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## Expression of Surface Markers on Mature Monocyte-Derived Dendritic Cells from Allergic Asthmatics

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Dendritic cells (DCs) are one type of important inflammatory cells in the pathogenesis of asthma for early response to allergen exposure. Monocyte-derived DCs (MoDCs) are characterized by a high antigen uptake capacity and poor T-cell stimulatory activity, both features of immature DCs. By stimulation with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or antigen capture, these cells differentiate into mature DCs with the disappearance of antigen-capturing regions, and increase in stimulatory activity. We measured the expression of some molecules on MoDCs before and after stimulation with TNF- $\alpha$  or house dust mite (HDM) antigen, from 9 house dust mite (HDM)-allergic asthmatic patients and 8 normal control subjects by flow cytometry. Primary MoDCs from HDM-allergic asthmatics showed a greater expression of histocompatibility leukocyte antigen (HLA)-DR and mannose receptor (MR), but not of CD80, CD86 or intercellular adhesion molecule-1 (ICAM-1), than those from normal subjects ( $P < 0.05$ ). After stimulation with TNF- $\alpha$  or HDM, DCs from asthmatic patients showed a greater expression of HLA-DR, CD86 and ICAM-1, than those from normal subjects. In HDM-allergic asthmatic patients, MR expression on DCs significantly declined after stimulation by HDM compared with stimulation by TNF- $\alpha$  ( $P < 0.05$ ). Results suggest that the reduction of MR expression may be characteristic on mature DCs after HDM exposure in allergic asthma.

**Key words:** asthma; dendritic cells; histocompatibility leukocyte antigen-deoxyribose; house dust mite; mannose receptor

Allergic asthma is a chronic inflammatory disease characterized by increased infiltration of eosinophils and allergen-specific Th2 lymphocytes into the airways (Lundgren et al., 1988; Ackerman et al., 1994). It is generally accepted that airway dendritic cells (DCs) are essential for stimulating naive T cells in a primary immune response to inhaled antigens and the most prominent cells involved in the presentation of inhaled antigens to Th2 lymphocytes (Ackerman et al., 1994; Bellini et al., 1993; Inaba et al., 1990). DCs are present in

the bronchial mucosa of both normal and asthmatic subjects. As assessed by immunohistochemistry, the airways of atopic asthmatics are known to contain increased numbers of CD1a + DCs (Moller et al., 1996).

The DC population in the airway epithelium is renewed every 48 to 72 h; a more rapid turnover reveals that DCs originate in the bone marrow and supposedly belong to the monocyte lineage (Holt et al., 1994; McWilliam et al., 1995). Romani and colleagues (1994) recently demonstrated that monocyte-enriched periph-

Abbreviations: DC, dendritic cell; FEV<sub>1.0</sub>, forced expiratory volume in one second; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; HDM, house dust mite; HLA, histocompatibility leukocyte antigen; ICAM-1, intercellular adhesion molecule-1; IgE, immunoglobulin E; IL-4, interleukin-4; MHC, major histocompatibility complex; MoDC, monocyte-derived dendritic cell; MR, mannose receptor; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; TNF- $\alpha$ , tumor necrosis factor- $\alpha$

**Table 1. Patients Characteristics**

Patient number	Sex	Age (year)	Total serum IgE (IU/mL)	FEV <sub>1.0</sub> (% predicted)	Minimum dose of methacholine (U)	Inhaled beclomethasone ( $\mu$ g/day)
1	Male	16	4300	80.9	3.387	—
2	Male	23	289	84.5	0.206	400
3	Male	24	1045	91.5	1.045	400
4	Male	30	11200	79.6	0.049	300
5	Male	34	170	89.3	1.358	—
6	Male	37	246	74.1	0.439	—
7	Female	19	2650	82.5	2.13	600
8	Female	22	6060	77.2	0.459	600
9	Female	26	1125	70.1	ND	600

