI. A significantly greater apoptotic change was observed in the monocytes from the IT-asthmatic group ($n = 12$) than in monocytes from the non-IT asthmatic group ($n = 12$) ($P < 0.001$) and in cells from healthy subjects ($n = 12$) ($P < 0.001$) (Fig. 3). Differences in levels of necrotic cells (annexin-V negative, vital-dye positive) or cells which have died via apoptosis (annexin-V positive, vital-dye positive) were not significant in any group (data not shown).

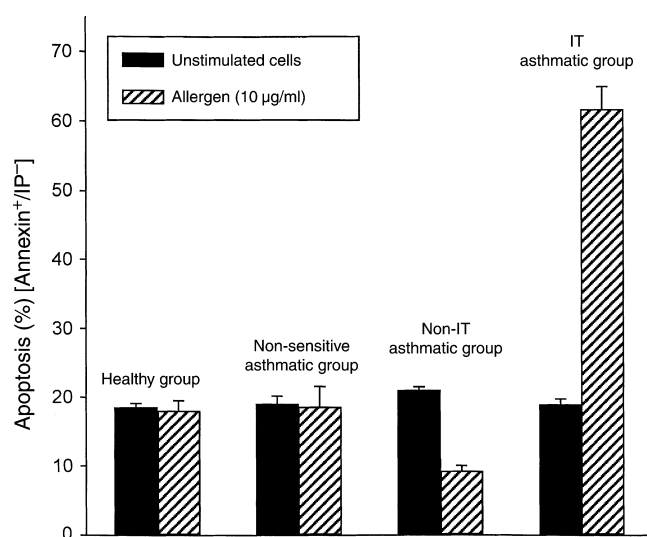


Figure 3. A significantly greater apoptotic change was observed in the monocytes from the IT asthmatic group than in those from the non-IT asthmatic group ($P < 0.001$), those from healthy subjects ($P < 0.001$), and those from the nonsensitive asthmatic group ($P < 0.001$). Data are given as mean $\pm$ SEM of 24 experiments in each group.

Discussion

The data presented herein provide the first evidence that allergens to which patients were sensitive up-regulate the expression of cell surface CD14 by human peripheral blood monocyte from non-IT asthmatic subjects. Our results indicate that *in vitro* activation of monocytes was very specific for allergens. Thus, allergens other than those that produced clinical symptoms did not stimulate the up-regulation of mCD14; furthermore, allergens were ineffective on monocytes from healthy donors. After sub-clinical doses of allergen were inhaled by asthmatic patients, alveolar macrophages showed a postchallenge altered phenotype pattern with increased expression of CD14 (21). Stimulation of monocytes with monocyte chemotactic protein resulted in up-regulation of the expression of CD14 on monocytes from symptomatic asthma patients (22). As earlier studies have shown that CD14 on monocytes mediates adhesion to activated vascular endothelial cells, the present findings that up-regulation of CD14 on monocytes by allergens may lead to an adhesion of monocytes to vascular endothelium, and their subsequent transendothelial migration into allergic sites (22). Monocytes have a limited life span, and their homeostasis is regulated by programmed cell death *in vivo*. The onset of apoptosis can be prevented by adding activating factors; monocytes receiving such signals differentiate into macrophages or dendritic cells. CD14 is involved in the regulation of monocyte apoptosis, because activating stimuli which are able to enhance CD14 expression rescue monocytes from apoptosis, whereas deactivating stimuli support programmed cell death (13). Thus, up-regulation of mCD14 by allergens could induce an enhanced monocyte life span, suppressing monocyte apoptosis. In accord with this possibility, we found a lower level of apoptosis in cells from the non-IT asthmatic group than in cells from healthy controls. This process may contribute to the establishment of chronic allergic disorders. After challenge with allergens, monocytes from IT asthmatic patients did not up-regulate mCD14, and presented significantly higher levels of apoptosis than did cells from non-IT asthmatic patients. Thus, IT may contribute to limiting the depth and the chronicity of allergic disorders by both abrogating transendothelial migration of monocytes into allergic sites and increasing monocyte apoptosis.

We have shown in this paper that a significantly higher sCD14 level was observed in the culture supernatant of the monocytes from the non-IT asthmatic group than in that of monocytes from the healthy group ($P < 0.001$). No significant differences was observed between the sCD14 level in the culture supernatant of the monocytes from the healthy group and that of monocytes from the non-IT asthmatic group when challenged with allergens to which the patients were not sensitive. The levels of

sCD14 in serum were significantly higher during acute asthma attacks than at recovery (23). In asthmatic atopic patients, sCD14 concentrations increased significantly in bronchoalveolar lavage 18 h following segmental allergen provocation (24). Other pro-inflammatory stimuli, such as LPS, elicit the shedding of sCD14 from human monocytes (16). Baldini et al. (11) reported an inverse correlation between sCD14 levels and total serum IgE. More recently, Arias et al. (12) reported that interaction of sCD14 with B cells resulted in a marked inhibition of IgE and interleukin-6 (IL-6) production. Previously, the same authors demonstrated (25) that sCD14 induces inhibition of T-cell activation and IL-4 production. The inhibitory effect on IL-4 and IL-6 should contribute to reducing IgE isotype switching. These findings show that sCD14 is capable of acting as physiological regulator of the immune response (25). It can be speculated that allergen-sCD14 could exert a negative feedback in the production of IgE and activation of T-lymphocytes, restricting the allergic response. Monocytes from the IT asthmatic group shed higher levels of sCD14. Thus, IT can contribute in the inhibition of the IgE-dependent allergic response. Together with the previously reported

effects on mCD14, the CD14 molecule can exert opposite effects in the development of atopic diseases.

In conclusion, we present evidence that the expression of CD14 and apoptosis in monocytes can be modulated by an allergen-dependent mechanism. These processes can be affected by IT. Although the study was performed solely in asthmatic patients, such modifications can be assumed to take place not only in asthmatic patients but generally in individuals with allergen sensitization, as with other allergen-specific cell responses in different types of cells (26). Together, all these findings possibly add a new facet to our understanding of the pathogenesis and treatment of allergic reactions.

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