FEV<sub>1.0</sub>, forced expiratory volume in one second; IgE, immunoglobulin E; ND, not done.

eral blood mononuclear cells (PBMCs) cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 can differentiate into DCs (MoDCs).

van den Heuvel and colleagues (1998) reported that MoDCs from allergic asthmatics showed phenotypic differences in the expression of surface markers. MoDCs have many of the same features as immature DCs, including the expression of molecules that enhance antigen capture and weak T-cell stimulation. If MoDCs are stimulated by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or antigens, these cells differentiate into mature cells with the disappearance of antigen-capturing regions and an increase in T-cell stimulatory function (Palucka et al., 1998). It has not been reported whether MoDCs from asthmatics develop into mature phenotypes after stimulation with any antigens.

The mannose receptor (MR) is a surface 175-kD C-type lectin and a membrane lectin or sugar-specific receptor, expressed by macrophages and DCs (Avrameas et al., 1996; Koning, 1997). MR enhances a histocompatibility leukocyte antigen (HLA) class II-restricted antigen presentation and specifically mediates the binding and endocytosis of mannose- and fucose-terminated glycoproteins, which are involved in the phagocytosis of pathogens such as the house dust mite (HDM) (Mitra and Chatterjee, 1990), because HDM is a complex of a glycoprotein with a high-mannose type of oligosaccharide (Uhlir, 1993).

In this study, we examined the expression of mature markers and T cell co-stimulating molecules on MoDCs isolated from asthmatics after stimulation with TNF- $\alpha$  or HDM.

## Materials and methods

### Subjects

Nine asthmatic patients (6 males and 3 females) aged 16 to 37 years (mean, 26 years) were studied (Table 1). Patients with asthma were defined as having a previous history of episodic wheezing and reversible airway obstruction, characterized by an increase in a forced expiratory volume in one second (FEV<sub>1.0</sub>) of >15% after inhalation of 40  $\mu$ g of procaterol. All patients were HDM-allergic, defined by a > 2-mm wheal response to HDM extract (Torii, Tokyo, Japan), and a raised serum concentration of total immunoglobulin E (IgE > 250 IU/mL) or specific IgE for HDM. None of the patients received oral corticosteroids within the 3 months preceding the study. Six of the asthmatic subjects were taking regular inhaled corticosteroids (range, 300 to 600  $\mu$ g of beclomethasone per day). Patients were excluded from the study if they experienced an asthmatic attack during the 2 months preceding the study. Eight healthy volunteers (mean age, 26.4 years; range, 21 to 35 years), who were HDM-non-allergic and had no history of asthma or allergic diseases, were

studied as a control group. All participating subjects were non-smokers and had no history suggesting a viral respiratory tract infection within 4 weeks preceding or during the study. All subjects gave their informed consent.

#### ***Determination of airway responsiveness***

Airway response was measured directly by examining the dose-response curve of respiratory resistance drawn by an Astograph (Chest, Tokyo) (Takishima et al., 1981) during continuous inhalation of methacholine administered in a stepwise incremental fashion. Briefly, methacholine aerosol in doubling concentrations (0.049–100 mg/mL) was inhaled continuously through the mouth during tidal breathing for each concentration. The aerosol was generated by a micronebulizer (Bird, Palm Springs, CA) with an output of 0.15 mL/min, and a particle size ranging from 0.5 to 4.0  $\mu$ m. Assessment of airway responsiveness was derived from a minimum dose of methacholine needed to increase the dose-response curve of respiratory resistance.

#### ***Preparation of DCs***

Sixty milliliters of peripheral blood were sampled from asthmatic patients and normal subjects. Peripheral blood mononuclear cells (PBMCs) were separated by standard Ficoll-Hypaque (specific gravity, 1.077 g/mL; Pharmacia, Uppsala, Sweden) density gradient centrifugation, and were washed twice with Mg<sup>2+</sup>- and Ca<sup>2+</sup>-free phosphate buffered saline (PBS, pH 7.4; Research Institute for Medical Disease, Osaka, Japan). The cells were resuspended in the following medium: RPMI 1640 with 25 mmol/L HEPES and 0.3 mmol/L glutamine (Bio Whittaker, Walkersville, MD) containing 10% fetal calf serum (Cansera International Inc., Rexdale, ON, Canada), 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Bio Whittaker), and then were incubated in culture dishes (Falcon 3002; Becton Dickinson, San Jose, CA) at 37°C with 5% CO<sub>2</sub> for 1 h. After non-adherent cells were decanted, DCs were prepared according to published methods

(Romani et al., 1994). In brief, adherent cells (monocyte-enriched peripheral blood mononuclear cells) were cultured in medium supplemented with 800 U/mL of GM-CSF (Genzyme, Cambridge, MA) and 500 U/mL of IL-4 (Genzyme). Cells were fed every other day (days 2, 4 and 6) with fresh culture medium containing the same levels of GM-CSF and IL-4. On day 7, cells were stimulated with and without 10  $\mu$ g/mL of HDM extract, supported by Torri (Tokyo) or 10 ng/mL TNF- $\alpha$  (Genzyme) for 24 h to induce maturation. At that point, the cells had become mostly non-adherent, and were removed from the plate and washed twice in RPMI 1640. They were then counted and analyzed. Viability was always above 95%. Cell yields were expressed as the number of DCs obtained from 10<sup>8</sup> PBMCs.

#### ***Immunofluorescence staining***

The expression of surface antigens on MoDCs was determined by the binding of monoclonal antibodies against CD1a (IgG2a; Cosmo Bio, Tokyo, Japan), CD1c (IgG1; Cosmo Bio), HLA-DR (IgG2a; Pharmingen, San Diego, CA), MR (IgG1; Pharmingen), CD80 (IgM; Serotec, Oxford, United Kingdom), CD86 (IgG1; Serotec), CD54 (IgG1; Coulter, Krefeld, Germany), CD11b (IgG1; Leinco, Ballwin, MO), CD83 (IgG1; Cosmo Bio), CD14 (IgG2a; Cosmo Bio), CD16 (IgG1; Nichirei, Tokyo) and CD3 (IgG1; Leinco) for 90 min on ice. After the cells were washed 3 times with PBS containing 0.1% sodium azide (PBS-azide), the expressions of these surface molecules were evaluated by indirect fluorescence after incubation for 30 min on ice in the dark with either fluorescein isothiocyanate (FITC)-conjugated antibodies (anti-IgG2a, IgG2b, IgM; Cappel, Durham, United Kingdom) or phycoerythrin-conjugated antibodies (anti-IgG1; Southern Biotechnology, Birmingham, AL). The cells were washed 4 times with PBS-azide, and were analyzed by flow cytometry.

### Flow cytometry

Flow cytometry was performed with 3,000 to 5,000 cells per sample by a FACS Calibur (Becton Dickinson). DCs were analyzed by selective gating based on forward- and side-scatter parameters with large cells in non-lymphoid areas (Fig. 1). Statistical markers were set using isotype-matched negative controls.

### Statistical analysis

Statistical analysis was performed with the Stat View 4.0 statistics package (Abacus Concepts, Berkeley, CA) and a Power Macintosh 7200/90 (Apple Computer, Cupertino, CA). All data are expressed as the mean  $\pm$  SD. The nonparametric Mann-Whitney *U* test and Wilcoxon's signed-rank test were used for statistical analysis of the unpaired data and paired data, respectively. The rate of induction of relative fluorescence intensity of MR on MoDCs stimulated with HDM or TNF- $\alpha$  was calculated by the following formula:

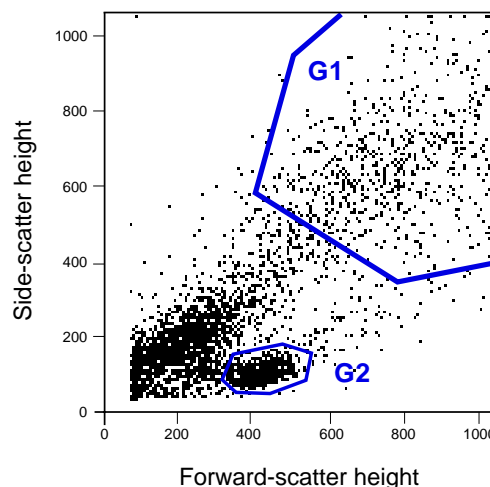
$$\left( \frac{\text{rFI of MR on MoDCs stimulated with HDM or TNF-}\alpha}{\text{rFI of MR on non-stimulated MoDCs}} - 1 \right) \times 100$$

where rFI stands for relative fluorescence intensity.

## Results

### Characteristics of primary MoDCs

To induce primary MoDCs, monocyte-enriched PBMCs were cultured with GM-CSF and IL-4 for 7 days. The cultured cells were a heterogeneous population containing  $16 \pm 6\%$  lymphocytes on morphology which was determined by using May-Grünwald-Giemsa staining. Whereas  $2.9 \pm 1.0 \times 10^6$  DCs were generated from  $10^8$  PBMCs from normal subjects,  $3.8 \pm 0.7 \times 10^6$  DCs could be obtained from PBMC from HDM-allergic asthmatics compared with normal subjects ( $P = 0.05$ ).



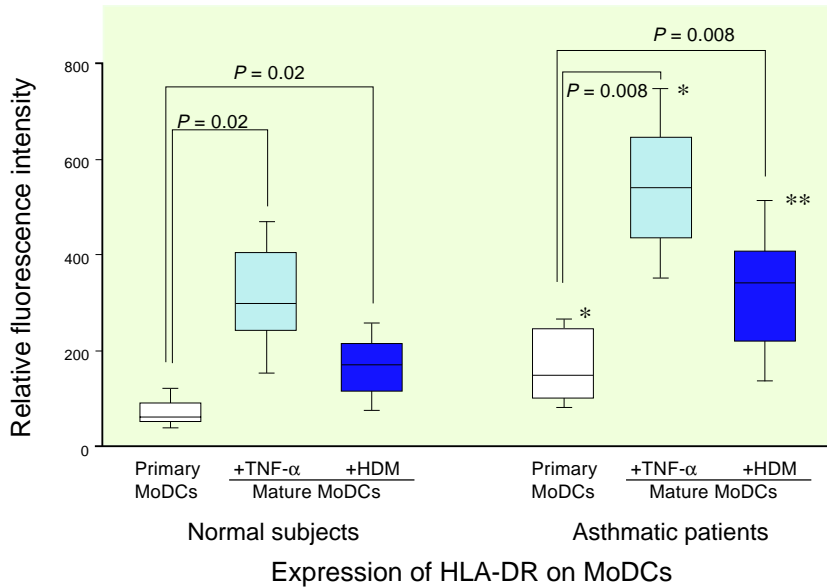
**Fig. 1.** Uniform high forward- and side-scatter properties of MoDCs (designated G1) and lymphocytes (designated G2) from representative HDM-allergic asthmatics (a). HDM, house dust mite; G1, large-cell population; G2, small-cell population; MoDC, monocyte-derived dendritic cell.

### Analysis of DCs by flow cytometry

Figure 1 shows representative forward- versus side-scatter density plots. On both the forward- and side-scatter, two subpopulations consisting of a large-cell population (G1) and a small-cell population (G2) were found in the cultured cells with GM-CSF and IL-4 for 7 days (Fig. 1). The cells in the small-cell population were CD3-positive and CD1a-negative lymphocytes. Using gating on the large cell population, we assessed the surface expression on DCs. The percentages of CD1a- or CD1c-positive cells on gating on the large-cell population did not differ between asthmatic patients ( $48.5 \pm 8.3\%$  and  $81.4 \pm 11.3\%$ , respectively) and control subjects ( $54.5 \pm 15.7\%$  and  $80.6 \pm 10.4\%$ , respectively). Less than 10% of DCs gating on the large-cell population expressed CD14.

### Expression of mature markers on DCs

To characterize mature DCs, we have used phenotypes emphasizing the presence of CD83 and HLA-DR. The expression of HLA-DR on primary MoDCs was significantly higher in HDM-



**Fig. 2.** Expression of HLA-DR as a mature marker on MoDCs of asthmatic patients and normal subjects. Primary MoDCs were derived from plastic-adherent peripheral blood mononuclear cells with 800 U/mL GM-CSF and 500 U/mL IL-4 for 7 days. Mature MoDCs were obtained from primary MoDCs by the stimulation of 10 ng/mL TNF- $\alpha$  or 10  $\mu$ g/mL HDM for 24 h. The lower, middle and upper lines in the boxes represent the 25th, 50th and 75th percentiles, respectively; the lower and upper limits of the bars represent the 10th and 90th percentiles, respectively. HDM, house dust mite; HLA-DR, histocompatibility leukocyte antigen-DR; IL-4, interleukin-4; GM-CSF, granulocyte-macrophage colony-stimulating factor; MoDC, monocyte-derived dendritic cell; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . \* $P < 0.05$ , compared with normal subjects. \*\* $P < 0.01$ , compared with normal subjects.

allergic asthmatic patients than in normal subjects (Fig. 2) ( $P < 0.05$ ), but that of CD83 was not (data not shown). The expression of CD83 and HLA-DR (Fig. 2) was enhanced after stimulation with TNF- $\alpha$  or HDM in the same manner with both asthmatics and control subjects.

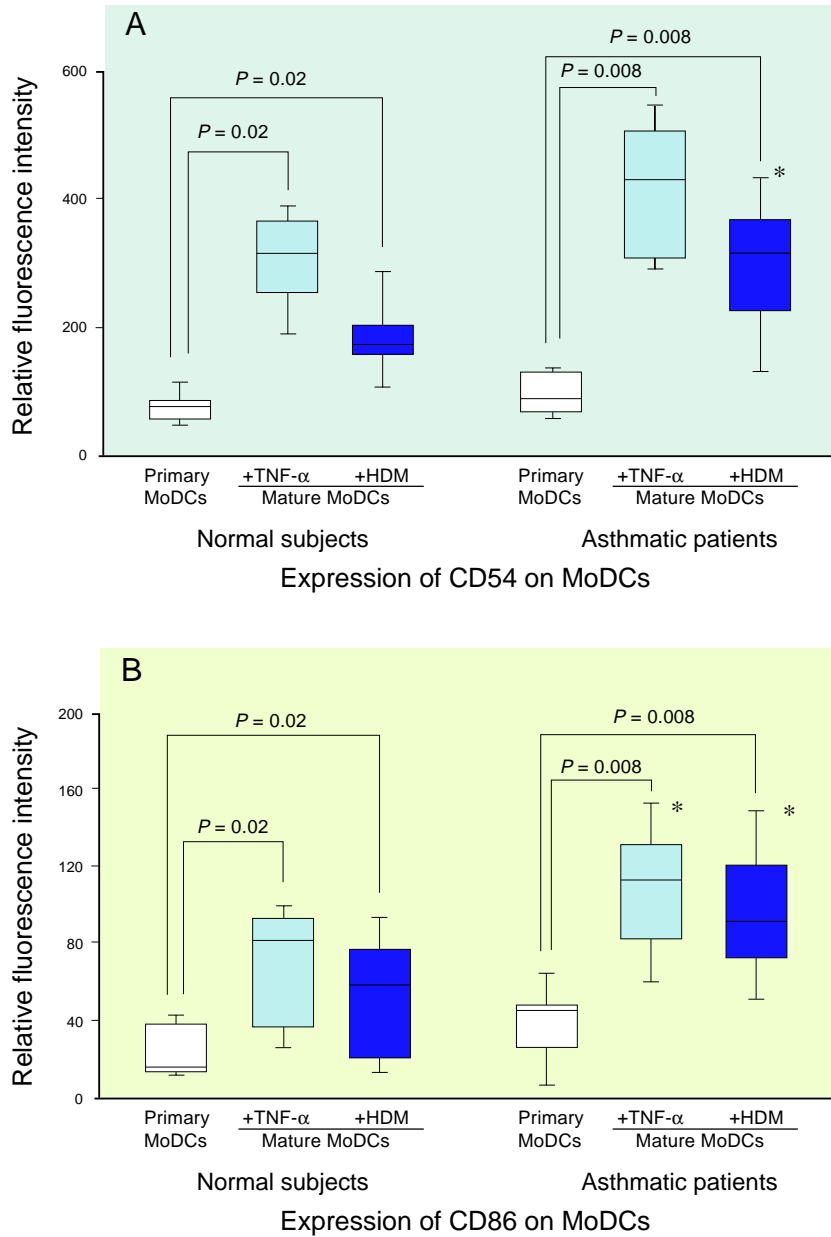
#### **Expression of T cell co-stimulating molecules on DCs**

When the expression of T cell co-stimulating molecules CD80 (B7-1), CD86 (B7-2), CD54 (ICAM-1), and CD11b (Mac-1) on primary MoDCs was assessed by flow cytometry, these molecules did not differ between asthmatics and control subjects. The expressions of CD54 and CD86 on MoDCs after stimulation with HDM were increased in the 2 groups of subjects, and the expression of these molecules on MoDCs from asthmatics was significantly

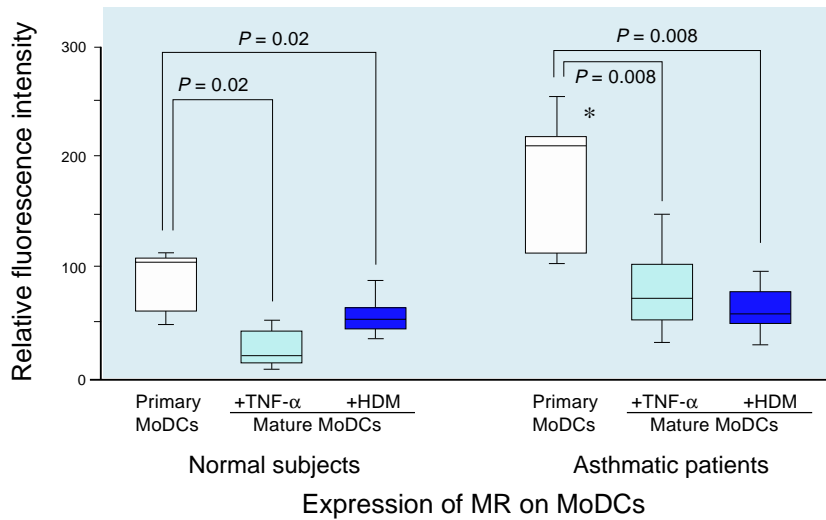
higher than that from control subjects (Fig. 3). There was no remarkable change in the intensity of CD80, or CD11b after stimulation with TNF- $\alpha$  or HDM in both groups.

#### **Expression of MR on DCs**

The expression of MR on primary MoDCs was significantly higher in HDM-allergic asthmatic patients than in normal subjects ( $P < 0.05$ ). MR expression on MoDCs from HDM-Allergic asthmatics was significantly reduced by stimulation with HDM as well as TNF- $\alpha$  (Fig. 4). When the rate of induction in the expression of MR was assessed, the MR of MoDCs from asthmatics were shown to be more reduced than that of DCs from control subjects after stimulation with HDM ( $P = 0.001$ ) (median reduction rate; asthmatic subjects, 65% and control subjects, 40%). However, after stimulation with TNF- $\alpha$ , there was no significant difference



**Fig. 3.** Expression of CD54 (**A**) and CD86 (**B**) as T cell co-stimulating molecules on MoDCs of asthmatic patients and normal subjects. Primary MoDCs were derived from plastic-adherent peripheral blood mononuclear cells with 800 U/mL GM-CSF and 500 U/mL IL-4 for 7 days. Mature MoDCs were obtained from primary MoDCs by the stimulation of 10 ng/mL TNF- $\alpha$  or 10  $\mu$ g/mL HDM for 24 h. The lower, middle and upper lines in the boxes represent the 25th, 50th and 75th percentiles, respectively; the lower and upper limits of the bars represent the 10th and 90th percentiles, respectively. HDM, house dust mite; IL-4, interleukin-4; GM-CSF, granulocyte-macrophage colony-stimulating factor; MoDC, monocyte-derived dendritic cell; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . \* $P < 0.05$ , compared with normal subjects.



**Fig. 4.** Expression of MR on MoDCs of asthmatic patients and normal subjects. Primary MoDCs were derived from plastic-adherent peripheral blood mononuclear cells with 800 U/mL GM-CSF and 500 U/mL IL-4 for 7 days. Mature MoDCs were obtained from primary MoDCs by the stimulation of 10 ng/mL TNF- $\alpha$  or 10  $\mu$ g/mL HDM for 24 h. The lower, middle, and upper lines in the boxes represent the 25th, 50th and 75th percentiles, respectively; the lower and upper limits of the bars represent the 10th and 90th percentiles, respectively. HDM, house dust mite; IL-4, interleukin-4; GM-CSF, granulocyte-macrophage colony-stimulating factor; MoDC, monocyte-derived dendritic cell; MR, mannose receptor; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . \* $P < 0.05$ , compared with normal subjects.

between the 2 groups in the rate of reduction of MR on stimulated MoDCs (median reduction rate; asthmatic subjects, 55% and control subjects, 75%).

### Discussion

We have studied the expression of surface markers on MoDCs from HDM-allergic asthmatics before and after stimulation with HDM or TNF- $\alpha$  in vitro. The expressions of HLA-DR and MR on primary MoDCs from asthmatics was higher than those from normal subjects, suggesting that these cells from asthmatics were differentiated into more mature DCs from the point of view of phenotypes as previously demonstrated (van den Heuvel et al., 1998). In response to HDM, MoDCs from asthmatics showed a greater expression of HLA-DR, ICAM-1 and B7-2 than those from normal subjects. In addition, the expression of MR on MoDCs reduced with stimulation with HDM, but not TNF- $\alpha$ .

To our knowledge this is the first study of phenotypical analysis of MoDCs stimulated with a specific antigen from allergic asthmatics. It has been reported that when DCs are induced to a mature state by TNF- $\alpha$ , CD40L or lipopolysaccharide, the expression of MHC class II, as well as co-stimulatory molecules ICAM-1 and B7, is up-regulated (Sallusto and Lanzavecchia, 1994; Morse et al., 1997; Palucka et al., 1998). Similarly, our data indicate that MoDCs from asthmatic patients showed a more mature-phenotype after stimulation with HDM than those from normal subjects. As to mature-phenotypic DCs from asthmatics, 2 reasons could be considered. First, a mature subpopulation of peripheral monocytes might affect to be derived into mature-phenotypic DCs from asthmatics. We have previously shown functional and phenotypic differences between monocytes from atopic asthmatics and those from normal subjects (Tomita et al., 1995, 1997). Second, exogenous cytokines such as IL-10, IL-4, GM-CSF or IL-12 might stimulate DCs to develop into mature cells.

In the airways, DCs are located in and just underneath the bronchial epithelium to allow for the uptake of inhaled antigens (Holt et al., 1997; van Haarst et al., 1994). In most tissues, DCs are present in a so-called “immature” state, unable to stimulate T cells. We demonstrated that immature MoDCs from asthmatic patients showed a higher expression of HLA-DR than those from normal subjects. Furthermore, this differentiated marker was up-regulated by stimulation with HDM as well as TNF- $\alpha$ . A recent study showed that immature DCs were active in the biosynthesis of class II MHC and in peptide loading, but the peptide-MHC class II complexes were recycled by accumulating in lysosomes and reaching the cell surface. If these cells started to mature, the increased biosynthesis of class II MHC was observed which maximizes the specific loading of antigens (Cella et al., 1997). So antigen presentation to class II-restricted T cells generally requires such newly synthesized class II MHC on mature DCs (Pinet et al., 1995). Thus, our findings may imply that the newly produced HLA-DR on “mature” DCs from asthmatics stimulated with HDM contributes to the presentation of inhaled antigens to T cells.

Recent studies have shown that Th2 cells play an important role in the generation of airway eosinophils in asthma through their ability to produce IL-5 (Ohnishi et al., 1993; Walker et al., 1994). The depletion of airway DCs suppressed Th2 cytokine-associated eosinophilic airway inflammation in a mouse model with asthma (Lambrecht et al., 1998) suggesting that DCs might play an important role in stimulating Th2 cells. ICAM-1/LFA-1 (King and Katz, 1989) and B7/CD28 (Lenschow et al., 1996; Reiser and Stadecker, 1996) interactions are major pathways for initiating naive T cell activation. DCs treated with TNF- $\alpha$  or lipopolysaccharide showed increased expression of the class II and class I molecules, B7-1 and ICAM-1 (Sallusto et al., 1995). Our data demonstrated that MoDCs from asthmatics treated with TNF- $\alpha$  or HDM develop into mature DCs, which show enhanced expression of ICAM-1 and B7-2. Kuchroo and colleagues (1995) reported that

B7-2 was one of the co-stimulating molecules for signaling Th2 cells.

We also demonstrated a significant reduction in MR on MoDCs from HDM-allergic asthmatics, after stimulation with HDM but not with TNF- $\alpha$ , in spite of a lack of significant differences in CD80, CD86 and ICAM-1 expressions. MR, which is expressed on cultured immature DCs (Reis e Sousa et al., 1993; Sallusto et al., 1995) is internalized to mediate phagocytosis of various microorganisms that expose mannosylated glycoproteins (Ezekowitz et al., 1991). Indeed, in DCs, MR allows the uptake and concentration of ligands such as FITC-labeled dextran and horseradish peroxidase (Sallusto et al., 1995). Engering and colleagues (1997) demonstrated that the MR pathway in DCs was ~100-fold more efficient in presenting antigens to T cells. Upon the maturation of DCs from HDM-allergic asthmatics, the uptake of mannosylated antigens such as HDM might play an important pathophysiological role in atopic asthma.

We examined the surface expression on DCs derived from peripheral blood. Airway DCs originate in peripheral blood monocytes from the bone marrow (Holt et al., 1994; McWilliam et al., 1995). McWilliam and colleagues (1995) hypothesized that the location of the development of DCs from peripheral monocytes after birth may influence the pathogenesis of immune regulated diseases such as asthma and allergic rhinitis. So, further studies are required to know the nature of MoDCs.

In conclusion, we have shown that immature DCs from patients with allergic asthma exhibit increased expression of mature markers, and more extensive development into a mature phase after stimulation with TNF- $\alpha$  or HDM. A reduction in MR after stimulation with HDM characterized DCs from patients with HDM-allergic asthma. These observations suggest that MR on “mature” DCs from atopic asthma might be used to specialize in capturing HDM antigens.



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